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A SIMPLIFIED DETERMINATION OF URINARY TESTOSTERONE UTILIZING COLUMN AND GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

A chromatographic analysis for urinary testosterone has been developed which requires only one purification by silica gel column chromatography prior to final separation and quantification by gas–liquid chromatography. Enzyme hydrolysis is achieved with 10% the customary enzyme concentration in 18 to 24 h since most of the enzyme-inhibiting debris is removed by preliminary precipitation of the steroidal material from the urine with ammonium sulfate. The free steroids extracted from the hydrolysate are acetylated before they are pre-purified by gradient elution from a small, disposable silica gel column.

INTRODUCTION

The measurement of total urinary 17-oxosteroids yields a dubious evaluation of endocrine disorders while the level of testosterone**, the most potent androgen known[1], is often a useful diagnostic parameter in both sexes. Most analytical technics require a "rough-cut" separation to remove the corticosteroids by thin-layer chromatography[2–6], paper chromatography[7–11], or column chromatography (florisil, silica gel, or alumina)[12–16]. All reported methods require one or two additional purification steps, depending on whether the final quantification is to be accomplished by absorption spectrometry[9,12,14,15], fluorimetry[4,7,8], hydrogen flame ionization[2,4–6,13,16], or double isotope dilution[11]. ZURBRUGG *et al.*[15] utilized CC exclusively to achieve excellent isolation in good yield through a lengthy procedure involving gradient elution from two of three different types of columns. SANDBERG *et al.*[13] combined the

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** Systematic nomenclature for steroids discussed: testosterone (17β-hydroxy-Δ^4-androsten-3-one), dehydroepiandrosterone (3β-hydroxy-Δ^5-androsten-17-one) epitestosterone (17α-hydroxy-Δ^4-androsten-3-one).

initial purification procedure (florisil followed by gradient elution from alumina) of CAMACHO AND MIGEON[12] with final purification by gas–liquid chromatography (GLC), which also affords quantification by hydrogen flame ionization.

All methods incorporate enzymatic hydrolysis (β-glucuronidase) to free the steroids which are conjugated with glucuronic acid in the urine; however, there is considerable variety in the use of the enzyme. In most cases an aliquot of urine is adjusted to pH 5 before the addition of 300 to 500 units of enzyme per milliliter of urine and incubation at 37° for 3 to 5 days. A notable exception is the recent work of VANDENHEUVEL[17] in which β-glucuronidase and sulfatases from *Helix pomatia* at high concentrations (namely, 4000 Fleishman units and 2000 Whitehead units, respectively, per milliliter of urine) are used to effect 99% enzymatic efficiency after incubation at 37° and pH 5.5 for 24 h. A disadvantage of performing the enzymatic hydrolysis in the raw urine, in addition to the time element, is the unpredictable amount of β-glucuronidase inhibitor found in different urine samples. Thus, it is nearly impossible to predict the optimum hydrolysis (or incubation) time for different urine samples. KENT *et al.*[18] reported a technic involving extraction with ethyl acetate prior to enzyme treatment and claimed near-maximum hydrolysis in 24 h for the extracted conjugates, while those in the untreated urine required 72 h. Recently CHATTORAJ AND SCOMMEGNA[19] described a method of avoiding enzyme inhibitors by precipitation of both free steroids and their conjugates from urine with 70% ammonium sulfate. Subsequent enzymatic hydrolysis of the precipitate gave maximum yields after 24 h at 37° with only 10% the usual dose of β-glucuronidase.

This paper describes an attempt to establish a simple, rapid, and reliable assay for urinary testosterone. CC is used instead of TLC to avoid, as much as possible, subjective decisions and sample handling (which affect sample recovery) on the part of the technician in the isolation of testosterone. The ammonium sulfate precipitation method has been extended to the testosterone determination because it renders a rapid and significant separation from enzyme-inhibiting debris in the urine. The steroids extracted from the hydrolysate are acetylated and then separated by gradient elution from a small silica gel column which retains the more polar corticosteroids. The fractions containing testosterone are then consolidated and allowed to evaporate; and finally, an aliquot is injected into a gas chromatograph for final separation and quantification of testosterone as the acetate derivative.

