## ABSTRACT

DETERMINATION OF THE MAJOR URINARY 17-KETOSTEROIDS INCLUDING PREGNANEDIOL AND 17-KETOGENIC STEROIDS BY GAS-LIQUID CHROMATOGRAPHY

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A gas-liquid chromatography(GLC) procedure is described for estimating major urinary 17-ketosteroids including pregnanediol and 17-ketogenic steroids without formation of their silylated derivatives. The separative and quantitative capabilities of GLC in determining certain urinary steroids are superior to the colorimetric methods in that; frequently, clinically significant individual alterations in steroids metabolites are masked by normal values obtained by analysis for total steroid groups. By making use of the resolving and measuring capabilities of GLC, several urinary steroid metabolites may be simultaneously assayed. The methods undertaken in the study can be applied to multiple samples, enabling the procedures to be used in routine clinical assay. Each analytical step has been made optimal, resulting in simple and rapid methods for determining the steroid metabolites in urine.

# ACKNOWLEDGEMENTS

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# CHAPTER I

# INTRODUCTION AND HISTORICAL

## Introduction

### Origin and Classification

The human adrenal cortex secretes a variety of steroid hormones that are intimately concerned with a wide range of metabolic processes. All steroids have in common a cyclopentanophenanthrene nucleus shown in Figure 1. The steroids

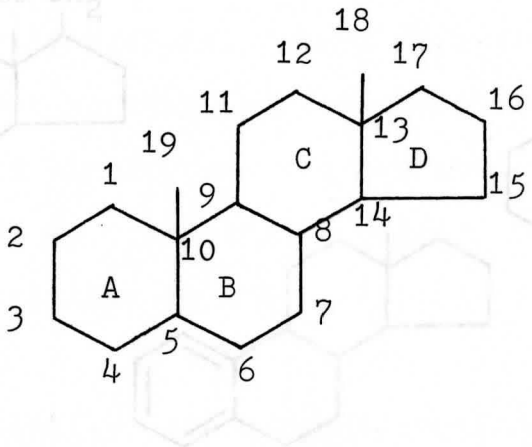



Figure 1. The Steroid Skeleton and Numbering System.

are distinguished from each other through a number of various substituted groups in the different positions on the ring and types of side-chain on C-17. The C-18 and C-19;

the angular methyl groups, are important reference points. The use of a solid line to attach them to carbons 10 and 13 indicates that carbons 18 and 19 are above the plane of the molecule. The use of a dotted line indicates that any constituent directly attached to the ring structure lies below the plane of the molecule. The general classification of all steroids secreted by the adrenal cortex is based upon the number of carbons present in the parent structural compounds. These are: pregnane, the C-21 steroids; androstane, the C-19 steroids; and estrane, the C-18 steroids. The structures of their parent molecules are shown in Figure 2. The

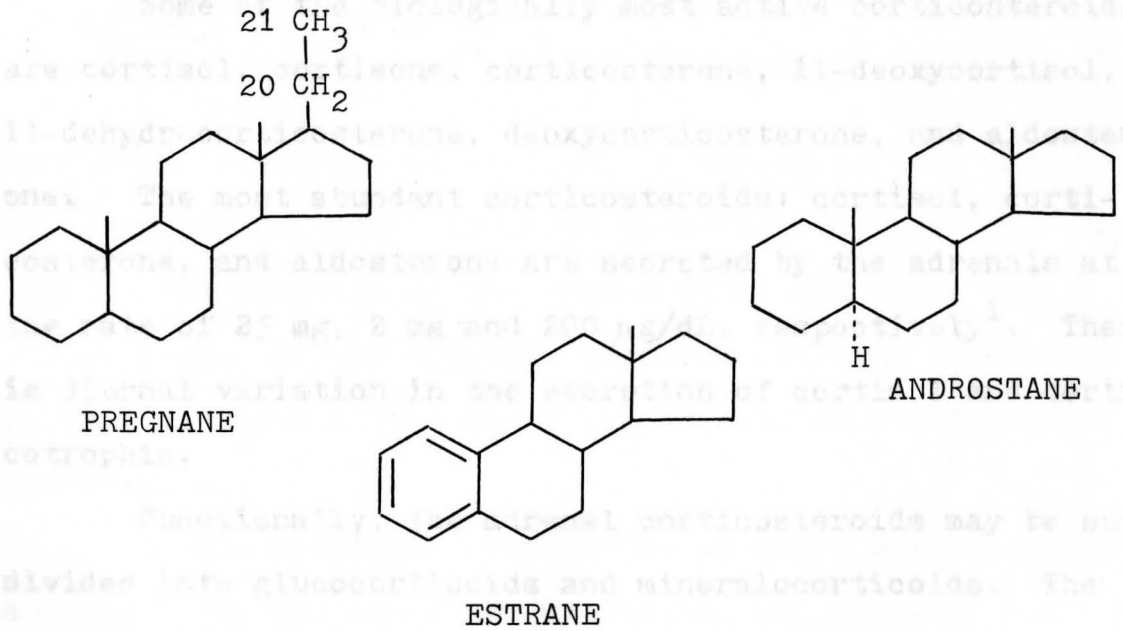



Figure 2. Parent Structural Compounds Secreted by Adrenal Cortex.

adrenocortical steroids include the corticosteroids, which are formed exclusively by the adrenals, in addition to the androgens, progestogens, and estrogens. The latter are also

secreted by the gonads. Adrenal activity is regulated by an anterior pituitary hormone, adrenocorticotrophic hormone (corticotrophin, ACTH). The corticosteroids are, from the physiologic as well as the quantitative point of view, the most important group of adrenal steroids. A consideration of the biological effects or actions of corticosteroids permits a diagnostic approach to evaluation of adrenocortical function; for example, cortisol is quantitatively the major circulating C-21 adrenocorticosteroid and exemplifies glucocorticoid effects with characteristic metabolic changes in carbohydrate, protein, and fat metabolism.

Some of the biologically most active corticosteroids are cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-dehydrocorticosterone, deoxycorticosterone, and aldosterone. The most abundant corticosteroids: cortisol, corticosterone, and aldosterone are secreted by the adrenals at the rate of 25 mg, 2 mg and 200 $\mu$g/dL, respectively[1]. There is diurnal variation in the secretion of cortisol and corticotrophin.

Functionally, the adrenal corticosteroids may be subdivided into glucocorticoids and mineralocorticoids. The glucocorticoids are hormones affecting carbohydrate metabolism. Mineralocorticoids regulate salt and water metabolism, and include the compounds 11-deoxycorticosterone and aldosterone.

The essential structural features of corticosteroids are unsaturation at C-4 and C-5, ketone group at C-3 and

another ketone group at C-20 as shown in Figure 3. The pres-

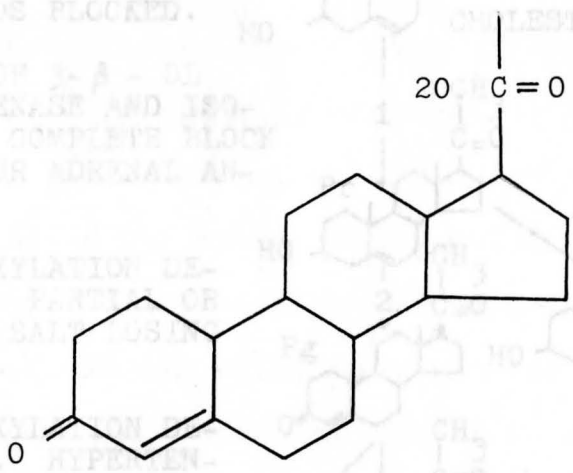



Figure 3. Essential Features of Corticosteroids.

ence of an -OH group at C-21 is a prerequisite for activity in carbohydrate metabolism, but most important for the function is the presence of the oxygen atom, either as -OH or =O at C-11. Figure 4 shows biogenesis of corticosteroids as well as sites of the major blocks causing adrenogenital syndromes[1].

Besides corticosteroids, the adrenals also secrete androgens, progesterone and estrogens, all of which are known to be produced by the gonads as well. The major androgens are: androsterone, etiocholanolone, androstenedione, testosterone, dehydroepiandrosterone(DHA or DHEA), and 11$\beta$-hydroxyandrostenedione. Of these; DHEA and androstenedione are, from the quantitative standpoint, the most important adrenal 17-ketosteroids. The former is believed to be pro-

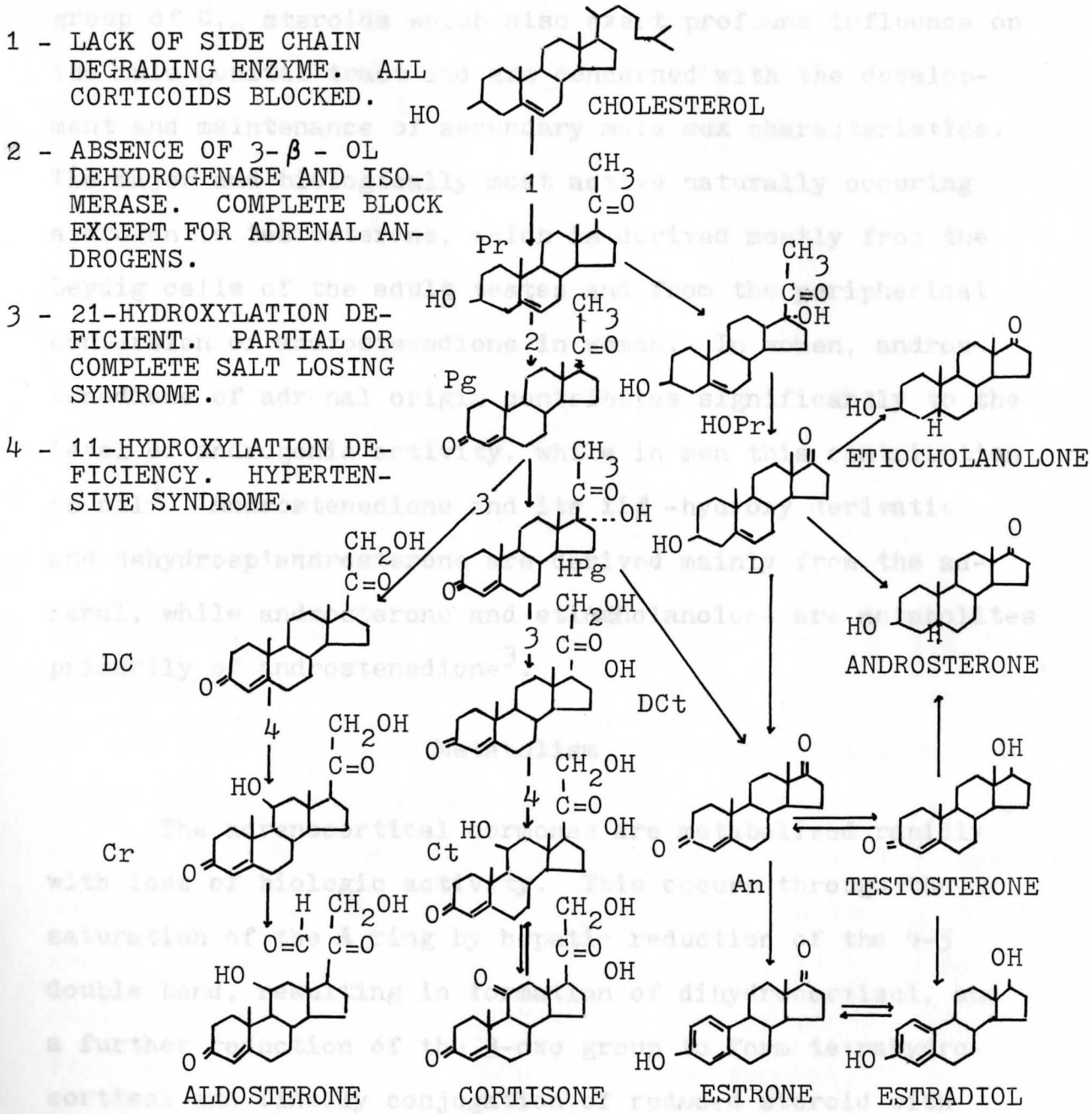



Figure 4. Biogenesis of Corticosteroids and Sites of the Major Blocks Causing Adrenogenital Syndromes. Pr, Pregnenolone; HOPr, 17-Hydroxypregnenolone; Pg, Progesterone; HPg, 17-Hydroxyprogesterone; D, Dehydroepiandrosterone; DC, 11-Desoxycorticosterone; DCt, 11-desoxycortisol; Cr, Corticosterone; Ct, Cortisol; An, Androstenedione.

duced exclusively by the adrenal[2]. The androgens are a group of $C_{19}$ steroids which also exert profound influence on the male genital tract and are concerned with the development and maintenance of secondary male sex characteristics. The major and biologically most active naturally occuring androgen is testosterone, which is derived mostly from the Leydig cells of the adult testes and from the peripherical conversion of androstenedione in women. In women, androstenedione of adrenal origin contributes significantly to the level of androgenic activity, while in men this contribution is nil[3]. Androstenedione and its 11$\beta$ -hydroxy derivative and dehydroepiandrosterone are derived mainly from the adrenal, while androsterone and etiocholanolone are metabolites primarily of androstenedione[3].

