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HIGH-RESOLUTION BIOMEDICAL GAS CHROMATOGRAPHY

DETERMINATION OF HUMAN URINARY STEROID METABOLITES USING GLASS OPEN TUBULAR CAPILLARY COLUMNS

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SUMMARY

Recently developed analytical procedures for the qualitative and quantitative analysis of human urine for major and minor steroid metabolites are described. Steroid profile samples were obtained by enzymic hydrolysis with β-glucuronidase and sulfatase. Methoxime-trimethylsilyl ether derivatives of steroid metabolites were prepared; the recommended procedure converts all ketone groups (except the 11-one group) into methoxime groups and all hydroxyl groups into trimethylsilyl ether groups. These derivatives are thermally stable, readily volatilized, not subject to dehydration or adsorption on gas chromatographic columns, and suitable for both qualitative and quantitative analytical studies.

Thermostable glass open tubular capillary columns, coated with the non-polar phase SE-30, and containing dispersed particles of silanized silicic acid, were used for the gas chromatographic separation. Illustrations of profiles for normal female and male subjects, and patients with a testosterone-secreting ovarian tumor, congenital adrenal insufficiency and a dehydroepiandrosterone-secreting adrenal tumor are included.

INTRODUCTION

Metabolic profiles of human urinary steroids obtained by gas chromatography (GC) reflect the production and metabolism of steroidal hormones. Their present use is largely for research purposes; in time, they will be used in diagnosis and possibly in establishing risk in disorders involving steroid biosynthesis and steroid metabolism.

The analytical procedures and technology described in this paper are the result of relatively recent advances both in chemical methods of derivative formation and in the preparation and use of high-resolution thermostable glass open tubular capillary columns. The chief problem in derivative formation is that of obtaining a single, thermally stable derivative from each steroid. This is best done by preparing methoxime-trimethylsilyl ether derivatives in a way that converts all ketone groups, except the 11-one group, into methoxime groups, and all hydroxyl groups into tri-

methylsilyl ether groups. The resulting derivatives are thermally stable, readily volatilized, not subject to dehydration or adsorption on GC columns (since 11- and 17-hydroxyl groups are derivatized) and suitable for qualitative and quantitative analytical studies. The separation problem is severe; human profiles contain many closely related steroids, and in our experience the best type of GC separation condition lies in the use of nonpolar thermostable glass open tubular capillary columns. We prefer the use of SE-30 (polymethyl siloxane) columns prepared with Silanox, and having 100,000 or more theoretical plate efficiency.

These procedures are being used for both qualitative and quantitative purposes. All designated compounds were identified through use of a gas chromatograph–mass spectrometer–computer analytical system (LKB 9000/PDP 12); it is not advisable to depend upon retention data alone for the identification of individual steroids.

Current experience suggests that these methods, which are based upon many advances made since the original observation of thin-film GC separation of steroids[1], can be used for the analysis of other types of biologic mixtures of steroids as well. The work described here was part of a study of both major and minor human urinary steroids; the results which are summarized are those relating to well known adult metabolites observed for normal subjects and for several types of patients.

EXPERIMENTAL

Isolation of "steroid profile" sample by enzymic hydrolysis

Urine sample. Urine samples (24 h) were collected in polyethylene containers. After recording the volume, all samples of less than 2 l of urine were diluted to 2 l using deionized water which had been glass-distilled from basic potassium permanganate. The diluted urines were stored at −14° if not immediately used. Analyses were normally carried out within 72 h after collection.

Enzymic hydrolysis. Sodium acetate trihydrate (1.0 g) was added to 50 ml of diluted urine in a 125 ml screw-capped erlenmeyer flask. The pH was adjusted to 4.5 using acetic acid, and 0.5 ml of enzymic solution (Glusulase, Endo Labs., Garden City, N.Y., U.S.A.; 1 ml contains 100,000 units of β-glucuronidase and 50,000 units of sulfatase) was added. After incubating at 37° (with gentle motion) for 24 h, the pH was adjusted to 5.5 using a few drops of 40% aqueous potassium hydroxide, and a second 0.5-ml portion of Glusulase was added. The incubation was continued for a second 24-h period at 37°. Flasks were stored overnight at 7° if the hydrolysate could not be extracted immediately.