MATERIALS AND METHODS

Urine collection

The urine specimens were cooled during the period of collection. At the end of the 24-h period (usually 0800 to 0800), the urine volume was measured, and a 100 cc aliquot was removed, acidified with 1 cc of concentrated HCl and refrigerated until analysis.

Radiochemistry

The radioactive testosterone conjugate ([7α-^{3}H]testosterone-β-D-glucuronide)* was used as a tracer to identify the chromatographic fractions which contained

* New England Nuclear Corp., Boston, Mass.

testosterone. Since the tritium atom was attached to the steroid moiety, the tracer also served as means to determine comprehensive recovery from precipitation, enzymatic hydrolysis, extraction, purification, and consolidation of the sample. The recovery determination was accomplished by adding 50 μl (approximately 0.01 μC) of [^{3}H]testosterone glucuronide stock solution (ethanol) to each urine sample as well as to each of three vials filled with 15 cc of scintillator solution and Cab-O-Sil*. The specific activity of the [^{3}H]testosterone glucuronide was 20 C/mmole, which means that the addition of 0.01 μC contributes approximately 1×10^{-10} g of testosterone to the urine aliquot. Thus, the tracer concentration could be increased by two orders of magnitude and still escape quantification on a mass basis by flame ionization detection. After the urine extract was purified on the silica gel column, the fractions containing testosterone (acetate derivative) were consolidated. An aliquot of this solution was transferred to a scintillation vial and evaporated to dryness before adding 15 cc of scintillator solution. All samples were corrected for quenching by the channels ratio method[20] which was based on the quenching of [^{3}H]toluene* by increasing amounts of chloroform.

It was necessary to use a small amount (approximately 5% of the empty vial volume) of Cab-O-Sil to prevent adsorption[21] of the steroid conjugate on the walls of the vial. Counts per minute were diminished less than 3% by a 10-fold increase in the amount of Cab-O-Sil used, so it was possible to use a spatula for the addition of Cab-O-Sil to the counting vials. The free testosterone (resulting from enzymatic hydrolysis) did not adsorb to the vial walls and thus did not require Cab-O-Sil. The scintillator solution was prepared by dissolving 5 g of 2,5-diphenyloxazole (PPO)* and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl POPOP)* in 1 l of toluene.

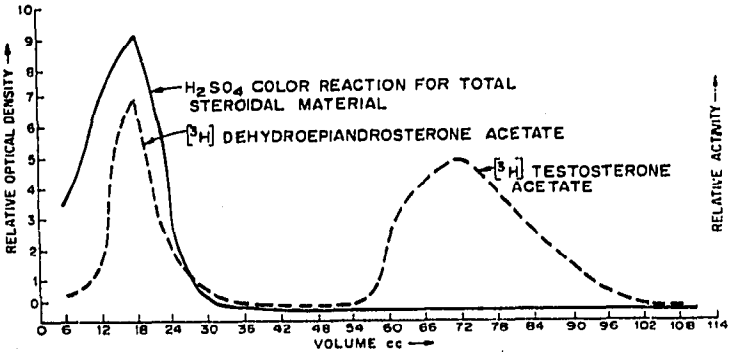
A Nuclear Chicago 720 series of scintillation spectrometer which was adjusted for channels ratio counting was used in this work. All counting was accomplished with a relative statistical error of $\pm$ 1% by accumulating at least 10 000 counts (error $= \pm \sqrt{N}$) for each sample. The radioactive materials were transferred with micropipettes with a precision of at least $\pm$ 1% (relative).