## Metabolism

The adrenocortical hormones are metabolized rapidly with loss of biologic activity. This occurs through the saturation of the A ring by hepatic reduction of the 4-5 double bond, resulting in formation of dihydrocortisol, and a further reduction of the 3-oxo group to form tetrahydrocortisol and finally conjugation of reduced steroid with glucuronic acid. The formation of a conjugate involves a 3-glucosiduronate or a 3-sulfate as shown in Figure 5. The conjugates are water soluble and therefore are readily excreted by the kidneys.[2] Cortisone and deoxycortisone give rise to essentially similar metabolites. They are excreted

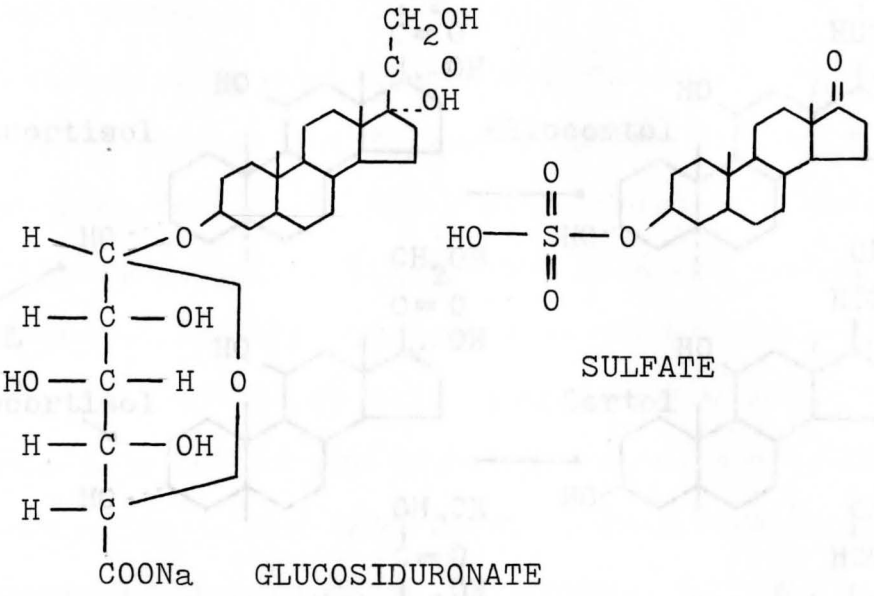



Figure 5. The 3-Glucosiduronate and 3-Sulfate Conjugates.

in the urine unchanged except for the largely conjugated glucuronide. Reduction at carbons 3, 4 and 5, and in part at C-20 ketone, yields a saturated A ring as dihydro and tetrahydro derivatives, plus cortolone and cortol; these are excreted in the urine as glucosiduronides. Cortisol is also converted to 11 $\beta$-hydroxy and 11-ketoetiocholanolone by a series of events, one of which is the removal of the side chain through the oxidation of the 17-hydroxy group. These 17-ketosteroids are then excreted in the urine mainly as sulfates and glucuronides. Corticosterone also undergoes reduction to dihydro and tetrahydro derivatives that are biologically inactive due to loss of the $\alpha, \beta$-unsaturated 3-ketone grouping in the A ring. Because it lacks a 17-hydroxy group, corticosterone cannot give rise to 17-ketosteroids. Figure 6 shows some metabolic products derived from

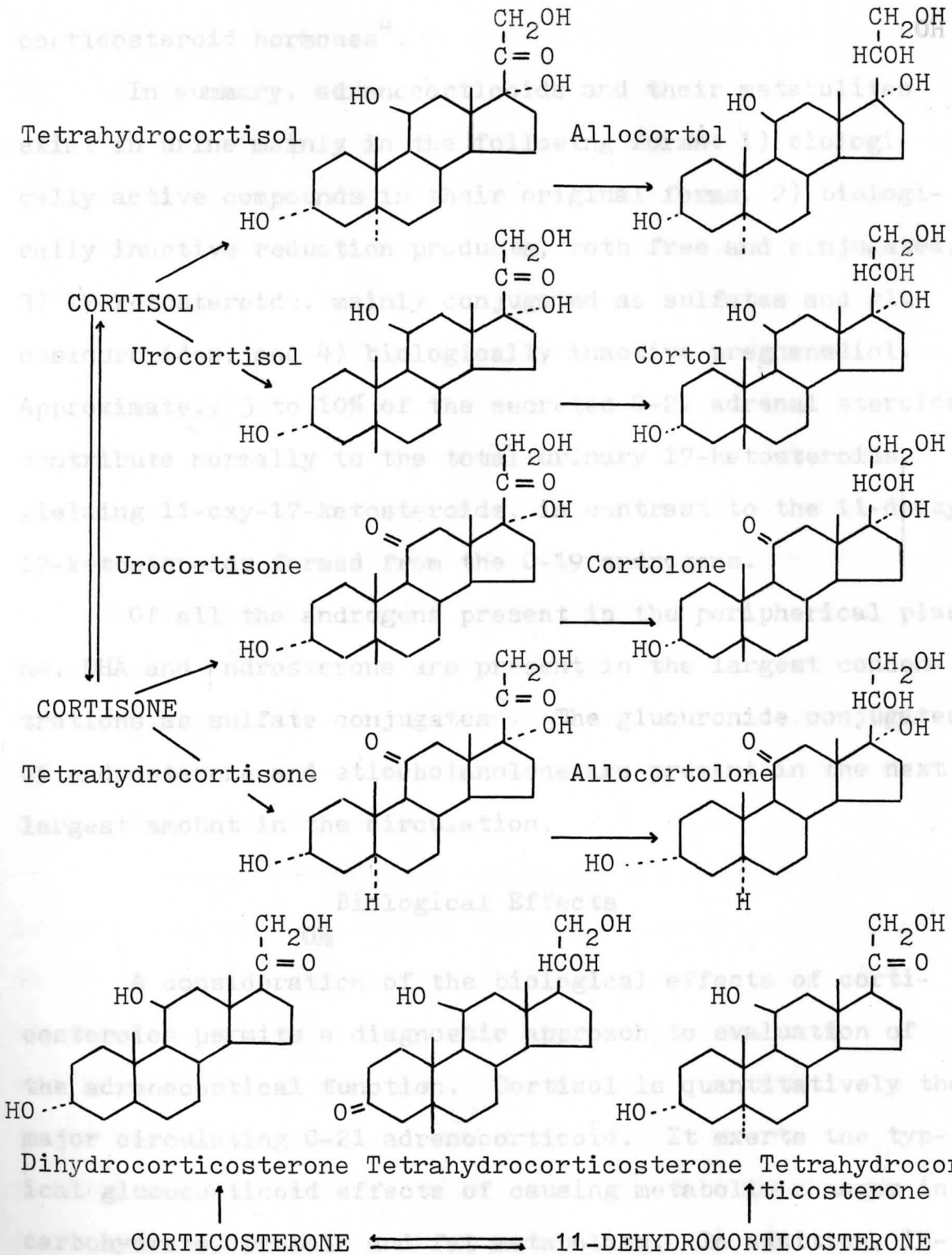



Figure 6. Some Metabolic Products Derived from Adrenal Cortical Hormones.

corticosteroid hormones[4].

In summary, adrenocorticoids and their metabolites exist in urine mainly in the following forms: 1) biologically active compounds in their original forms, 2) biologically inactive reduction products, both free and conjugated, 3) 17-ketosteroids, mainly conjugated as sulfates and glucosiduronides, and 4) biologically inactive pregnanediol. Approximately 5 to 10% of the secreted C-21 adrenal steroids contribute normally to the total urinary 17-ketosteroids, yielding 11-oxy-17-ketosteroids, in contrast to the 11-deoxy-17-ketosteroids formed from the C-19 androgens.

Of all the androgens present in the peripherical plasma, DHA and androsterone are present in the largest concentrations as sulfate conjugates[3]. The glucuronide conjugates of androsterone and etiocholanolone are present in the next largest amount in the circulation.

## Biological Effects

A consideration of the biological effects of corticosteroids permits a diagnostic approach to evaluation of the adrenocortical function. Cortisol is quantitatively the major circulating C-21 adrenocorticoid. It exerts the typical glucocorticoid effects of causing metabolic changes in carbohydrate, protein and fat metabolism. In addition, insulin may be antagonized by glucocorticoids, since an increased sensitivity to insulin is noted under conditions of hypoadrenocorticism that are produced by pancreatectomy or

alloxan[2]. Administration of cortisol or hyperadrenocorticism aggravates diabetes mellitus.

In addition, cortisol may be antagonistic in terms of physiologic effects with two other hormones. Such a consideration at least helps one to understand the clinical features of Addison's disease, an insidious and usually progressive disease resulting from adrenocortical hypofunction. The release of posterior pituitary antidiuretic hormone; ADH, appears to be opposed by cortisol, perhaps through plasma volume expansion. A similar reciprocal relationship between plasma cortisol concentration upon release of melanocyte-stimulating hormone(MSH) from anterior pituitary also seems to occur. Thus, increased skin pigmentation in Addison's disease may be due to high plasma concentrations of MSH in the presence of decreased cortisol levels that fail to suppress MSH release.

Another clinical manifestation of the glucocorticoids is osteoporosis, an absolute decrease in bone tissue mass; while the remaining bone is morphologically normal. This condition is observed with administration of glucocorticoids and in hyperadrenocorticism, and may be attributed to the following: 1) a possible decrease in synthesis of acid mucopolysaccharides(chondroitin sulfate), 2) protein catabolism and antianabolism, 3) hypercalciuria, secondary to the loss of bone matrix from the above effects, and 4) a decrease intestinal absorption of calcium, secondary to interference with the effect of the reactive metabolite of

vitamin D on the mucosal transport mechanism[5].

In the measurement of corticosteroids, low or low-normal values are present in Addison's disease, myxedema, and hypopituitarism. The most striking elevations are observed in Cushing's syndrome(not all cases); a constellation of clinical abnormalities due to chronic exposure to excesses of cortisol or related corticosteroids, in which diurnal plasma cortisol variations is absent, and in extreme stress, eclampsia and acute pancreatitis. Moderate elevations may be associated with stress of disease, surgery and burns. Slight increases are seen in the first trimester of pregnancy, virilism, obesity, hyperthyroidism, and in severe hypertension[6,7].

Since C-19 type steroids of clinical interest possess very weak androgenic properties, measurements of urinary 17-ketosteroids may not be completely informative. However, the 17-ketosteroids serve as a rough guide to androgenic activity because androsterone and dehydroepiandrosterone stimulate secondary male characteristic. Urinary 17-ketosteroid assay does not directly measure androgenic hormones but instead their metabolites. Only a fraction of these metabolites are excreted as urinary 17-ketosteroids. Increased values are most striking with adrenocortical tumors, especially malignant forms. In adrenal carcinoma there is an increase in the beta fraction; derived after treatment with digitonin during the extraction procedure, usually due to an increase in DHEA. Relatively low levels are often

associated with benign tumors. If hyperplasia develops before puberty, the elevation of 17-ketosteroids is greater than if hyperplasia develops after puberty. The Cushing's syndrome or adrenogenital syndrome may show adenoma, hyperplasia or rarely, carcinoma as the pathologic lesion responsible for functional derangement. Hyperplasia is the most frequent cause of the adrenogenital syndrome. In Addison's disease; the most profound depression of 17-ketosteroid levels is observed, as they are also in panhypopituitarism.

## Historical

### Standard Experimental Methods

Some analytical steps are common to most steroid assays. Some steroids are excreted largely as sulfate or glucuronide conjugates, thus require conversion to the free, reactive forms by either acid or enzyme catalyzed hydrolysis. The latter approach, using enzymes such as sulfatase and glucuronidase(derived from various animal sources), is the preferable one. This is because the acid methods tend to produce interfering substances.

Solvent extraction, another means of partial purification, is only reliable when the optimum conditions are met for low interferences and maximum recovery. Interfering substances are removed by washing solvent extracts with alkali or other reagents, or in some cases by carefully controlled chromatography. Since most steroids are heat-labile, a potential source of loss during assay is the solvent evap-

oration step.

In spectrophotometric assay, the effect of interfering chromogens is minimized; although not completely removed, by such methods as taking absorbance readings at three wavelengths and applying an Allen-type correction. The Allen correction is accomplished by measuring absorbance at the peak wavelength and at two other wavelengths equidistant from the peak. Values for the latter are averaged to obtain a baseline under the peak, which is then subtracted from the peak reading. The value thus obtained is known as a "corrected" absorbance and can be related to the concentration, provided that the background absorbance is linear with wavelength over the region in which readings are made[1]. Interfering chromogens can also be minimized by the extraction of the final chromogens into a suitable solvent[8].

## Porter-Silber Method

C-21 adrenocorticosteroids are usually determined using the Porter-Silber method[9]. The procedure involves the reaction of phenylhydrazine with C-21 adrenocorticoids containing dihydroxyacetone groups at C-19, C-20, and C-21. This determination is a sensitive index of adrenocortical function, since it measures a major portion of corticoid secretion in the adrenal gland. The method, however, does not measure all the 17-hydroxycorticosteroids excreted in certain pathologic states, i.e., pregnanetriol and other 20-hydroxy-compounds, which may be strikingly increased and

comprise a major fraction of the total 17-hydroxycorticosteroids. Also not measured are the C-20 reduced derivatives of cortisol, cortisone, 11-deoxycortisol and abnormal elevations of the following corticoids frequently seen in adrenogenital syndrome and occasionally in Cushing's syndrome, pregnanetriol, 17-hydroxyprogesterone, and 17-hydroxypregnenolone. Although the Porter-Silber method is sensitive, it involves troublesome extraction and purification procedures and is susceptible to interferences by other chromogens and drugs[10,11,12].

## Total 17-Ketogenic Steroids Method

A more convenient and comprehensive method that include a greater assesment of adrenocorticoid secretion was developed as a urinary 17-ketogenic steroid assay[13]. The bismuthate oxidation of the 17-hydroxy group employed in the assay has been helpful, but reproducible results have been difficult to obtain[14]. Many of these difficulties may be attributed directly to steps in the procedures which cannot be adequately controlled. Consequently, undesirable reaction products may be formed.

Introduction of sodium metaperiodate oxidation in place of bismuthate seemed to solve many of these problem[15]. Periodate oxidation has been shown not to oxidize 11$\beta$-hydroxy group except in acid solution, and in the absence of 11-ketoetiocholanolone[16]. Once oxidized and extracted, the final product of either oxidation is treated to a color

reaction in the same way as 17-ketosteroids of androgenic origin.