Extraction of steroids. Into a 1-l rotary extractor were placed 75 ml of dichloromethane and 100 μg of an internal reference compound, obtained from a stock solution (1 mg/ml) of cholesteryl butyl ether (Sigma, St. Louis, Mo., U.S.A.) in pyridine. The hydrolysate was then added. The extraction step required 10 min of the swirling action of the rotary extractor. Another extraction with 75 ml of dichloromethane, and a final extraction with 75 ml of ethyl acetate (all solvents were Nanograde, from Mallinckrodt, St. Louis, Mo., U.S.A.), led to a combined organic phase of 225 ml which was collected in a 500-ml round-bottomed flask and evaporated (Rotovap; temperature kept below 40°). The residue was transferred to a 125-ml separatory funnel using 15 ml of ethyl acetate. The organic layer was washed three times with 10-ml portions of aqueous 5% sodium bicarbonate–10% sodium chloride, and then

three times with saturated aqueous sodium chloride. The water used to make the wash solutions was deionized and glass-distilled from basic potassium permanganate. The ethyl acetate solution was drained into a 50-ml Erlenmeyer flask, dried over 0.5 g anhydrous magnesium sulfate, filtered into a 15-ml conical test tube, and evaporated using a nitrogen stream. The residue was transferred using ethyl acetate to a 1-ml Reacti-vial (Pierce, Rockford, Ill., U.S.A.) and stored at −14° if not immediately processed.

Derivative formation

Preparation of methoxime-trimethylsilyl ether (MO-TMS) derivatives. After the ethyl acetate solution of steroids was evaporated with the aid of a nitrogen stream, 100 μl of a stock solution of methoxyamine hydrochloride (Eastman-Kodak, Rochester, N.Y., U.S.A.) in pyridine, 100 mg/ml, was added, and the vial was fitted with a PTFE-lined screw cap. The solution was heated at 70° for 15 min, then evaporated using a nitrogen stream. After the addition of 100 μl of N-trimethylsilylimidazole (TSIM; Pierce), the solution was heated at 100° for 2 h. The final solution was used directly for GC analysis, using 2–5-μl samples in most instances.

Gas chromatography

Column preparation. Sixty-meter borosilicate glass capillaries (1.0 mm O.D., 0.3 mm I.D.) were drawn from 1.25-m tubes (7.8 mm O.D., 3.8 mm I.D.) using a Shimadzu Model GDM-1 glass drawing and coiling apparatus. Pyrex tubes to be drawn were successively rinsed with acetone, methylene chloride, 1 % aqueous potassium hydroxide, and methanol, and dried under vacuum. The diameter of the capillary coil was 10.5 cm.

Using the method of German and Horning[2], glass capillaries silanized with dimethyldichlorosilane and coated with SE-30 containing Silanox 101 (Cabot, Boston, Mass., U.S.A.) were prepared. These were conditioned under carrier gas (nitrogen) flow by temperature programming at 1°/min from 25° to 280°, holding at 280° for 2 h, lowering to 200°, programming at 1°/min to 300° and holding at 300° for 1 h. The theoretical plate efficiencies of the resulting columns (for *n*-tetracosane at 250°) ranged from 125,000 to 150,000.

Instrumentation. Separation of the MO-TMS derivatives of urinary steroid metabolites was carried out using a Tracor Model 550 gas chromatograph which had been modified to include a previously described glass inlet system[3] and to accept glass capillary columns. A Fisher Recordall Series 5000 recorder was employed. Hydrogen was supplied by a Hewlett-Packard Model 18591A hydrogen generator. All gas flows (except air) were controlled by Brooks Model 5840 flow controllers. The flame ionization detector was extensively modified[4] for use with glass capillaries.

Retention behavior. Methylene unit (MU) values[5,6] were determined through use of *n*-alkanes co-injected with the sample using an initial temperature of 200° and programming at 1°/min. Other GC conditions included: split ratio, 5:1; temperature of pre-column inlet splitter, 270°; detector bath temperature, 300°; column inlet pressure, 10 p.s.i., resulting in a carrier gas (helium) linear velocity of 18–20 cm/sec (200°); hydrogen flow-rate, 37.5 ml/min; air flow-rate, 200 ml/min; nitrogen make-up gas to the detector, 50 ml/min. The order of elution of steroid derivatives with an

SE-30 phase is the same for packed and open tubular capillary columns, but the observed MU values are usually slightly different.