Silica gel

Silica gel was used to remove the corticosteroids and effect a reasonable separation of the 17-oxosteroids. The tracing in Fig. 1 shows the separation of [^{3}H]-testosterone-acetate from [^{3}H]dehydroepiandrosterone-acetate which represents the elution pattern of most of the major 17-oxosteroids. The silica gel was held in a mini-column made from a small disposable pipette (15 cm/capillary) which had been plugged with glass wool.

The silica gel (Anasil**, type S, 200–250 mesh) must be purified to remove contaminants which would otherwise contribute to a high background current in the subsequent gas chromatographic analysis. The purification was accomplished by shaking the silica gel with water for 2 h and drying at 100° for 10–16 h. The dry silica gel was washed successively with chloroform–methanol (1:1), methanol, and finally with methylene chloride. The silica gel was again dried for 10 to 16 h at 110°

* Packard Instrument Company, Downers Grove, Ill.
** Analabs, Inc., Hamden, Conn.



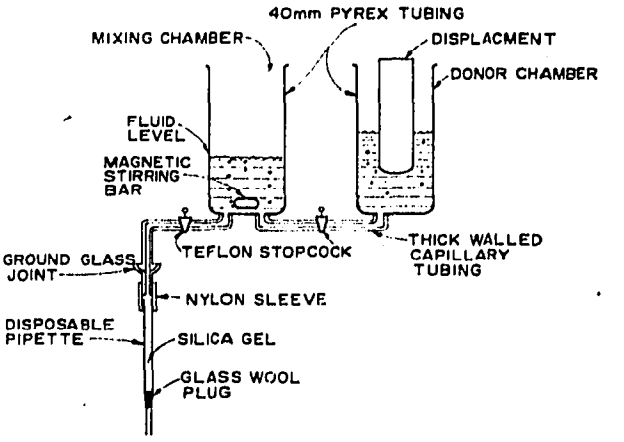


Fig. 1. Elution profile from silica gel mini-column *versus* eluent volume.

Fig. 2. Schematic of gradient elution device with silica gel mini-column attached.

and stored under methylene chloride to prevent contact with moisture in the atmosphere. Furthermore, the silica gel stored in this manner is immediately available for preparing the mini-columns as they are needed.

Elution from the silica gel column is particularly sensitive to the amount of moisture in the methylene chloride. Since the moisture content was difficult to control in the redistillation process, it was necessary to remove all the water from the solvent with molecular sieves* (5A, 8–12 mesh). A standard quantity of water was then added; 600 p.p.m. water in the methylene chloride was found to be optimum in our work.

Gradient elution system

It was necessary to incorporate gradient elution[22] from the silica gel mini-column in order to effect a reasonable separation of testosterone acetate from the other 17-oxo-acetylated steroids (Fig. 1). A gradient elution device, having two chambers of equal volume connected by a Teflon stopcock, was joined to the mini-column via a ground glass joint as shown in Fig. 2. The mini-column was connected to the detachable portion of the ground glass joint by a sleeve of nylon tubing of the appropriate diameter to effect a seal against the hydrostatic pressure-head of the solvents in the gradient elution device. The displacement tube at the top of the donor chamber lessens the volume of the donor fluid at the beginning of the elution to effect a better separation from the 17-oxosteroids and also to sharpen the elution of testosterone acetate. The magnetic stirring bar arrangement ensures a uniformly enriched composition of the eluting solvents.

Sample-handling procedure

Ammonium sulfate precipitation. Aliquots of refrigerated 24-h urine samples were treated in a manner analogous to that of CHATTORAJ AND SCOMMEGNA[19]. A measured amount (50 μl or about 0.01 μC) of a standard stock solution of [7α-^{3}H]-testosterone-β-D-glucuronide in ethyl alcohol was added to each 30 cc aliquot of

* Davison Chemical Co., Baltimore, Md.

urine before it was shaken with 22 g of $(NH_4)_2SO_4$ in a 50 cc ground glass centrifuge tube. After the $(NH_4)_2SO_4$ had dissolved, the samples were centrifuged at 3000 r.p.m. for 1 h.