The 17-ketogenic steroid procedure is suited to analysis of multiple samples without requiring specialized equipments and appears to be a distinct advantage for clinical diagnosis[13].

## Zimmermann Method

The colorimetric method most widely used for the determination of the 17-ketosteroids is modifications of the basic reaction discovered by von Bitto[17], and adapted to steroid ketones by Zimmermann[18]. The reaction depends upon the development of a red-purple color with an absorption maximum at 520 nm when 17-ketosteroids are reacted with m-dinitrobenzene in alkali. Extensive extraction and purification procedures, and interference by other chromogens[19,20] make this method very inconvenient as a routine assay for adrenal and gonadal function.

## Fractionation Methods

The estimation of total neutral 17-ketosteroids now serves as a screening test for the diagnosis of adrenal or gonadal disease. To derive detailed information, of course, the determination of individual components of this group of steroids would be necessary. For example, when the 17-ketosteroids are measured as a group, no distinction is made between the metabolites derived mainly from the testes and

those derived primarily from the adrenals[21]. It is known that androsterone and etiocholanolone are primary metabolic products of testosterone. The increased excretion of these two compounds in a male, without proportionate changes of DHEA and 11-oxygenated-17-ketosteroids, will yield a positive indication of testicular dysfunction. When only total 17-ketosteroids are estimated, such specific changes will go unobserved. Similarly, in both adrenocortical hyperplasia and carcinoma, the excretion value of 17-ketosteroids is quite high. The fractionated estimation shows that whereas in hyperplasia all components of 17-ketosteroids are elevated, in carcinoma the increased excretion value is mainly the result of the presence of excessive DHEA.

Fractionation also provides the opportunity to determine the ratio of $C_{19}O_2$(androsterone, etiocholanolone, DHEA) to $C_{19}O_3$(11-oxygenated-17-ketosteroids). The importance of this ratio lies in differentiating the type of adrenogenital syndromes, each of which has a characteristic value. Furthermore, changes in the pattern of excreted steroid groups may occur while the total group value remains constant. Thus administration of metyrapone, a steroid 11 $\beta$-hydroxylase inhibitor, leads to a radical alteration in the pattern of excreted steroids which is not always accompanied by a change in the level of urinary 17-ketogenic steroids. Clearly further fractionation, in addition to providing estimates of the urinary 17-ketosteroids and 17-ketogenic steroid levels, would largely eliminate such uncertainties[22].

The first method for the fractionation of 17-ketosteroids was published by Dingemanse and her collegues[23,24]. It was improved by Kellic and Wade[25]. In the revised method, the individual 17-ketosteroids were separated from each other by chromatography on alumina. These time-consuming methods were succeeded by the development of methods for the gas-liquid chromatographic analysis of urinary steroids which include reports by Sparagana, et al[26], Kirschner and Hipsett[27,28], Wotiz[29], Creech[30], Kinoshita and Isurugi[31], Raman, et al[32], and Rivera, et al[33].

The majority of these GLC methods for 17-ketosteroid determination follow the similar procedures after extraction: 1) partial purification of the extract, i.e., washing with sodium hydroxide and water, 2) conversion of steroids into their trimethylsilyl derivatives, and 3) the use of highly polar column such as XE-60 and neopentyl glycol succinate[34]. It also has been found that the comparison between the colorimetric total group assays for urinary 17-ketosteroids and 17-ketogenic steroids , and the estimation of individual urinary steroids using GLC are very consistent[35].

The advantages of the GLC method over colorimetric assays of 17-ketosteroids are that GLC: 1) permits in a single trial the quantitation of three groups of urinary steroids of diagnostic importance, 2) allows a greater differentiation between normal and abnormal states than is obtained with the recommended colorimetric methods, 3) is accurate

and precise for low titre urines, 4) minimizes interference from drugs and their metabolites, and 5) assists in the interpretation of the results of a metyrapone test[36].

# CHAPTER II

## STATEMENT OF THE PROBLEM

The present methods employed at Youngstown Hospital Association for urinary 17-ketosteroids and corticosteroids are long, involved and use extensive extraction procedures. Furthermore they measure only total 17-ketosteroids and corticosteroids and are easily interfered with artifacts and drugs[37]. Although the precision and accuracy are good, the difficulty of the procedures is such that new methods would be more convenient and perhaps more comprehensive. Gas-liquid chromatography is convenient and a plausible candidate for routine clinical use.

There are already a great many methods for GLC of urinary 17-ketosteroids and 17-ketogenic steroids. Most of them, however, are very complex, and require large volumes of samples and reagents and involve many isolation procedures. The availability of a rapid, reliable, and economic assay for steroid metabolites would greatly simplify the clinical evaluation of endocrine dysfunction.

This study was undertaken to find, such efficient methods for the assay of clinically significant steroids in urine, and to develop their methodology in routine clinical work.

# CHAPTER III

# EXPERIMENTAL

## Materials

### Steroids

Pregnanediol(5β-pregnane-3α, 20α-diol), pregnanetriol(5β-pregnane-3α, 17α, 20α-triol), androsterone (3α-hydroxy-5β-androstan-17-one), etiocholanolone(3α-hydroxy-5α-androstan-17-one), dehydroepiandrosterone(3β-hydroxy-5-androsten-17-one), 11-oxoetiocholanolone(3α-hydroxy-5β-androstane-11, 17-dione), 11-oxoandrosterone(3α-hydroxy-5α-androstane-11, 17-dione), 11β-hydroxy-androsterone(3α, 11β-dihydroxy-5α-androstan-17-one) and 11β-hydroxy-etiocholanolone(3α, 11β-dihydroxy-5β-androstan-17-one) were obtained from Sigma(Sigma Chemical Co., St. Louis, MO 63178). Stock solutions(final concentration 1 g/L) were prepared by dissolving steroids in methanol. The solutions are stable for 6 months when kept at 4°C.

Dehydroepiandrosterone glucuronide(3β-hydroxy-5-androsten-17-one glucuronide) and dehydroepiandrosterone sulfate(3β-hydroxy-5-androsten-17-one sulfate) were obtained from Sigma.

As an internal standard, progesterone was purchased from Mann(Mann Research Laboratories, Inc., New York, N.Y. 10006). Stock solutions(final concentration 1 g/L) were

prepared by dissolving progesterone in methanol.

## Chemicals

Propane-1, 2-diol, methylene chloride, ethyl acetate, n-heptane, benzene, chloroform, ethanol, methanol and acetic acid were A.R. grade. Ethyl acetate and methanol were distilled to eliminate impurities before use. Disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, sodium carbonate, sodium hydroxide, sodium chloride, sodium borohydride, sodium metaperiodate, sulfuric acid were also A.R. grade. Bacterial $\beta$ -glucuronidase, type I & II from E. coli., was obtained from Sigma.

The following antibiotics were used: Streptomycin sulfate, 1.25 g is dissolved in 1.3 mL distilled sterile water (final concentration 500 g/L streptomycin). Nebacitin; an amount corresponding to 50 mg neomycin sulfate and 2500 U Bacitracin, is dissolved in 1.0 mL of 9.0 g/L sodium chloride solution. Penicillin G, $10^6$ U is dissolved in 2.5 mL distilled sterile water. All antibiotics were purchased from Sigma.

Phosphate buffer(0.45 mol/L; pH 7.0) was prepared by dissolving 61.24 g potassium dihydrogen orthophosphate in 1000 mL distilled water and 80.12 g disodium hydrogen orthophosphate in 1000 mL distilled water. 41.30 mL of the potassium salt solution was mixed with 58.70 mL of the sodium salt solution to give 100 mL of the buffer solution. Phosphate buffer(0.075 mol/L; pH 7.0) was prepared by diluting

1 mL of the 0.45 mol/L phosphate buffer with 5 mL distilled water. All buffer solutions were stored at 10°C and were kept for not longer than one month.

## Apparatus

### Column Chromatography

Amberlite XAD-2, coarse 0.5-1.0 mm diameter(Rohn & Haas, Philadelphia, PA 19105) was first washed with water( five times with the threefold volume of the resin) to remove the fines and then washed with methanol(twice with the two-fold volume of the resin) to remove impurities. The purified resin was kept under water at room temperature before use. The column(20 x 1.0 cm internal diameter) were fitted at the bottom with a sintered glass filter and a capillary outlet. At filling time, the capillary exit was closed by means of a metal plug and the aqueous suspension of Amberlite(5 g) is poured slowly into the column. The resin was allowed to settle under gravity. The upper end of the filled column was covered with glass wool and glass beads (3 mm diameter). After use, the resin was discarded.

### Gas-Liquid Chromatography

The gas-liquid chromatograph used was a Toxichrome with a flame ionization detector(Bendix Corporation, Ronceverte, W. VA 24970). It contained a siliconized glass column (1.0 m x 2.4 mm internal diameter), packed with 3% OV-7 on Chromosorb WHP 100-120 mesh(Supelco Inc., Bellefonte,

PA 16823). Hewlett-Packard digital integrator, model 3380A, with total-area board, and printer was used to collect chromatograms and perform calculations(Hewlett-Packard, Avondale, PA 19406). In automatic operation the 3380A integrator operates according to the preset programs, enabling the detection and coded event-marking of the beginning, apex, and end of a peak as well as the rejection of noise and correction of baseline. The digital area and retention time print-out are to four significant figures. The integrator's performances in peak area determination are: precision 0.05%, linearity 0.1%, and dynamic range $1 \times 10^6$ to 1. Results are expressed in concentration(mg/mL).

## Accessories

Centrifuge(Ivan Sorvall, Inc., Newton, Connecticut 06470), Metrion IV pH meter(Coleman, Maywood, Illinois 60153), rotavapor(Büchi, Zürich, Switzerland), and Dubnoff waterbath(Precision Scientific, Chicago, Illinois 60648).

## Assay

### 17-Ketosteroids and Pregnanediol

The method of Külpmann[38] was followed with some modifications. These modifications will be subsequently discussed in Chapter V.

After urine has been collected over a period of 24 h, its volume is measured and then made up with water to 1200 mL or, if more has been excreted, to a larger multiple of

100 mL. The dilute urine can be kept indefinitely at 4°C without any detectable change in its concentrations of 17-ketosteroids and pregnanediol[38].

10 mL of diluted urine is used for the assay. The pH is adjusted to 7.0 with 2 mol/L sodium hydroxide and 0.45 mol/L phosphate buffer. Preliminary chromatographic separation is carried out according to Bradlow[39] as follows: the neutralized urine is poured through the XAD-2, followed by 40 mL distilled water and then 20 mL methanol. The methanol fraction; containing the conjugates of 17-ketosteroids and pregnanediol, is collected. 2 drops of propanediol are added and the methanol and traces of water are evaporated under vacumn. The residue remains dissolved in propanediol. 5 mL n-heptane is added to the propanediol solution. The steroid conjugates are then extracted once with 3.0 mL of distilled water, following twice with 1.0 mL of distilled water extractions.

To the combined aqueous extracts, the following additions are made: 1.0 mL of 0.45 mol/L phosphate buffer, 2500 U β-glucuronidase dissolved in 1.0 mL of 0.075 mol/L phosphate buffer, 2.5 μL of the penicillin G solution, 10 μL of the Nebacitin solution, 7.0 μL of the streptomycin solution and 2 drops of chloroform. The mixture is incubated for 24 h at 37°C in a waterbath. The free steroids are extracted with 10.0 mL ethyl acetate. The extract is transferred to a 50 mL round-bottom flask and the organic phase evaporated under vacumn(Fraction $E_1$).

Solvolysis is carried out according to Burstein and Lieberman[40]. The remaining aqueous phase is adjusted to pH 1.0 by the addition of 1.0 mol/L sulfuric acid. 1.4 g sodium chloride is added and dissolved by shaking. The mixture is then extracted three times with 5.0 mL ethyl acetate. The extracts are combined by transferring them separately to the round-bottom flask containing Fraction $E_1$. This solution is incubated for 24 h at 37°C in a waterbath. At the end of the solvolysis, the ethyl acetate phase is washed with 1.0 mL sodium carbonate solution. The aqueous solutions are discarded, and the organic phase is evaporated under vacumn.

The residue is dissolved in 2.0 mL methanol and transferred to 15 mL conical tube and the organic phase evaporated to dryness under a slow-jet of nitrogen at 50°C. The residue is then taken up in 100 μL methanol containing 100 μg progesterone as internal standard. 3 μL of the sample is injected into the column. The following settings were used: column temperature, 225°C; inlet temperature, 250°C; and detector temperature, 275°C. Purged nitrogen was used as a carrier gas at a flow rate of 35 mL/min. The flows of hydrogen(45 mL/min) and air(225 mL/min) were adjusted for maximal sensitivity.

Retention times of the emerging peaks are calculated relative to the internal reference peak. The amount of individual steroid is estimated from the known relationships between area and mass for each compound and also by taking

into account any fluctuation in the area of the reference steroid injected with each specimen. A standard mixture of steroids is injected prior to, and after, every six samples, and at the end of a day's run of routine specimens.

## 17-Ketogenic Steroids

The method of Trafford and Makin[22] was followed with few modifications. These modifications will be discussed in Chapter V.

10 mL urine is used for the assay. 2.0 mL 10% sodium borohydride in 0.01 mol/L sodium hydroxide is added and the mixture is incubated at 50°C for 15 minutes. Excess borohydride is destroyed by the addition of 0.5 mL glacial acetic acid and further incubation at 50°C for 15 minutes. The pH of the solution is carefully adjusted to 6.5 using a pH meter and 8.0 mL of 10% sodium metaperiodate are added and the urine is incubated at 50°C for 20 minutes. The urine is then cooled under running tap water for 5 minutes. Steroids are then extracted with 15.0 mL methylene chloride. The extracts are washed with 1.0 mL 2 mol/L sodium hydroxide and evaporated to dryness under a slow-jet of nitrogen at 50°C. The residue is then taken up in 100 μL methanol containing 50 μg progesterone as internal standard. 5 μL of the sample is injected into the column. The following settings were used: column temperature, 215°C; inlet temperature, 250°C; and detector temperature, 275°C. Purged nitrogen was used as a carrier gas at a flow rate of 30 mL/min.