Quantitative analyses. Calculations were made by hand; area measurements were made by multiplying the height of the peak by the width at half height. The chart speed and pen line width are variables that affect the results; in our work the height was measured to the outside of the pen line, but the width at half height was measured to the inside of the pen line. A magnifying scale was used for the width measurement. Usually a chart speed of 1.25 cm/min was used for quantitative calculation charts and 0.5 cm/min for illustrations. The precision of this method is about 2% (standard deviation).

The calculations were based on individual peak area responses compared to the area response exhibited by a known amount of internal reference compound, cholesteryl butyl ether. A study of mass response factors of steroid metabolites will be published separately.

Mass spectrometry

The identity of each steroid indicated in the illustrations was confirmed by comparisons of retention behavior and mass spectra for authentic samples. The mass spectra were obtained with an LKB 9000/PDP 12 analytical system in the usual way, using an SE-30 column. The mass spectral studies, and a discussion of problems involved in the identification of minor components of samples, will be published separately.

RESULTS AND DISCUSSION

Derivatives for gas chromatography

MO-TMS derivatives are the most satisfactory derivatives now known for gas-phase analytical studies of steroids. For special purposes, *n*-butyloxime-trimethylsilyl ethers[7], benzyloxime-trimethylsilyl ethers[8] and trimethylsilyloxime-trimethylsilyl ethers[9] are also useful; and persilylated trimethylsilyl[10] (hydroxyl and enol) ethers have been used in some applications. Very few problems are associated with the preparation of derivatives of steroids with two or three functional groups; the major difficulties lie in working with steroid mixtures of biologic origin containing compounds with many (up to six) functional groups.

Three principal problems are present. The purpose of converting ketone groups to methoximes[11] is to prevent enol ether formation during the silylation procedure. Ketone groups in the usual unhindered positions (3-, 16-, 17-, 20-) react rapidly under a variety of conditions, but a hindered ketone (11-one) group will react very slowly. The reaction condition described here was established through an investigation of conditions designed to result in rapid derivative formation for 3-, 16-, 17- and 20-one groups, with negligible reaction of the 11-one group in tetrahydrocortisone and related compounds[11–14]. A second problem involves the fact that conversion of the 17α-hydroxyl group of tetrahydrocortisol (and of related compounds with the same type of functional groups) to an ether is required to provide thermal stability. Otherwise, the side chain is lost during the vaporization process. Conditions for the preparation of derivatives of steroids with a tertiary 17-hydroxyl group were developed by Gardiner and Horning[15] and for some time this procedure was used in our work. An

additional problem, however, is posed by the fact that steroids with a free hydroxyl group usually show evidence of adsorption or dehydration when separated under conditions involving relatively high temperatures (280–320°). For this reason, studies were carried out of relative rates of reaction of steroidal hydroxyl groups with different silylating reagents and different conditions of reaction. This work[14,16,17] resulted in the establishment of conditions for the conversion of all hydroxyl groups to trimethylsilyl ether groups. The rates of reaction of unhindered, moderately hindered and highly hindered hydroxyl groups are quite different; the condition described here was established by Thenot and Horning[14] and was chosen in order to convert all hydroxyl groups in human urinary steroids, alone and in combination, to trimethylsilyl ethers while restricting enol ether formation for the 11-one group of tetrahydrocortisone to 2% or less. (This method has been modified for use with dexamethasone[18].)

The resulting MO-TMS derivatives are thermally stable, easily volatilized and show no indication of dehydration or compound loss when good GC analytical practices are followed. The methods can be used for many other types of steroid mixtures of biologic or synthetic origin, providing suitable precautions are taken if steroidal hormones with a 3-one-4-ene structure are involved. All 17-one steroids that have been studied yield a single MO derivative, but 3-one-4-ene steroids form *syn-* and *anti*-isomers which may or may not separate when packed columns are employed[12,13]. The isomeric derivatives are stable and are usually formed in characteristic ratios for each steroid. It is usually preferable to employ methods yielding a single derivative whenever possible; in this instance we have found that isomer formation is acceptable for compounds of interest including cortisone[19], cortisol[19] and 6β-hydroxycortisol[20].