Enzymatic hydrolysis. The supernatant was drawn off by aspiration and the remaining sediment dissolved in 5 cc of 1 *N* sodium hydroxide. After complete dissolution, 8 cc of water and 0.3 cc of concentrated hydrochloric acid were added to the centrifuge tube. The solution was still slightly basic and the pH was adjusted to 5 (as indicated by a pH meter) with dropwise addition of glacial acetic acid.

The volume of the solution was noted and sufficient 1 *M* sodium acetate buffer (pH 5) added to make a final concentration of 0.1 *M* sodium acetate buffer. One hundred Fishman units of Ketodase per cubic centimeter of final solution were added, and the solution incubated at 37° for 24 h. All steps of ammonium sulfate precipitation and enzymatic hydrolysis were accomplished in the centrifuge tube, thereby avoiding sample transfer to this point.

Extraction. The hydrolysis solution was extracted three times with 15 cc aliquots of peroxide-free diethyl ether*. The ether fractions were consolidated and washed twice with 20 cc aliquots of 1 *N* sodium hydroxide and then washed to neutrality with 25 cc aliquots of water. The ethereal portion was evaporated to dryness on a flash evaporator.

Derivatization for chromatography. The extract residue was transferred to a conical centrifuge tube with peroxide-free diethyl ether which was evaporated under a stream of nitrogen. The residue was dissolved in 0.25 cc of pyridine and 0.25 cc of acetic anhydride and allowed to react overnight at room temperature or for 2 h at 60°. After acetylation, the excess reagents were evaporated under a stream of nitrogen.

Gradient elution from silica gel mini-column. The mini-column was prepared by adding approximately 1 g of silica gel (Anasil, type S, 200–250 mesh) to a capillary disposable pipette. The silica gel was stored under methylene chloride (after purification), making it especially easy to transfer some of the silica gel slurry to the mini-column. The acetylated sample residue was transferred to the mini-column with three 1-cc aliquots of methylene chloride. The mini-column was then connected to the gradient elution device.

A typical gradient elution requires about 60 cc of methylene chloride in the mixing chamber and 120 cc of 60% diethyl ether in methylene chloride in the donor chamber. Care is taken to ensure that the fluid levels (or heights) are appropriately established in the two chambers before opening the two stopcocks to start the elution. For example, the level of the 60% ether solution must be significantly higher than that of the pure methylene chloride because the methylene chloride is almost 1.4 times as dense. The mini-column is discarded after each elution (approximately 2.5 h) and a new sample may be chromatographed within a few minutes by simply connecting a new mini-column to the gradient elution device.

Collection of chromatographic fractions. The gradient elution device and mini-column were mounted above a fraction collector which consistently delivered a desired volume (6 cc) to each fraction tube. A small quantity from each of 5 or 6 fractions was tested for activity in an effort to define the leading and trailing edge of

* Mallinkrodt Chemical Works.

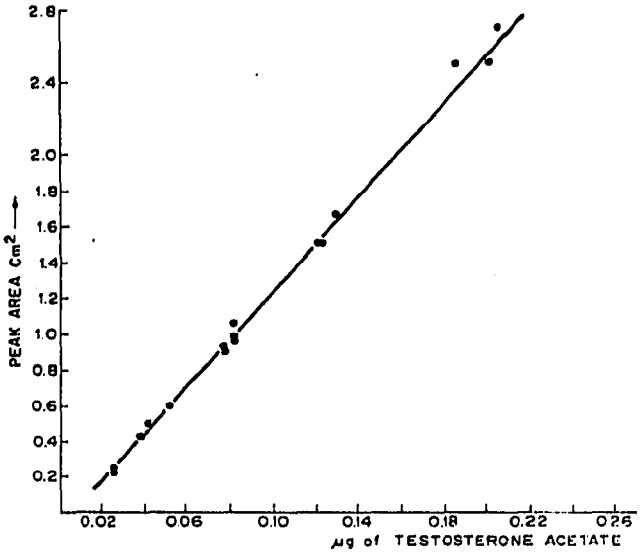


Fig. 3. Response of flame ionization detector to testosterone acetate.