The flows of hydrogen(35 mL/min) and air(175 mL/min) were used.

Calculations and the use of a mixture of pure steroids are done in the same fashion as that for 17-ketosteroids.

## Calculation

$$\text{Amount of Y} = \frac{\text{Area}_Y \times \text{Response}_Y}{\text{Area}_{IS} \times \text{Response}_{IS}} \times (\text{IS AMT}) \times (\text{XF})$$

Response = AMT/AREA in calibration

IS AMT = The amount of internal standard added to the samples.

XF = The multiplying factor that applies to every reported results and can serve as a dilution factor.

During the integration, the 3380 A has automatic tangent correction, up/down drifting baseline correction and a self-adjusting baseline reset delay. A slope value which is set in manually or selected automatically is applied to both the up and down slopes. Eight consecutive(filtered) data points, all increasing above the slope setting relative to the current baseline, are required before start integration is established. Data is sampled every 0.2 seconds.

If a new peak begins before the last peak ends, it will be recognized as a peak when the eight data points exceeding the slope sensitivity criteria is met.

# CHAPTER IV

# RESULTS

## 17-Ketosteroids and Pregnanediol

Figure 7 shows a typical chromatogram of the standard mixture of etiocholanolone, dehydroepiandrosterone, androsterone, pregnanediol, 11-ketoandrosterone, 11-ketoetiocholanolone, 11 β-hydroxy-androsterone, 11 β-hydroxy-etiocholanolone, and progesterone. The position of each steroid was established through the analysis of a pure individual compound. Since they have relative retention times, androsterone and dehydroepiandrosterone; as well as pregnanediol and 11-ketoandrosterone, emerge from the column as single peaks. The total time elapsed for a GLC run is about 20 minutes. Figures 8.1 and 8.2 show a reproduction of a typical chromatogram and a printout from the integrator for calibration purposes. Figure 9 shows a reproduction of a typical determination.

Figures 10-12 show the linearity of the peak-area responses with increasing concentrations of 17-ketosteroids, pregnanediol and progesterone. It can be seen that the sensitivity of the flame ionization detector permits the detection of steroids in the microgram range.

Recovery of pure standards taken through the extraction procedure and estimated by GLC is shown in Table 1. In

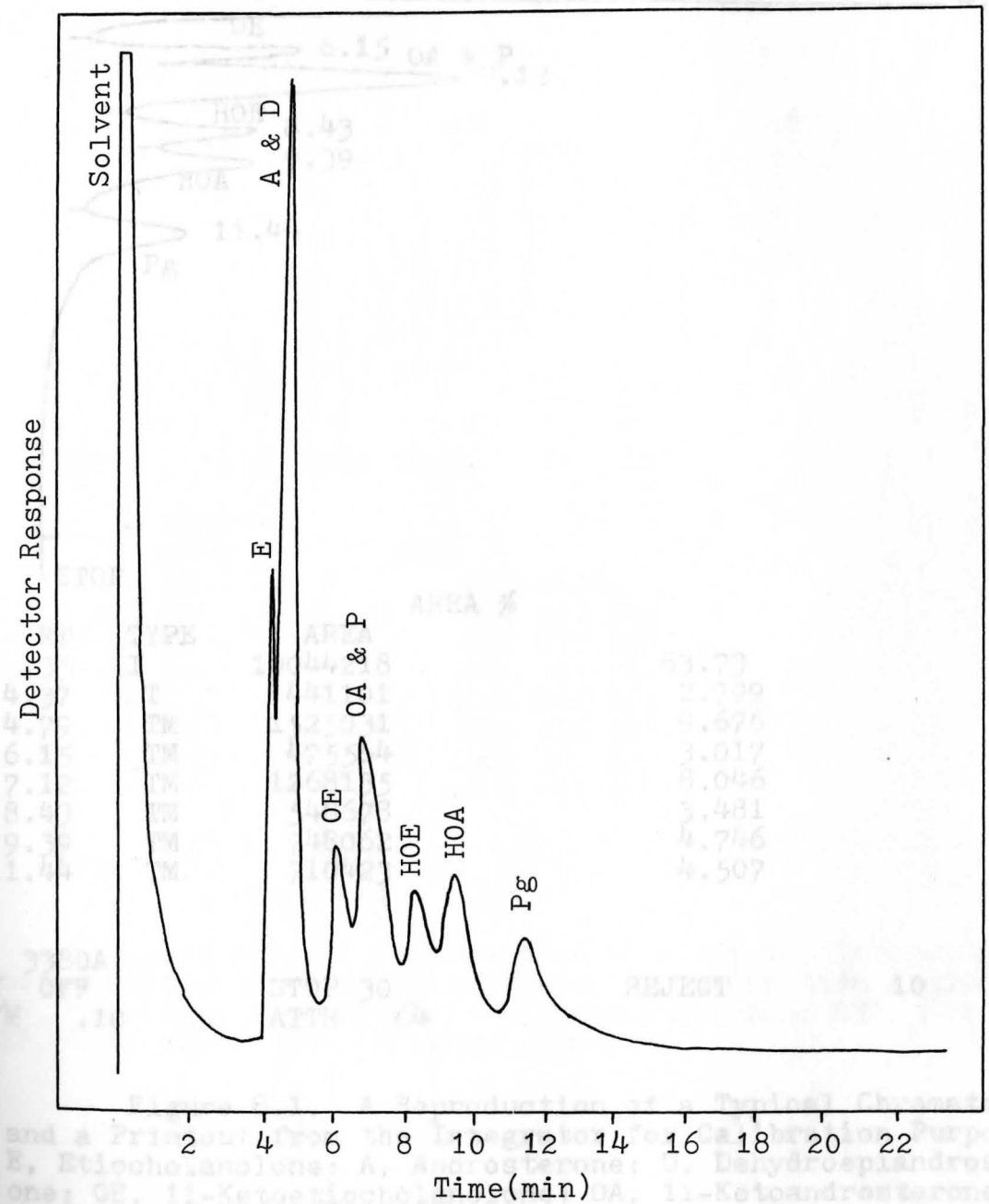
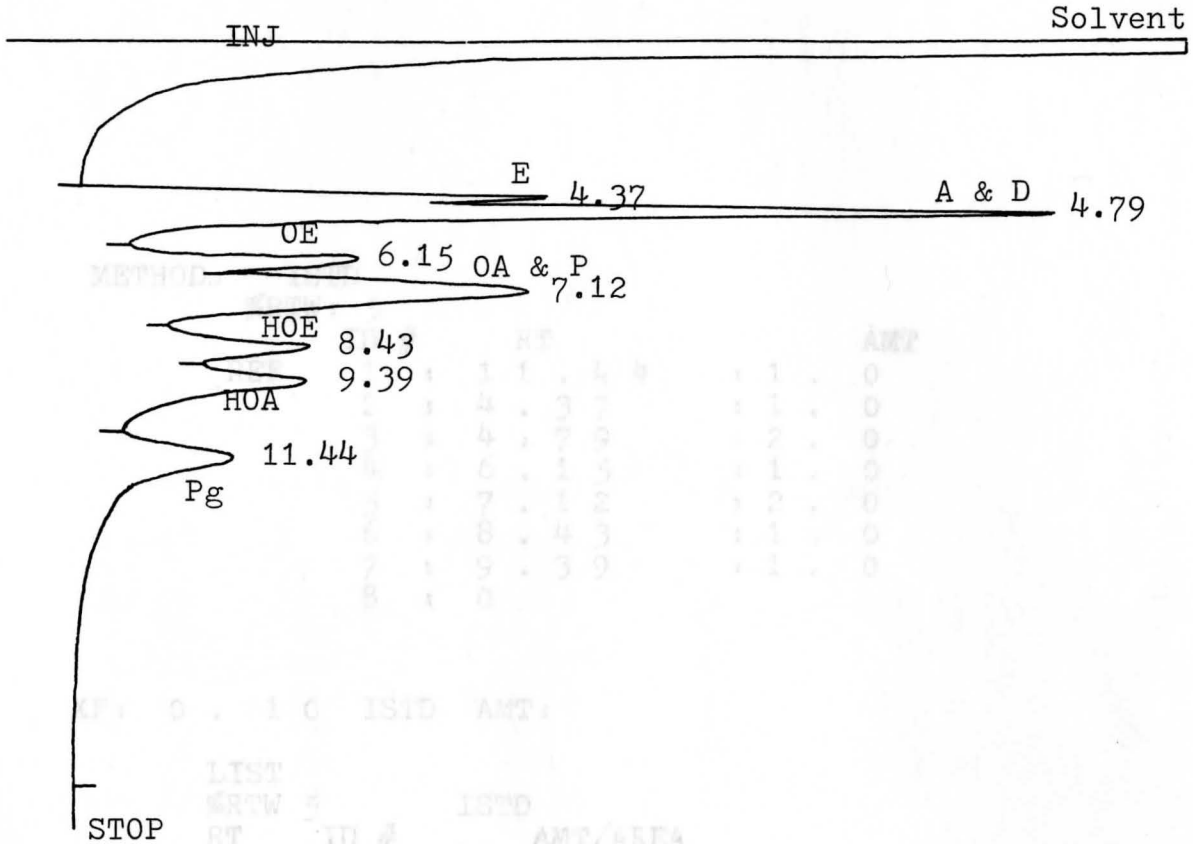



Figure 7. Gas Chromatogram of a Standard Mixture of Steroids. E, Etiocholanolone; A, Androsterone; D, Dehydroepiandrosterone; OE, 11-Ketoetiocholanolone; OA, 11-Ketoandrosterone; P, Pregnanediol; HOE, 11$\beta$ -Hydroxy-Etiocholanolone; HOA, 11$\beta$ -Hydroxy-Androsterone; Pg, Progesterone.




| RT | TYPE | AREA | AREA % |
|---|---|---|---|
| .35 | I | 10044218 | 63.73 |
| 4.37 | T | 441101 | 2.799 |
| 4.79 | TM | 1525031 | 9.676 |
| 6.15 | TM | 475564 | 3.017 |
| 7.12 | TM | 1268135 | 8.046 |
| 8.43 | TM | 548678 | 3.481 |
| 9.39 | TM | 748062 | 4.746 |
| 11.44 | TM | 710423 | 4.507 |

HP 3380A
DLY OFF STOP 30 REJECT 10
MV/M .10 ATTN 64

Figure 8.1. A Reproduction of a Typical Chromatogram and a Printout from the Integrator for Calibration Purposes. E, Etiocholanolone; A, Androsterone; D, Dehydroepiandrosterone; OE, 11-Ketoetiocholanolone; OA, 11-Ketoandrosterone; P, Pregnanediol; HOE, 11$\beta$-Hydroxy-Etiocholanolone; HOA, 11$\beta$-Hydroxy-Androsterone; Pg, Progesterone. INJ, Injection; RT, Retention Time; I, Incomplete; T, Tangent; M, Merge; DLY, Delay; ATTN, Attenuation; MV/M, Millivolt per Minute.

```
METHOD:   ISTD
        %RTW: 5
              ID #      RT                AMT
        REF    1   :  1 1 . 4 4    : 1 .  0
               2   :  4 . 3 7      : 1 .  0
               3   :  4 . 7 9      : 2 .  0
               4   :  6 . 1 5      : 1 .  0
               5   :  7 . 1 2      : 2 .  0
               6   :  8 . 4 3      : 1 .  0
               7   :  9 . 3 9      : 1 .  0
               8   :  0

XF:  0 .  1 0  ISTD  AMT:

      LIST
      %RTW 5          ISTD
      RT     ID #       AMT/AREA
   11. 44      1        .000 001 408
    4. 37      2        .000 002 267
    4. 79      3        .000 001 311
    6. 15      4        .000 002 103
    7. 12      5        .000 001 577
    8. 43      6        .000 001 823
    9. 39      7        .000 001 337

 XF     .  1  ISTD  AMT  1.
```

Figure 8.2. A reproduction of a Typical Chromatogram and a Printout from the Integrator for Calibration Purposes. RTW, Retention Time Window; RT, Retention Time; AMT, Amount; REF, Reference; ISTD, Internal Standard.

| RT | TYPE | AREA | ISTD ID # | AMT |
|---|---|---|---|---|
| . 27 | I | 6982234 | | . |
| 2. 27 | T | 1259 | | . |
| 4. 33 | T | 60411 | 2 | .146 3 |
| 4. 74 | TM | 171555 | 3 | .240 2 |
| 6. 14 | TM | 53331 | 4 | .119 8 |
| 7. 05 | TM | 139945 | 5 | .235 7 |
| 8. 42 | TM | 58094 | 6 | .113 1 |
| 9. 40 | TM | 69473 | 7 | .099 21 |
| 11. 57 | TM | 66502 | 1 | . |
| | | | TOTAL | .954 31 |

XF . 1 ISTD AMT 1.

HP 3380A
DLY OFF STOP 30 REJECT 10
MV/M . 10 ATTN 64

Figure 9. A Reproduction of a Typical Determination. T, Tangent; M, Merge; RT, Retention Time; AMT, Amount; I, Incomplete; ATTN, Attenuation.