Caution should be exercised in the separation of all MO-TMS derivatives with respect to column conditions. In a study of the problem of acid-catalyzed decomposition or alteration of MO derivatives, Thenot and Horning[19,21] found that under acidic conditions 17-MO steroids were converted to a nitrile by D-ring opening, but that 3-MO derivatives were lost by decomposition except for 4,4-dimethyl steroids, which were transformed to other products by A-ring opening. When the MO-TMS procedure described here is employed, there is no evidence of residual acidity (due to hydrogen chloride) in the reagent–sample mixture, and the use of a short (1 cm) initial segment of 10% SE-30 column packing is recommended[19] to prevent the development of acidic active sites at the head of the column. When the inlet system of German and Horning[3] is used, this segment is placed in the fore-column.

The derivatization procedure employed in this work is suitable for many types of urinary steroid samples. When excessive amounts of steroids are excreted, however, the urine sample should be diluted as required to approximate the range of steroid concentrations usually encountered.

Glass open tubular capillary column separations

The initial studies of Golay[22] on open tubular columns suggested that highly complex mixtures of biologic origin might well be separated in this way with a degree of effectiveness not achievable by other types of procedures. When attempts were made during 1960–1965 to prepare glass open tubular capillary columns, however, only ephemeral success was attained. The major problem in preparing and using columns of this kind lies in the relatively rapid conversion of thin films of liquid phase

on a smooth glass surface to microdrops when the column is heated and cooled over the range 200–300° (ref. 23). Glass columns suitable for general use were first prepared by Grob[24,25]; the process involved surface modification by etching. These columns were evaluated for use in steroid separations by Völlmin and co-workers[26–28] and Ros and Sommerville[29]. Later etching procedures include those of Novotny and Tesarik[30,31] and Alexander and Rutten[32–34]. The Novotny–Tesarik method was evaluated for steroid separations by Novotny and Zlatkis[35]. These procedures, and others which involve surface modification by etching, are based on the concept that stabilization of the liquid film results when the coating is applied to a glass surface with micro-irregularities.

The procedure of German and co-workers[2,36,37] employed in this study is based on the concept that thermostable columns can also be prepared by coating a thin film of liquid phase containing microparticles of silanized silicic acid on a smooth glass surface. The surface is silanized, to decrease solute adsorption, when a nonpolar phase is used. For polar phases, the glass is not silanized. The stabilization of the film which results when this method is employed may also be due to microirregularities arising from the particulate material which is present, but the glass surface is not etched and there is no chemical interaction or anchoring process involved in the stabilization. Continued experience indicates that they can be used in many applications[37–39]. The temperature limit is determined by the phase; SE-30 columns can be used to about 320°, and columns containing the phase described by Mathews *et al.*[40] can be used to about 320–350°.

Other methods of glass capillary column preparation and use have been described in the past few years. These include the methods of Ilkova and Mistryukov[41–43] and Schomburg and co-workers[44–46]. It is evident that glass open tubular capillary columns can be made in a variety of ways and with different phases. Further work will be required to establish the relative merit of competitive procedures. The columns used in this work show high capacity, long life and high resolution (100,000 or more theoretical plates for 60-m columns), and they can be used in many types of biologic studies. The use of a fore-column in the inlet system[3,19] may contribute to the long life of the column when used with mixtures of biologic origin.

The order of elution of steroid derivatives when a nonpolar phase is used is largely determined by molecular size and shape. Although retention data for steroid derivatives are reproducible, the identification of individual metabolites should be made on the basis of mass spectral studies with a gas chromatograph–mass spectrometer. All compounds indicated in the figures were identified in this way. Searches for minor compounds are best made through techniques based on selective ion detection (mass fragmentography) or on computer-based data analysis of repetitive scans (mass chromatography).

The addition of an internal reference compound permits the quantification of major metabolites. Cholesteryl butyl ether is a satisfactory compound.