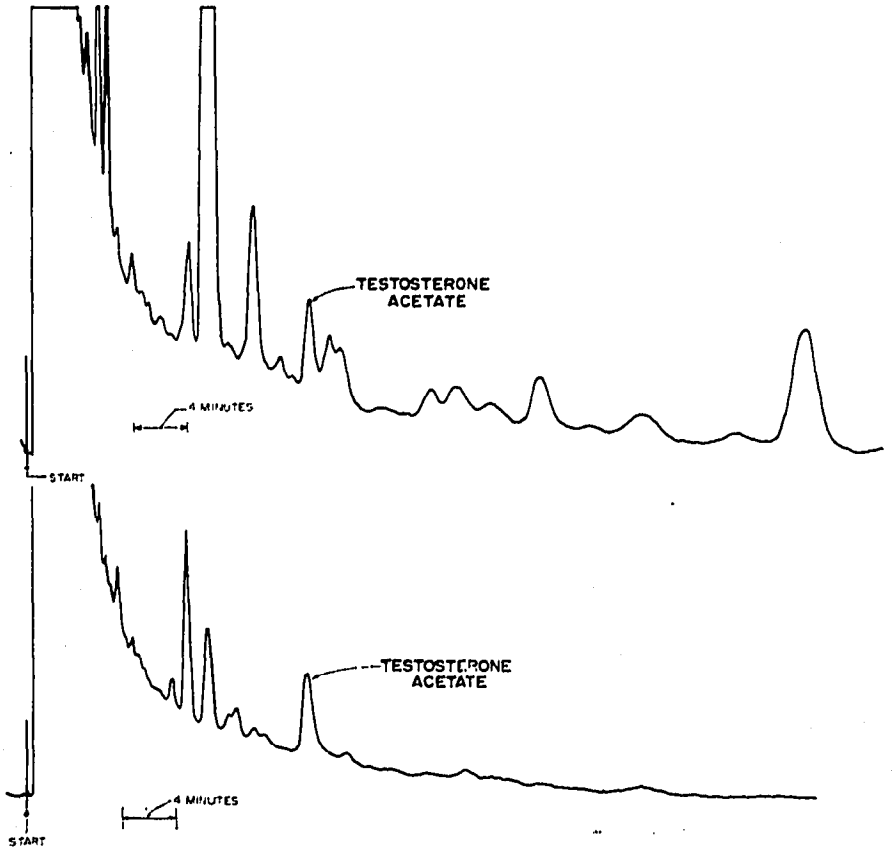


Fig. 4. Comparison of gas chromatograms of a typical urine extract before (top) and after (bottom) purification on mini-column of silica gel; column isothermal at 218° (for other GLC conditions see text).

the testosterone elution profile. All fractions whose activity was at least twice background (30 c.p.m.) were consolidated. An aliquot of this consolidated solution was removed and subjected to scintillation analysis to determine the methodology recovery. The remaining solution was evaporated to dryness and then dissolved in 50 μl of benzene for final analysis by GLC.

Gas–liquid chromatography. GLC was used for final separation of the remaining steroid derivatives and for quantification of testosterone acetate by a flame ionization detector. A F&M model 402 gas chromatograph equipped for on-column sample injection was used in this work. The 3.67 m (12 ft.) glass U-shaped columns and associated connections were silanized with 5% dimethyldichlorosilane in toluene. The solid support and liquid phase coating were treated and prepared according to the recommendations of HORNING *et al.*[23].

The samples were chromatographed isothermally on a 3.67 m × 6.3 mm (1/4 in.) O.D. column packed with 1% SE-30 on silanized Gas Chrom P at a flow rate of 60 cc/min of helium. The operating temperatures were: injector, 260°; column, 218°; detector, 260°. Under these conditions testosterone acetate exhibited a KOVATS index[24] of 2652.