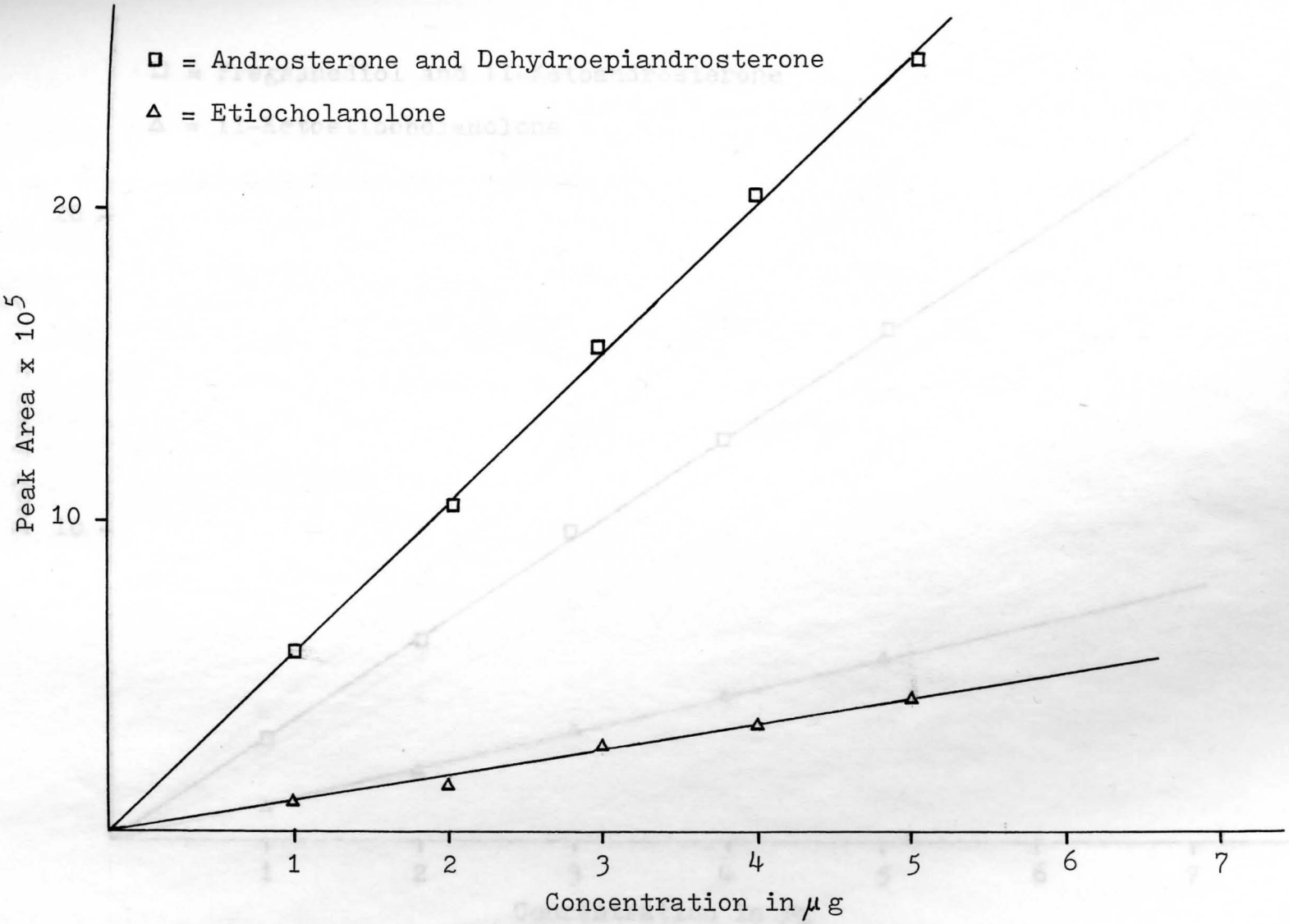



Figure 10. Linearity Study of Steroid with Increasing Concentration.

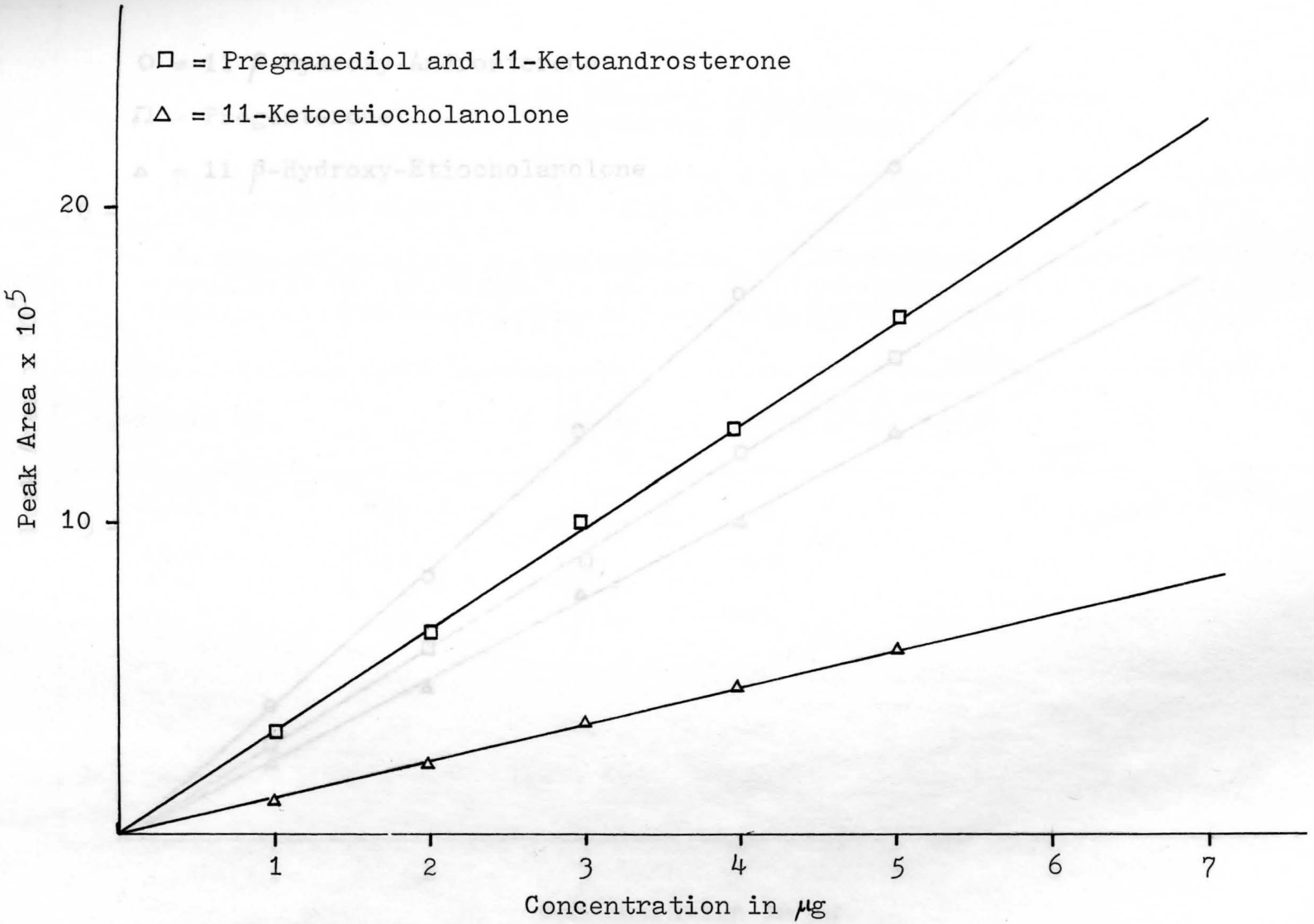



Figure 11. Linearity Study of Steroid with Increasing Concentration.

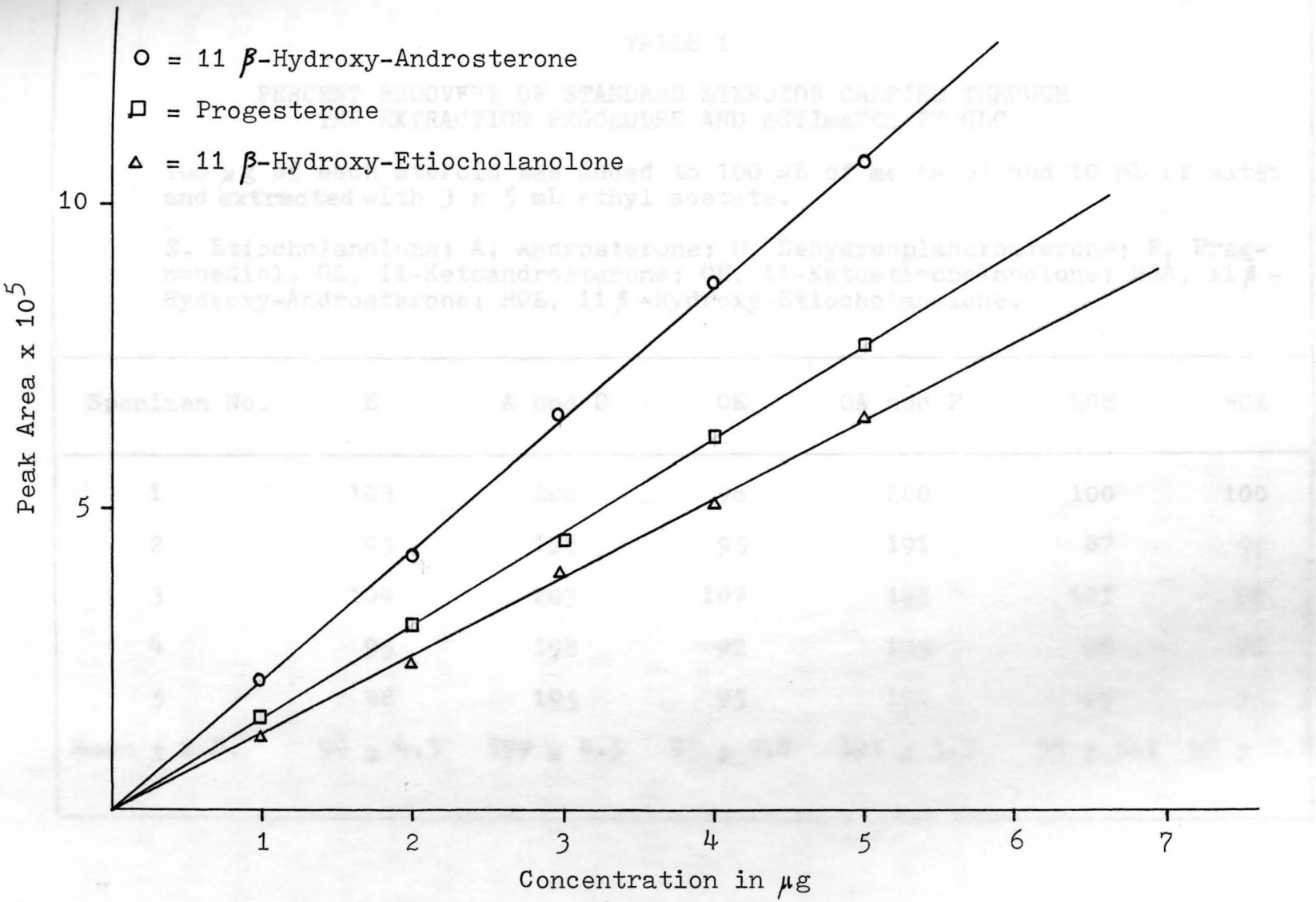



Figure 12. Linearity Study of Steroid with Increasing Concentration.

TABLE 1

PERCENT RECOVERY OF STANDARD STEROIDS CARRIED THROUGH THE EXTRACTION PROCEDURE AND ESTIMATED BY GLC

100 μg of each steroid was added to 100 μL of methanol and 10 mL of water and extracted with 3 x 5 mL ethyl acetate.

E, Etiocholanolone; A, Androsterone; D, Dehydroepiandrosterone; P, Pregnanediol; OA, 11-Ketoandrosterone; OE, 11-Ketoetiocholanolone; HOA, 11β-Hydroxy-Androsterone; HOE, 11β-Hydroxy-Etiocholanolone.

| Specimen No. | E | A and D | OE | OA and P | HOE | HOA |
|---|---|---|---|---|---|---|
| 1 | 103 | 200 | 98 | 200 | 100 | 100 |
| 2 | 95 | 198 | 95 | 191 | 87 | 95 |
| 3 | 104 | 207 | 107 | 196 | 101 | 98 |
| 4 | 95 | 198 | 92 | 195 | 96 | 98 |
| 5 | 98 | 195 | 95 | 194 | 95 | 93 |
| Mean ± S.D. | 99 ± 4.3 | 199 ± 4.5 | 97 ± 5.8 | 195 ± 3.3 | 96 ± 5.5 | 97 ± 2.7 |

each instance the average recovery was greater than 95%.

In addition to the urine specimens, the hydrolytic procedure was applied to 100 $\mu$g samples of dehydroepiandrosterone sodium sulfate and dehydroepiandrosterone glucuronide. Recoveries for the equivalent free steroids from its sulfate or glucuronide conjugate are 90% or better. Figure 13 shows the chromatogram of a typical hydrolysis of the conjugated standard steroids.

Reproducibility of the method was tested by fifteen analyses in duplicate of 10.0 mL of pooled urine subjected to the procedure on different days during the period of investigation. The results of the steroid recoveries from the pooled urine are summarized in Table 2.

Precision of the method under the condition of routine use was determined according to Snedecor[41]. Random specimens as received from Youngstown Hospital Association were analyzed in duplicate and a difference(d) between the two values was obtained for each specimen. An estimate of the standard deviation(S.D.) of the procedure in daily use was calculated using the formula $\sqrt{\sum d^2/2n}$, where n = number of duplicate assays. The S.D. of these differences and the mean concentration of each steroid were, etiocholanolone (1.80 mg, n = 15) ± 0.8; androsterone and dehydroepiandrosterone(4.0 mg, n = 15) ± 0.5; 11-ketoetiocholanolone(0.02 mg, n = 15) ± 0.006; 11-ketoandrosterone and pregnanediol(0.5 mg, n = 15) ± 0.2; 11-hydroxy-etiocholanolone(0.01 mg, n = 15) ± 0.005; 11-hydroxy-androsterone(1.5 mg, n = 15) ± 0.5.