Human urinary steroid metabolic profiles

Fig. 1 shows a metabolic profile of a normal post-pubertal pre-menopausal female. All of the usual components are present: androsterone (An), etiocholanolone (Et), dehydroepiandrosterone (DHEA), 11β-hydroxyandrosterone (11-HAn), 11β-hydroxyetiocholanolone (11-HEt), pregnanediol (Pd), pregnanetriol (Pt), 5-androsten-

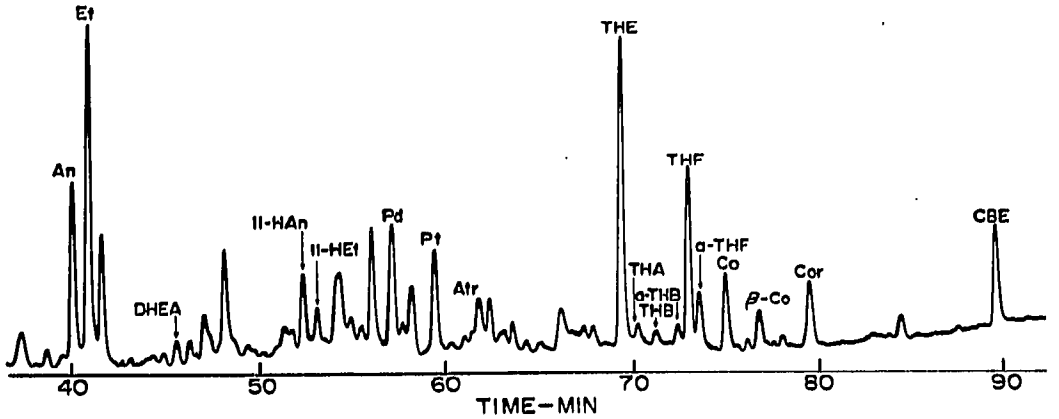

Fig. 1. Metabolic profile of a normal post-pubertal pre-menopausal female. Separation of the MO-TMS derivatives of the steroid metabolites is carried out using a 60-m SE-30/Silanox glass open tubular capillary column, temperature-programmed at 1°/min from 200°. Abbreviations defined in the text.

3β,16α,17β-triol (Atr), tetrahydrocortisone (THE), tetrahydro-11-dehydrocorticosterone, 5β-pregnan-3α,21-diol-11,20-dione (THA), tetrahydrocorticosterone, 5β-pregnan-3α,11β,21-triol-20-one (THB), *allo*-tetrahydrocorticosterone, 5α-pregnan-3α,11β,21-triol-20-one (a-THB), tetrahydrocortisol (THF), *allo*-tetrahydrocortisol (a-THF), cortolone, 5β-pregnan-3α,17α,20α,21-tetrol-11-one (Co), β-cortolone, 5β-pregnan-3α,17α,20β,21-tetrol-11-one (β-Co), and cortol, 5β-pregnan-3α,11β,17α,20α,21-pentol (Cor). The last component appearing in the chromatogram is the internal reference compound, cholesteryl butyl ether (CBE).

Androsterone, etiocholanolone, tetrahydrocortisol and *allo*-tetrahydrocortisol are products of liver enzymic reduction of the 3-one-4-ene group of the steroid A/B ring system. Specific 5α-reductases convert testosterone to androsterone and cortisol to *allo*-tetrahydrocortisol; specific 5β-reductases convert testosterone to etiocholanolone and cortisol to tetrahydrocortisol. The relative abundance of these enzymes leads to the ratios of metabolites observed for each individual. Normal females usually excrete etiocholanolone (5β-H) and androsterone (5α-H) in a ratio of about 2:1; their usual ratio of tetrahydrocortisol (5β-H) to *allo*-tetrahydrocortisol (5α-H) is about 3:1. Marked deviations from these two sets of usually observed ratios may be indicative of an increased state of risk of cystic disease[47] (polycystic disease of the ovary or fibrocystic disease of the breast). In Fig. 1, the ratios and the amounts of both androgen and adrenocorticosteroid metabolites are typical.

Fig. 2 shows a metabolic profile of a normal post-menopausal female. Although the Et:An ratio is about 3:1 and the THF:a-THF ratio is about 5:1, this is not regarded as a significant deviation from the usual female ratio.

The lower amount of androgen metabolite excretion observed here is typical for the post-menopausal female; a steady decline in androgen metabolites can be observed with advancing years. The amounts of adrenocorticosteroid metabolites seen here are comparable to those of the pre-menopausal female.