The response of the flame ionization detector was linear with standard quantities of testosterone acetate as shown in Fig. 3. Fig. 4 shows a chromatogram of a urine sample before (top) and after (bottom) gradient elution from the silica gel column.

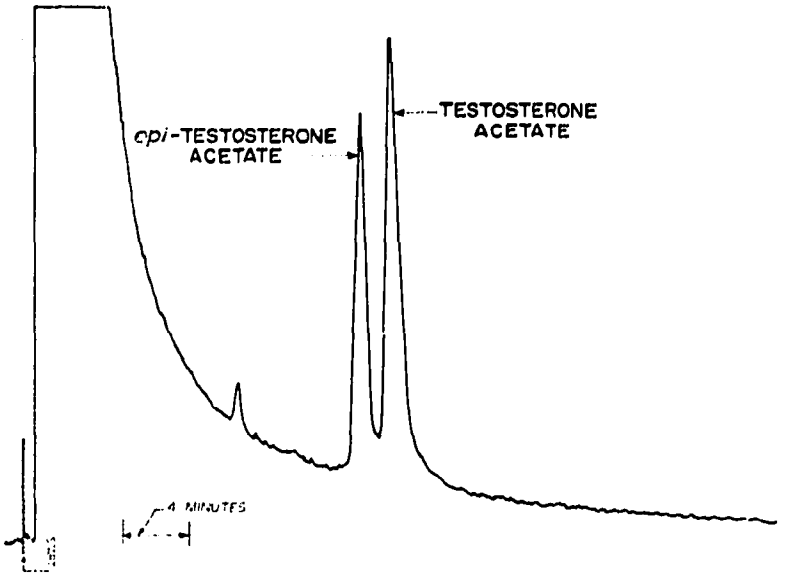
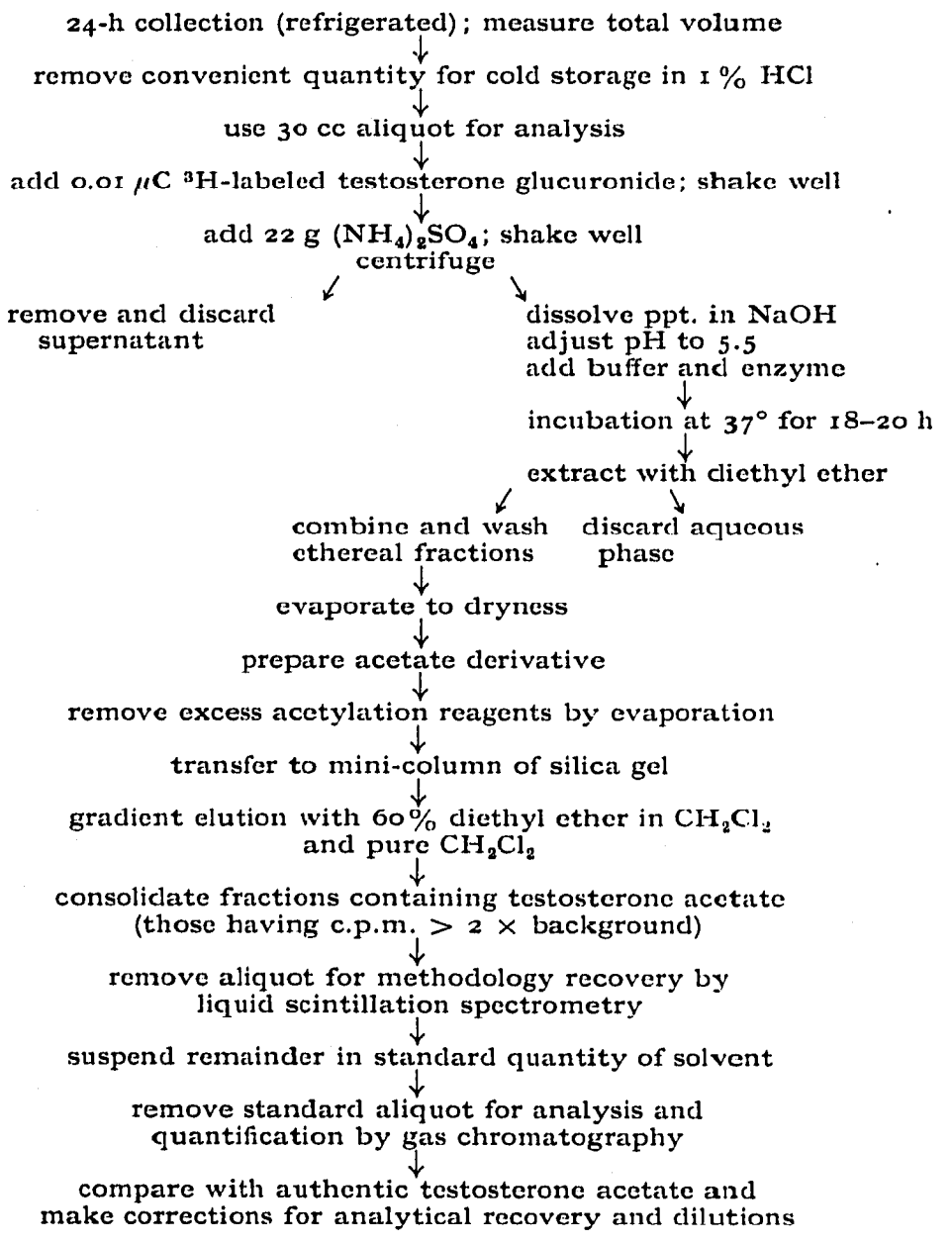