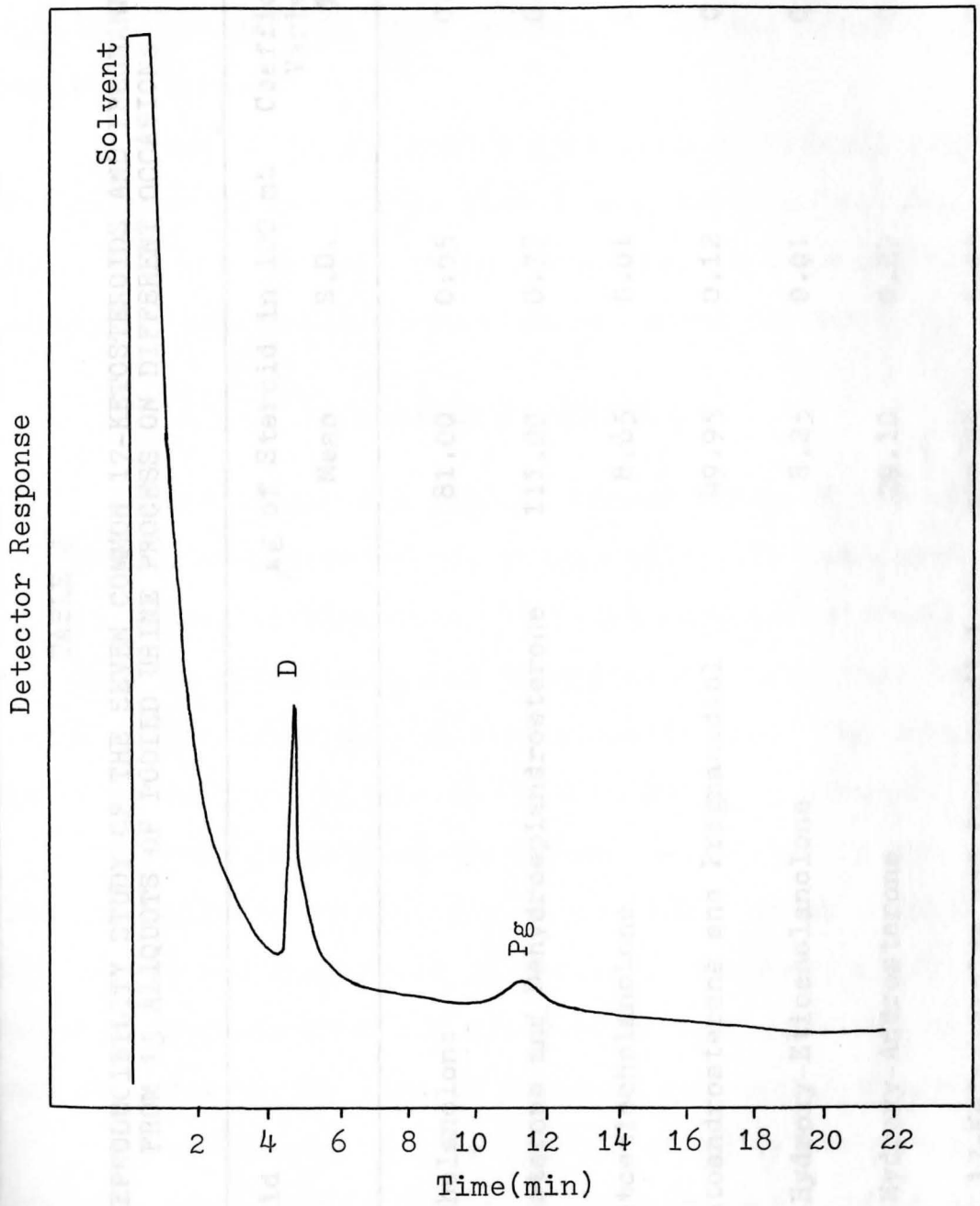



Figure 13. GLC Pattern of Standard Dehydroepiandrosterone Sodium Sulfate and Glucuronide. D, Dehydroepiandrosterone; Pg, Progesterone.

TABLE 2

A REPRODUCIBILITY STUDY OF THE SEVEN COMMON 17-KETOSTEROIDS AND PREGNANEDIOL FROM 15 ALIQUOTS OF POOLED URINE PROCESS ON DIFFERENT OCCASIONS

| Steroid | μg of Steroid in 100 mL | | Coefficient of Variation |
|---|---|---|---|
| | Mean | S.D. | % |
| Etiocholanolone | 81.00 | 0.65 | 0.8 |
| Androsterone and Dehydroepiandrosterone | 111.00 | 0.77 | 0.7 |
| 11-Ketoetiocholanolone | 8.65 | 0.01 | 0.1 |
| 11-Ketoandrosterone and Pregnanediol | 49.95 | 0.12 | 0.2 |
| 11 β -Hydroxy-Etiocholanolone | 8.25 | 0.01 | 0.1 |
| 11 β -Hydroxy-Androsterone | 29.10 | 0.27 | 0.9 |
| Total 17-Ketosteroids and Pregnanediol | 287.95 | 1.23 | 0.4 |

Figures 14-16 and Table 3 are representative chromatograms and values of the steroids obtained with this method. Figures 14 and 15 show the urinary steroid pattern of healthy male and female subjects without obvious adrenal abnormality. The last peak in the chromatogram is the internal standard progesterone.

Figure 16 is the gas chromatogram of urinary steroids of a representative sample from Youngstown Hospital Association. For this patient etiocholanolone, 11-keto and 11 $\beta$ -hydroxy-etiocholanolone are clearly absent in the urine.

## 17-Ketogenic Steroids

Figure 17 shows a typical chromatogram of the standard mixture of androsterone, pregnanediol, 11-ketoandrosterone, 11-ketoetiocholanolone, 11 $\beta$ -hydroxy-androsterone, 11 $\beta$ -hydroxy-etiocholanolone, and progesterone. The last peak to emerge is the internal standard progesterone. The total time elapsed for a single GLC run is about 25 minutes.

Reproducibility of the method was tested by ten analyses in duplicate of a single normal adult urine sample. The results are summarized in Table 4. Recovery study for the free steroids from its glucuronide conjugate has not been done due to the lack of standard conjugated steroids.

Normal values of 17-ketogenic steroids are established by analyzing in duplicate fifteen samples of pooled urine on different days during the course of the experiment. The representative chromatogram and results are depicted in

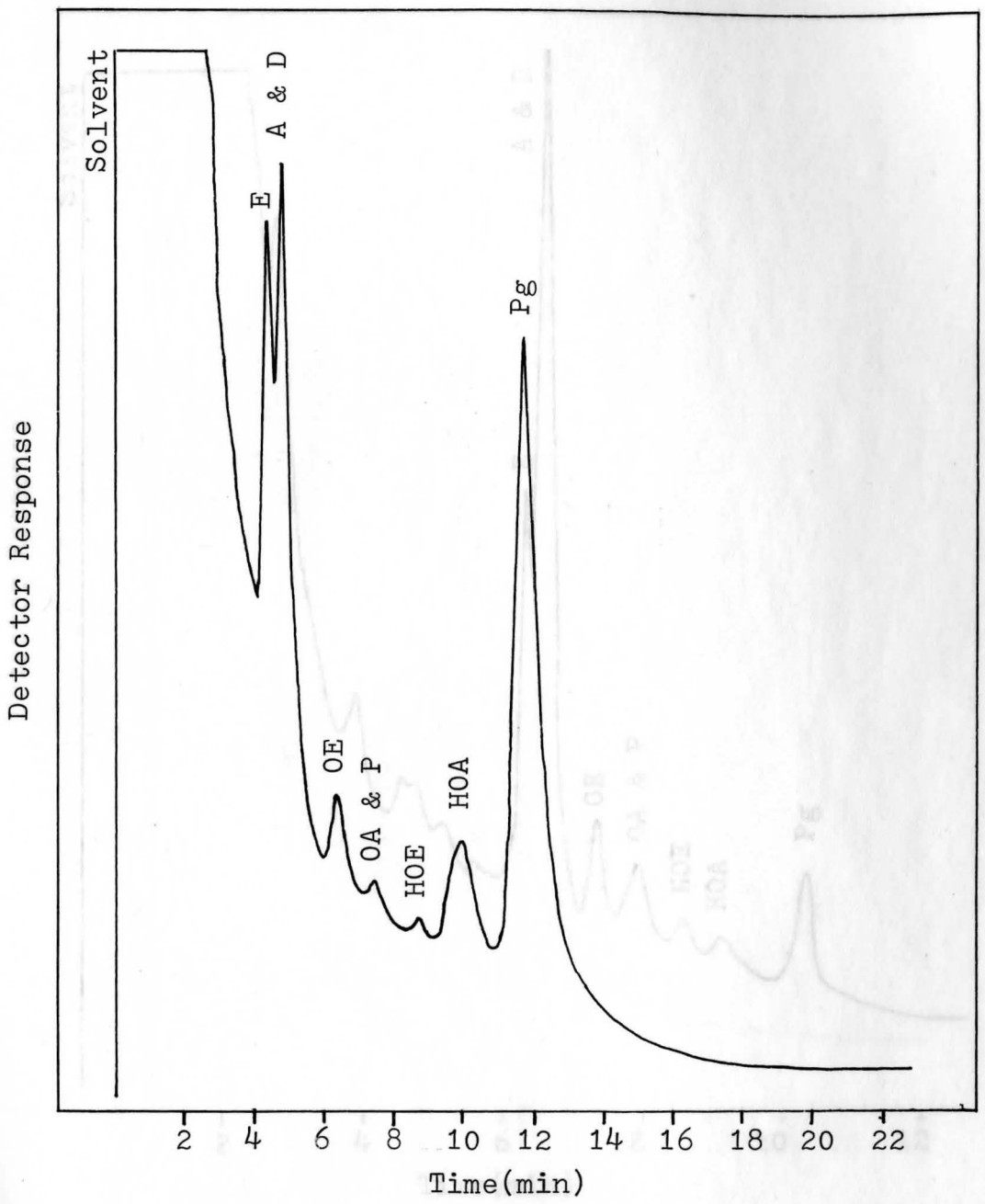



Figure 14. GLC Pattern of Urinary Steroids from a Healthy Male. E, Etiocholanolone; A, Androsterone; D, Dehydroepiandrosterone; OE, 11-Ketoetiocholanolone; OA, 11-Ketoandrosterone; HOE, 11β-Hydroxy-Etiocholanolone; HOA, 11β-Hydroxy-Androsterone; Pg, Progesterone; P, Pregnanediol.

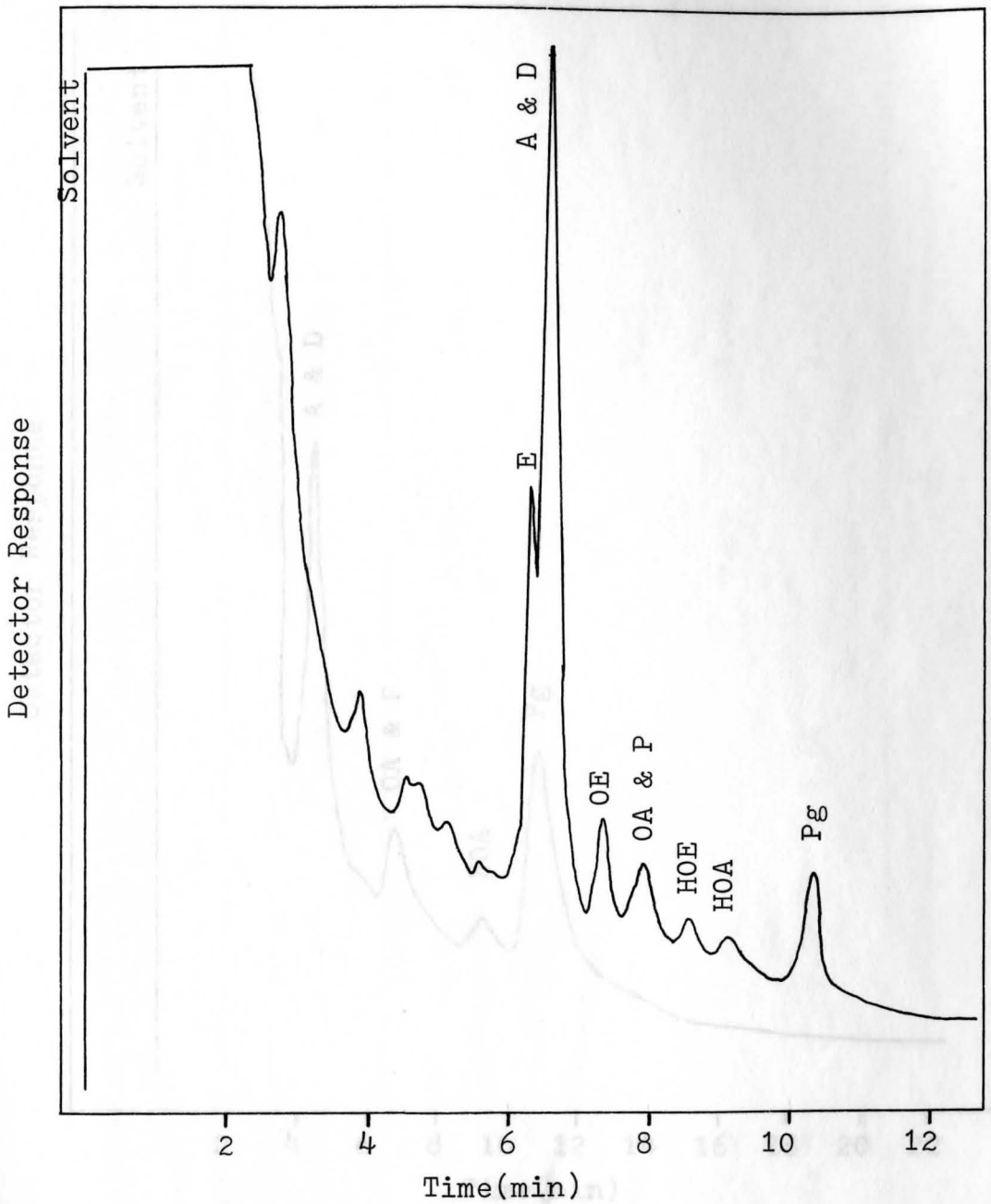



Figure 15. GLC Pattern of Urinary Steroids from a Healthy Female. E, Etiocholanolone; A, Androsterone; D, Dehydroepiandrosterone; OE, 11-Ketoetiocholanolone; OA, 11-Ketoandrosterone; P, Pregnanediol; HOE, 11$\beta$-Hydroxy-Etiocholanolone; HOA, 11$\beta$-Hydroxy-Androsterone; Pg, Progesterone.