Fig. 3 shows a metabolic profile of a female entering the second trimester of

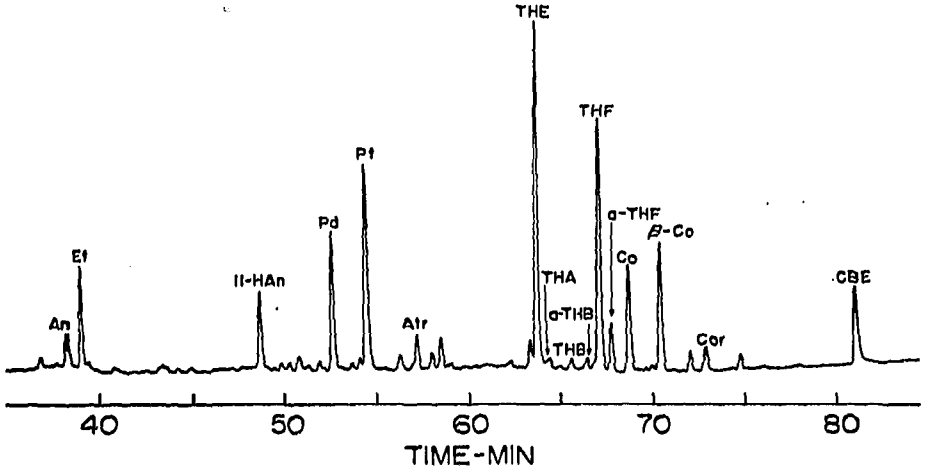

Fig. 2. Metabolic profile of a normal post-menopausal female. Analysis conditions are similar to those used in Fig. 1.

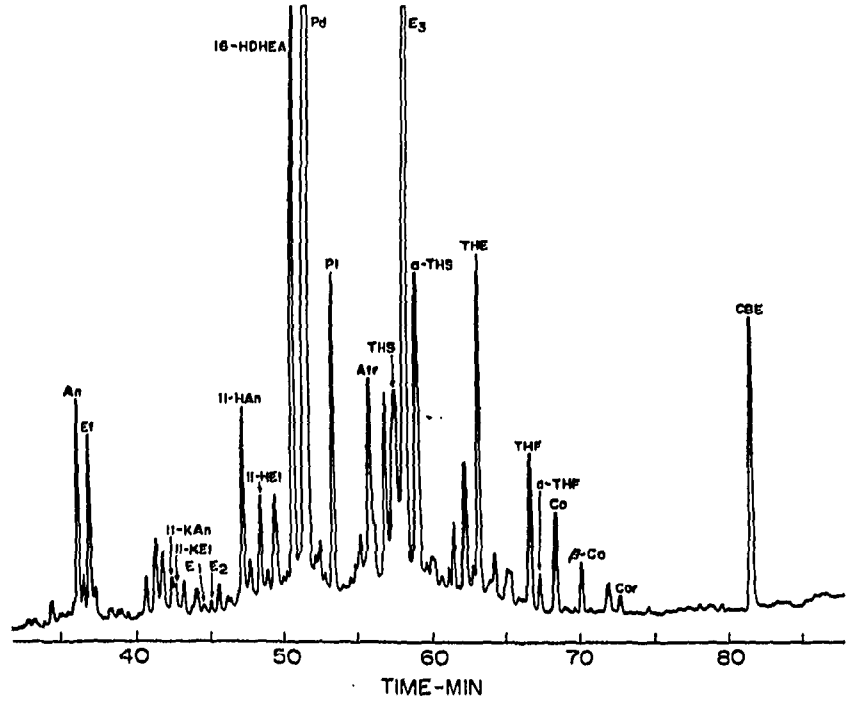

Fig. 3. Metabolic profile of a female entering the second trimester of pregnancy. Analysis conditions are similar to those used in Fig. 1.

pregnancy. Significant increases in the excretion of 11β-hydroxyandrosterone (11-HAn), 11β-hydroxyetiocholanolone (11-HEt), 16α-hydroxydehydroepiandrosterone (16-HDHEA), pregnanediol (Pd), androstenetriol (Atr), tetrahydrocortexolone, 5β-pregnan-3α,17α,21-triol-20-one (THS), estriol (E_3) and *allo*-tetrahydrocortexolone, 5α-pregnan-3α,17α,21-triol-20-one (a-THS) are present. Small amounts of 11-keto-androsterone (11-KAn), 11-ketoetiocholanolone (11-KEt), estrone (E_1) and estradiol (E_2) are also excreted.