Fig. 5. Chromatogram showing separation of acetate derivatives of testosterone isomers; column isothermal at 218° (for other GLC conditions see text).

Epitestosterone does not survive the purification procedure. If it were present in the final sample mixture, it would be discernible as shown in Fig. 5 which indicates that the acetate derivatives of authentic epitestosterone* and testosterone** are clearly resolved under these GLC conditions.

* Kindly donated by Southwest Research Foundation, San Antonio, Texas.
** Applied Science Labs, State College, Pa.

FLOW DIAGRAM FOR DETERMINATION OF URINARY TESTOSTERONE



RESULTS

Precision and recovery

In an effort to document the recovery and precision of the method, five 30-cc aliquots were taken from the same 24-h urine collection and processed through the purification step. As shown in Table I, 70 ± 5% of the urinary testosterone was recovered through precipitation from the urine, enzymatic hydrolysis (18–24 h), extraction, purification, and consolidation. Approximately 95% of conjugated (glucuronide) and virtually all the free testosterone was precipitated with $(NH_4)_2SO_4$.

TABLE I

PRECISION OF METHODOLOGY: REPLICATE ANALYSES OF SAME 24-h URINE

Replicate Number	*Recovery (%)*	*Quantity (μg/day)*
1	70.0	95.2
2	64.6	89.0
3	67.0	84.4
4	78.0	95.0
5	68.0	84.8
Averages	69.5 ± 5.1 (S.D.)	89.7 ± 5.3 (S.D.)

The yield of the enzymatic hydrolysis was approximately 80% after 18 h with 100 Fishman units of enzyme per cubic centimeter of solution. There was no appreciable increase in the yield with increased hydrolysis time or with enzyme concentrations above 100 units/cc of hydrolysate.

The precision of the overall technic including the final quantification by GLC is indicated by the third column of data in Table I. Independent analysis of these five replicate aliquots of the same 24-h urine collection gave results which varied with a relative standard deviation of ± 5.9%. The brief summary of 24-h urinary testosterone excretions (Table II) shows agreement with other reported values[6,9,13,25–27].