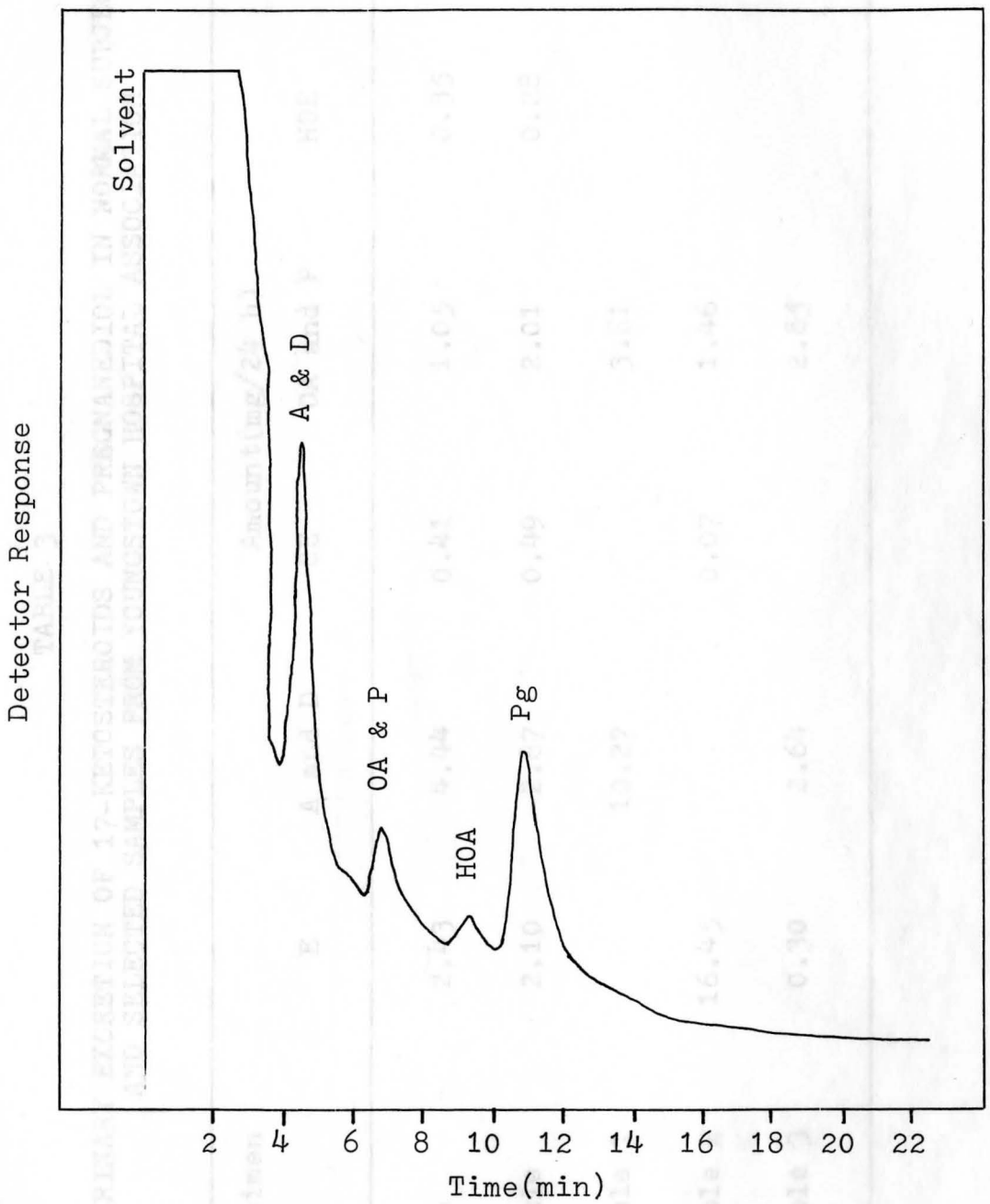



Figure 16. GLC Pattern of Urinary Steroids from a Patient of Youngstown Hospital Association. A, Androsterone; D, Dehydroepiandrosterone; OA, 11-Ketoandrosterone; P, Pregnanediol; HOA, 11 β -Hydroxy-Androsterone; Pg, Progesterone.

TABLE 3

URINARY EXCRETION OF 17-KETOSTEROIDS AND PREGNANEDIOL IN NORMAL SUBJECTS AND SELECTED SAMPLES FROM YOUNGSTOWN HOSPITAL ASSOCIATION

| Specimen | Amount(mg/24 h) | | | | | |
|---|---|---|---|---|---|---|
| | E | A and D | OE | OA and P | HOE | HOA |
| Male | 2.43 | 4.44 | 0.41 | 1.05 | 0.35 | 1.11 |
| Female | 2.10 | 2.87 | 0.49 | 2.01 | 0.28 | 0.65 |
| Sample 1 | | 10.27 | | 3.81 | | 1.94 |
| Sample 2 | 16.45 | | 0.07 | 1.46 | | 1.01 |
| Sample 3 | 0.30 | 2.64 | | 2.85 | | 0.04 |

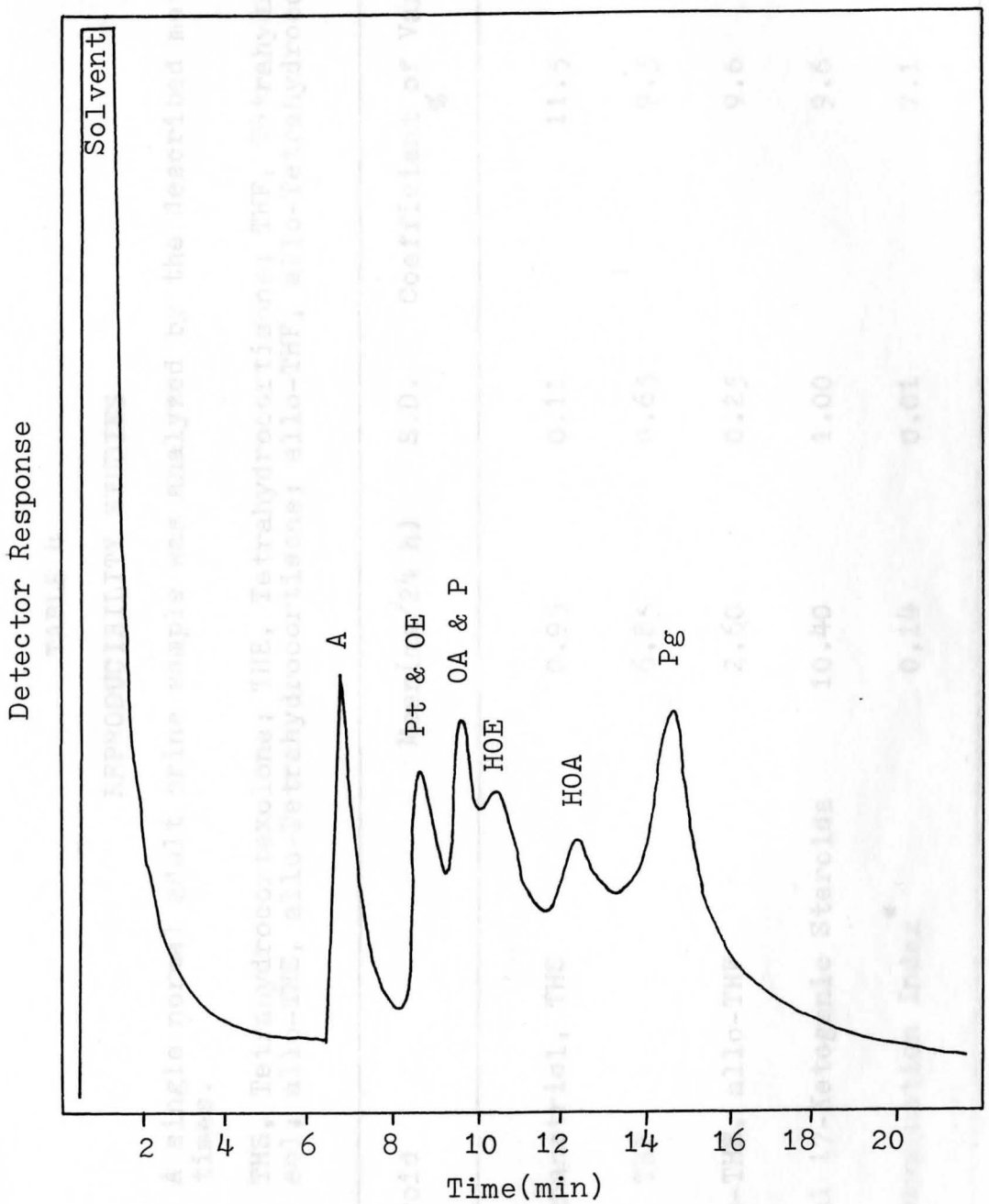



Figure 17. Gas Chromatogram of a Standard Mixture of Steroids. A, Androsterone; Pt, Pregnanetriol; OE, 11-Keto-etiocholanolone; OA, 11-Ketoandrosterone; P, Pregnanediol; HOE, 11 $\beta$ -Hydroxy-Etiocholanolone; HOA, 11 $\beta$ -Hydroxy-Androsterone; Pg, Progesterone.

TABLE 4

REPRODUCIBILITY STUDIES

A single normal adult urine sample was analyzed by the described method 10 times.

THS, Tetrahydrocortexolone; THE, Tetrahydrocortisone; THF, Tetrahydrocortisol; allo-THE, allo-Tetrahydrocortisone; allo-THF, allo-Tetrahydrocortisol.

| Steroid | Mean(mg/24 h) | S.D. | Coefficient of Variation % |
|---|---|---|---|
| Pregnanetriol, THS | 0.95 | 0.11 | 11.5 |
| THE, THF | 6.85 | 0.65 | 9.5 |
| allo-THE, allo-THF | 2.60 | 0.25 | 9.6 |
| Total 17-Ketogenic Steroids | 10.40 | 1.00 | 9.6 |
| 11-Oxygenation Index* | 0.14 | 0.01 | 7.1 |

* Pregnanetriol, THS : THE, THF

Figure 18 and Table 5.

22 samples from Youngstown Hospital Association were run. Figure 19 and Table 6 are representative chromatogram and values of the steroids obtained with this method.

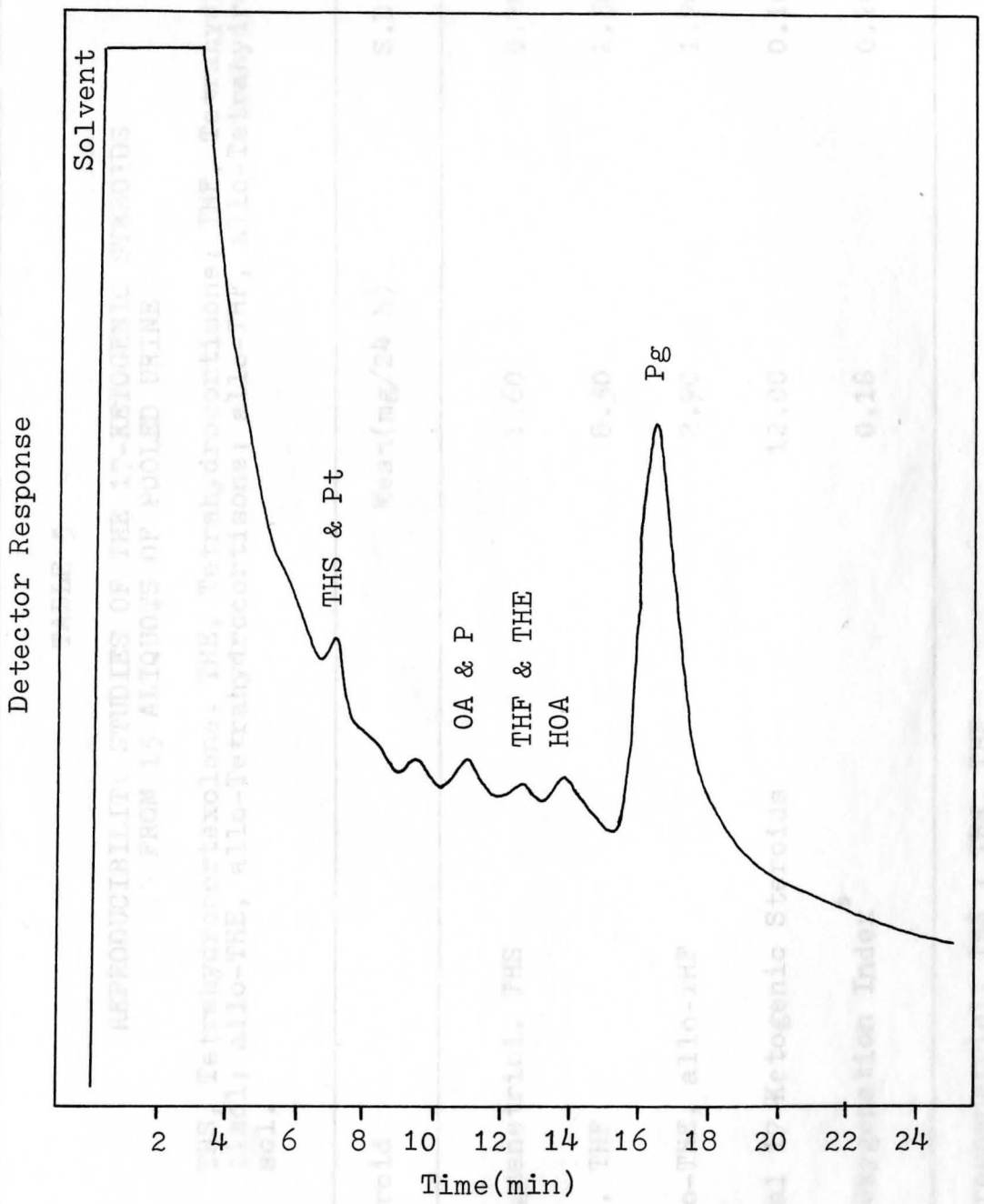



Figure 18. GLC Pattern of Urinary Steroids of Pooled Urine. THS, Tetrahydrocortexolone; Pt, Pregnanetriol; OA, 11-Ketoandrosterone; P, Pregnanediol; THF, Tetrahydrocortisol; THE, Tetrahydrocortisone; HOA, 11 β-Hydroxy-Androsterone; Pg, Progesterone.