As early as 1946, Venning[48] found only a slight rise in 17-ketosteroid excretion during pregnancy. Her observations were later confirmed by both Schuller[49] and Birke *et al.*[50] who detected no appreciable rise in androsterone or etiocholanolone excretion until late in the third trimester. A steady increase in the excretion of 11-oxygenated 17-ketosteroids, primarily 11-hydroxyandrosterone and 11-hydroxyetiocholanolone, was observed.

The greatly elevated excretion of estriol during pregnancy is due to feto-placental processes[51]. Fetal 16α-hydroxylation, which is required for estriol formation, is also responsible for increased production of 16α-hydroxydehydroepiandrosterone. Because 16α-hydroxylation is primarily a fetal adrenal, rather than a placental, hydroxylation[52], estriol has been regarded as a specific indicator of fetal well-being. The excretion of less than 1 mg daily in late pregnancy usually indicates fetal death. Klopper[53] regards less than 3 mg daily indicative of probable fetal death; Green *et al.*[54] considered an excretion of 4–12 mg daily a warning that the fetus is at risk.

Increased pregnanediol excretion (always found in pregnancy) results from maternal liver reduction of progesterone produced by placental tissue.

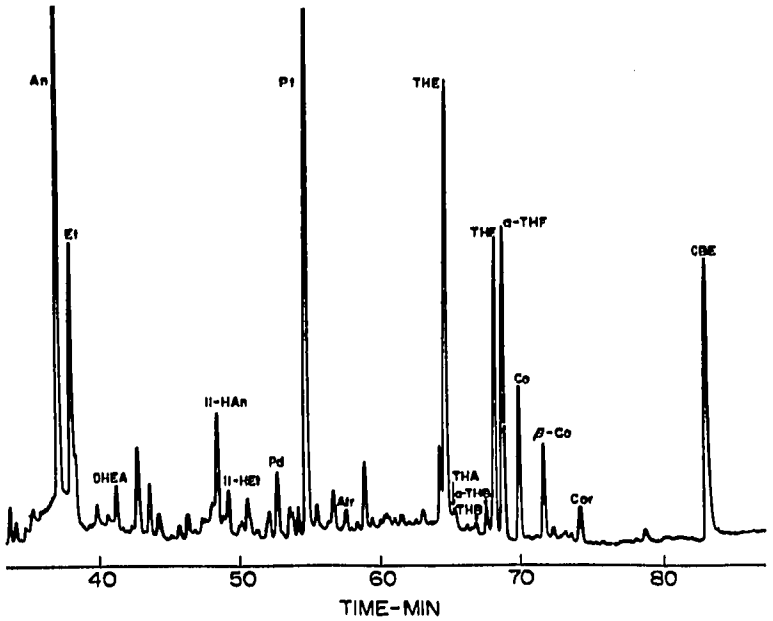

Fig. 4. Metabolic profile of a normal adult male. Analysis conditions are similar to those used in Fig. 1.

Dässler[55] found significant rises in cortexolone (4-pregnen-17α,21-diol-3,20-dione) excretion during pregnancy, an observation consistent with the elevated excretion of tetrahydrocortexolone and *allo*-tetrahydrocortexolone indicated in the profile. These metabolites (THS and a-THS) are usually present only in trace amounts under ordinary circumstances.

Fig. 4 shows a metabolic profile of a normal adult male. As in Fig. 1, all of the usual urinary steroid metabolites are present. Normal males excrete etiocholanolone and androsterone in a ratio (5β-H:5α-H) of about 1:2; the usual ratio of tetrahydrocortisol to *allo*-tetrahydrocortisol (5β-H:5α-H) is about 1:1. These ratios reflect the liver 3-one-4-ene reductase distribution found in males.

Fig. 5 shows a metabolic profile of a pre-menopausal female with a testosterone-secreting ovarian tumor. (The urine was diluted 20× with glass-distilled water before the enzymic hydrolysis step.) Large amounts of etiocholanolone and androsterone were excreted (ratio about 2:3). That an ovarian tumor can produce very large quantities of testosterone (leading to greatly elevated excretion of androsterone and etiocholanolone) was demonstrated in 1964 by Rosner *et al.*[56]. Their patient excreted forty-five times the amount of urinary testosterone glucuronide normally found for females. Greatly elevated excretion of 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone, and of pregnanetriol, is evident from the profile. Adrenocorticosteroid metabolites are present in larger amounts than are observed for normal subjects.