DISCUSSION

This method is simple and reliable in that it minimizes the number of sample transfers and virtually eliminates analytical decisions on the part of the operator. The use of $(NH_4)_2SO_4$ to precipitate the steroids yields a fairly "clean" urine extract as shown by the chromatogram (top) in Fig. 4. While this particular chromatogram

TABLE II

SUMMARY OF NORMAL AND PATHOLOGICAL URINARY TESTOSTERONE

Age	*Sex*	*Condition*	*Quantity (μg/24 h)*
19	M	Normal	90
21	M	Normal	79
18	M	Normal	73
19	M	Normal	87
37	M	Normal	60
21	M	Normal, but superficial hirsutism	135
17	M	Pituitary hypogonadism	8.5 (baseline)
17	M	Pituitary hypogonadism	21 (treatment[a] day No. 14)
17	M	Pituitary hypogonadism	88 (treatment[a] day No. 27)
21	F	Normal	21
21	F	Normal	4.7
26	F	Normal	15
39	F	Normal	21
45	F	Hirsute	38

[a] Treatment with 5000 units of human chorionic gonadotropin 3 times weekly.

may be adequate for quantification, the number and size of other peaks near that of testosterone acetate in the chromatograms of urine extracts are quite variable. Furthermore, there are several peaks in the chromatogram (not all shown in Fig. 4) which have retention times between 60 and 100 min. However, a single partitioning on silica gel consistently removed peaks of any significant proximity to testosterone acetate and diminished those compounds which have gas chromatographic retention times greater than 45–60 min. Thus, even though some urine extracts may give useful gas chromatograms, the preliminary purification on silica gel ensures a consistently cleaner and more reliable sample for quantification.

Several samples are processed simultaneously, but a minimum of 36 h is required to complete the analysis of any given urine sample. This would involve overnight hydrolysis and accelerated derivatization (at 60°). We currently purify three samples per normal working day on silica gel columns. Thus, if sample processing, hydrolysis, extraction, and derivatization are properly scheduled, it is possible to analyze fifteen urine samples per week. Of course, the gradient elution device could be easily modified to accommodate several mini-columns simultaneously, thereby increasing the analytical capacity.

The acetate derivatization is reasonably foolproof. An excess of reagents is added to avoid problems with traces of moisture in the urine extract. The acetate derivatives are quite stable even when stored for weeks in the open laboratory and they are also thermally stable under GLC conditions. It is very important, however, to completely remove all the excess pyridine and acetic anhydride by evaporation prior to transferring the sample to the silica gel column. Any of these residual reagents will affect the retention of the steroid derivatives on the column, presumably because of a temporary, localized increase in the polarity of the eluting solvent.

In the ammonium sulfate precipitation, 22 g of salt/30 cc of urine produces a saturated solution at ambient temperature. There may be a few crystals of excess salt in the precipitate; however, this does not interfere with the remaining steps of the procedure. Some urine samples take on a very cloudy appearance when saturated with ammonium sulfate; it has been shown that this does not affect the analytical yield significantly. After centrifugation, the supernatant urine is routinely drawn off with an aspirator. Some urines do not form a solid or compact precipitate and some pieces may actually float on the supernatant and may therefore be inadvertently removed. The comprehensive recovery determination which is based on the addition of a standard quantity of tritium-labeled testosterone glucuronide to the initial urine sample will correct for this inadvertent loss. Of course, the yield also corrects for losses due to incomplete hydrolysis and extraction.

The ammonium sulfate precipitation, ether extraction, and silica gel purification prepare a relatively simple mixture for analysis by GLC as shown at the top of Fig. 4. The gas chromatogram (bottom of Fig. 4) contains an isolated peak which corresponds (in retention time) to authentic testosterone acetate. This was later confirmed by mass spectrometry.

The described assay for urinary testosterone represents a trend toward simplicity. Various technics have been incorporated to increase laboratory efficiency and reduce analysis time. The data presented establish the reliability and validity of the methodology.

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