TABLE 5

REPRODUCIBILITY STUDIES OF THE 17-KETOGENIC STEROIDS FROM 15 ALIQUOTS OF POOLED URINE

THS, Tetrahydrocortexolone; THE, Tetrahydrocortisone; THF, Tetrahydrocortisol; allo-THE, allo-Tetrahydrocortisone; allo-THF, allo-Tetrahydrocortisol.

| Steroid | Mean(mg/24 h) | S.D. |
|---|---|---|
| Pregnanetriol, THS | 1.60 | 0.70 |
| THE, THF | 8.50 | 2.50 |
| allo-THE, allo-THF | 2.90 | 1.70 |
| Total 17-Ketogenic Steroids | 12.00 | 0.10 |
| 11-Oxygenation Index* | 0.18 | 0.10 |

* Pregnanetriol, THS : THE, THF

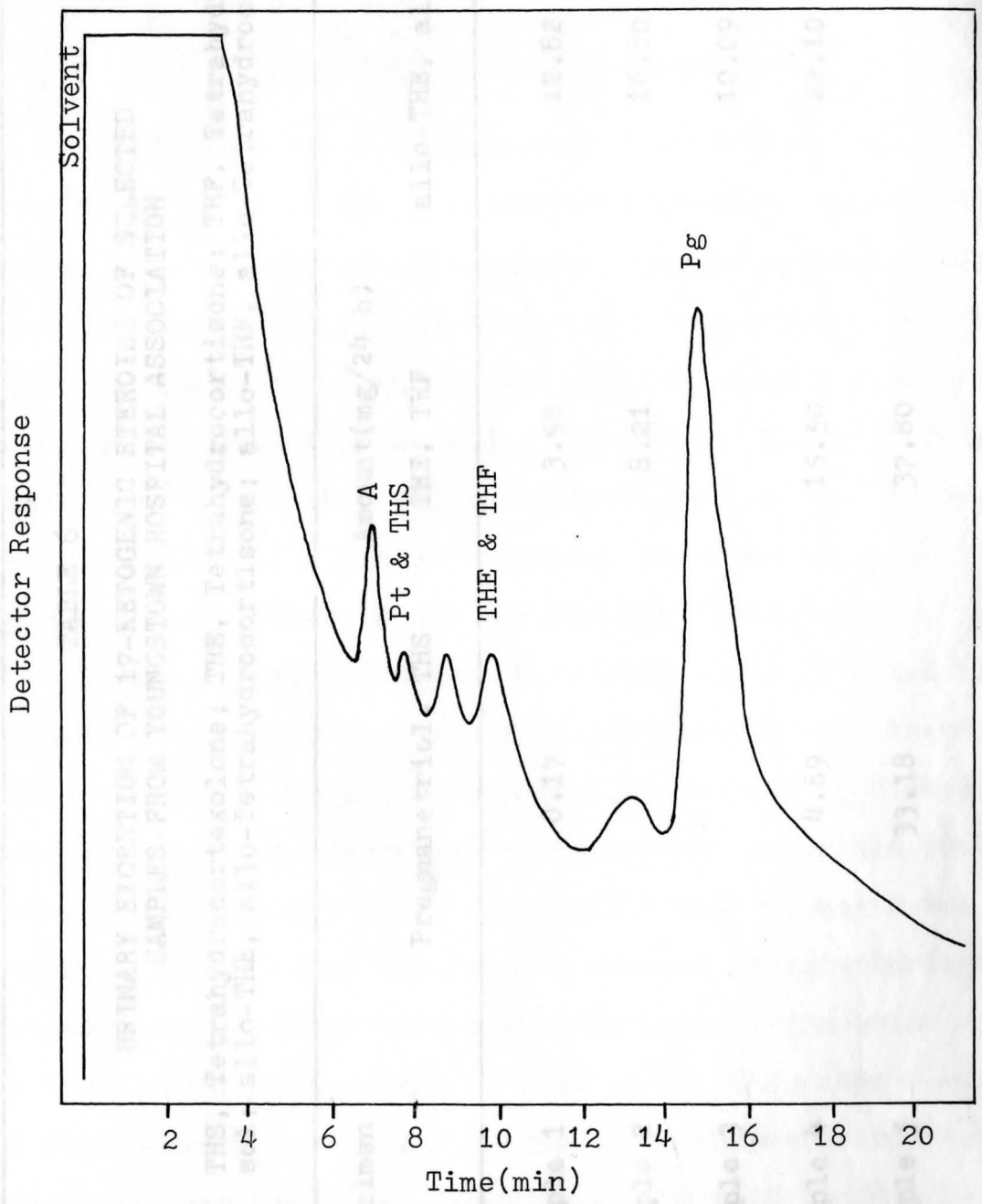



Figure 19. GLC Pattern of Urinary Steroids from a Patient of Youngstown Hospital Association. A, Androsterone; Pt, Pregnanetriol; THS, Tetrahydrocortexolone; THE, Tetrahydrocortisone; THF, Tetrahydrocortisol; Pg, Progesterone.

TABLE 6

URINARY EXCRETION OF 17-KETOGENIC STEROIDS OF SELECTED SAMPLES FROM YOUNGSTOWN HOSPITAL ASSOCIATION

THS, Tetrahydrocortexolone; THE, Tetrahydrocortisone; THF, Tetrahydrocortisol; allo-THE, allo-Tetrahydrocortisone; allo-THF, allo-Tetrahydrocortisol.

| Specimen | Amount(mg/24 h) | | |
|---|---|---|---|
| | Pregnanetriol, THS | THE, THF | allo-THE, allo-THF |
| Sample 1 | 0.17 | 3.98 | 12.82 |
| Sample 2 | | 8.21 | 16.80 |
| Sample 3 | | | 10.09 |
| Sample 4 | 4.89 | 15.50 | 22.10 |
| Sample 5 | 33.18 | 37.80 | |

# CHAPTER V

# DISCUSSION

## 17-Ketosteroids and Pregnanediol

Of the total 17-ketosteroids excreted in the urine, 70-85% is found as the glucuronide conjugates containing measurable amounts of all compounds except dehydroepiandrosterone. The remainder of the urinary steroids(15-30%) appear as the sulfate conjugates, of which dehydroepiandrosterone is frequently the major component. Since no feasible methods are available for the measurement of the glucuronides of 17-ketosteroids, these compounds must be hydrolyzed, a process which often causes certain problems.

The formation of artifacts can only be avoided if enzymes are used for the hydrolysis instead of hot mineral acids. To ensure complete enzymatic hydrolysis of steroid conjugates, it is essential to remove all materials present in the urine sample which may inhibit the enzymatic reactions. An efficient way for the removal of interfering substances is the adsorption chromatography of the urine on Amberlite XAD-2 according to Bradlow[39]. This step leads to a reduction of the volume of the urine sample which facilitates further handling. Even under the mild conditions of enzymatic hydrolysis, some artifacts may arise, whose formation can be successfully prevented by the addition of antibi-

otics and traces of chloroform to the urine sample[38].

In contrast to enzyme preparations of different origin, solvolysis leads to a complete cleavage of steroid sulfates as was found with dehydroepiandrosterone sulfate as test compound. Using the well-known method of Burstein and Lieberman[40], no formation of artifacts during solvolysis was noted as reported by other authors[21,33,42,43].

The utilization of two-dimensional thin layer chromatography(TLC) to reduce the amount of nonspecific material which may otherwise intefere with GLC determination as done in the original work by Külpmann[38] was found to be unnecessary. In the present work it was found that without TLC, the nonspecific substances appeared early and did not interfere with the 17-ketosteroids and pregnanediol determination.

Without derivatization of the extracted steroids prior to GLC, the method does not resolve dehydroepiandrosterone from androsterone. However, much clinically useful information can be gained without separating these two steroids. The method has been used succesfully to provide corroborative evidence in the diagnosis of virilizing syndrome in a young female based on the ratios of etiocholanolone to the mixture of androsterone and dehydroepiandrosterone following ACTH stimulation and dexamethasone suppression tests[44]. The method also does not resolve pregnanediol from 11-ketoandrosterone. However, this resolution would not provide any further information of the patient's adrenal status than was available by other means.

With the proposed method, 20-30 analyses can be perform in one working week. The method described is also less susceptible to drug interferences as compared with methods which are based on the determination of 17-ketosteroids by Zimmermann reaction. However, it should be stressed that a number of compounds, particularly synthetic steroids; such as dexamethasone and meprednisone[45], may directly or indirectly interfere with the determination and thereby falsify the result.

## 17-Ketogenic Steroids

The method used here is based on chemical procedures already described[15,22,46,47] with the modification in that the extracted steroids are not derivatized prior to GLC. The completeness of periodate oxidation of the glucuronide residues is still a matter of some controversy. Few[15] claimed 92% oxidation at pH 6.5 whereas Wade[48] showed only a 50% liberation of pregnanediol at pH 6.5 but at pH 3.5 he obtained recoveries comparable to those of Few[15]. During the incubation periods of both borohydride reduction and periodate oxidation, the urine samples should be done under the hood due to the strong unpleasant odor.

The individual urinary steroid values obtained using the present procedure agree well with values given by other workers using different techniques, and 17-ketogenic steroid values calculated from these individual values are not significantly different from values obtained by the total pro-

cedure carried out on the same urine sample. It is, however, fully appreciated that the specificity of the procedure described is open to criticism since the identification of each peak rests on the criterion of the retention time in a single GLC system.

Demonstration of the high specificity in individual urine samples does not invalidate objections on the grounds of lack of specificity in the urine samples since each urine is unique and when analyzed can give rise to peaks of steroids that have the relative retention times as that of certain 17-ketogenic steroids. The appearances of these steroids may then be identified incorrectly as 17-ketogenic steroids. It is clearly of little value to present the routine clinical laboratory with a complex and time-consuming fractionation procedure of high specificity which cannot be applied to every patient because of the expertise required and the length of time it takes. The GLC method described here is simple and quick, 15 urine specimens and a standard can be prepared in 5 h and the GLC carried out the following morning. The method should be found to be suitable for the routine measurement of urinary steroids in a busy clinical laboratory. The method also appears to be particularly useful as an aid to the rapid diagnosis of congenital adrenal hyperplasia, the results being available within 24 h from the receipt of the specimen.

# CHAPTER VI

## CONCLUSION

The normal urinary excretion of 17-ketosteroids varies with age, rising slowly from birth to puberty and then rapidly to a maximum in the mid-twenties(males: 8-26 mg/24 h, mean 15 mg/24 h; females: 4-17 mg/24 h, mean 10 mg/24 h)[49]. Although the average for males is higher than that of females of comparable age, the scatter is quite large, and there is considerable overlap of values. The daily excretion of 17-ketosteroids in the young adult varies marginally from day to day and the total amount of the steroids excreted in urine per 24 h does not depend on the intake of fluid[50].

The simultaneous determination of 17-ketosteroids and pregnanediol is of great value in the diagnosis of the adreno-genital syndrome and in the differentiation of the various forms of enzyme deficiency, underlying this disease. In patients with a 3$\beta$-hydroxy-steroid-oxidoreductase deficiency, the excretion of dehydroepiandrosterone is elevated, whereas that of the other 17-ketosteroids and of pregnanediol drops to undetectable values. In 11$\beta$-hydroxylase deficiency, the excretion of 11-oxygenated-17-ketosteroids is markedly decreased and that of the 11-deoxy-17-ketosteroids as well as of pregnanediol increased. In 21-hydroxylase deficiency, the excretion of 17-ketosteroids is significantly elevated. The method described in this work will greatly

aid with the detection of these different states of enzyme deficiencies which otherwise is impossible with the Zimmermann reaction.

Furthermore, the differential determination of 17-ketosteroids may be useful in the evaluation of, 1) hypothalamic-hypophyseal disorders, such as dystrophia adiposo-genitalis(Frohlich's syndrome), anorexia nervosa and hypopituitarism, and 2) gonadal disorders, such as Stein-Leventhal syndrome and arrhenoblastoma.

The methods described, particularly that for 17-ketogenic steroids, render themselves to a considerable degree of automation and can be used to screen the patients at approximately 1/3rd of the cost of 17-ketosteroid and 17-ketogenic steroid group estimations.

In conclusion, there is clearly no doubt that determination of 17-ketosteroids and pregnanediol and of 17-ketogenic steroids will contribute to the characterization of many endocrinological disorders which are at present poorly understood.

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