Fig. 6 shows a metabolic profile of an adult male with congenital adrenal insufficiency. In the absence of adrenal cortex function, adrenocorticosteroid metabolites are not observed as excretion products. Androgen excretion levels are normal in this

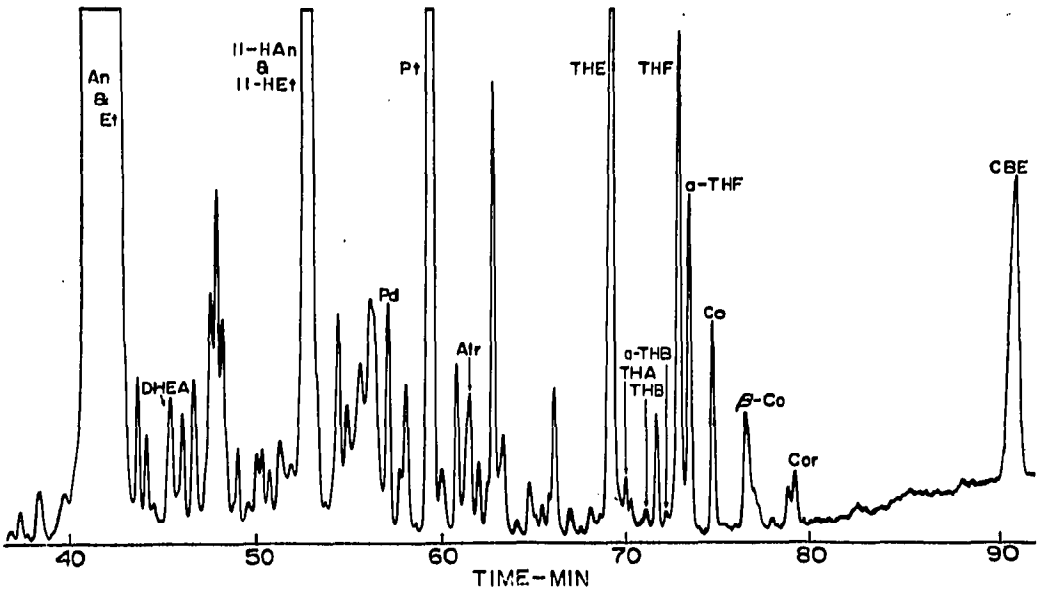

Fig. 5. Metabolic profile of a pre-menopausal female with a testosterone-secreting ovarian tumor. Analysis conditions are similar to those in Fig. 1.

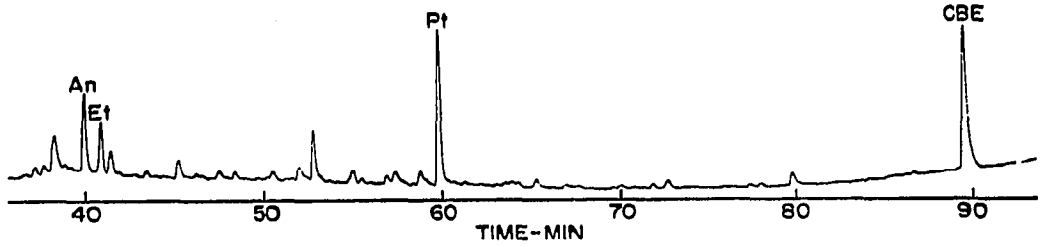

Fig. 6. Metabolic profile of an adult male with congenital adrenal insufficiency. Analysis conditions are similar to those in Fig. 1.

patient; the Et:An ratio is about 2:3. Pregnenolone, the precursor of progesterone, leads to pregnanetriol via 17α-hydroxylation and subsequent reduction of both the 20-one and the 3-one-4-ene moieties of 17α-hydroxyprogesterone. The component which elutes about 53 min after injection is a pregnanediolone, presumably 5β-pregnan-3α,17α-diol-20-one, which would result from reduction of the 3-one-4-ene system of 17α-hydroxyprogesterone[57].

Fig. 7 shows the metabolic profile of a post-menopausal female with a dehydroepiandrosterone-secreting adrenal tumor.

Dehydroepiandrosterone was isolated from adrenal tumor tissue by Plantin *et al.*[58] in 1957, and is found in increased amounts in the urine of patients with adrenal carcinoma. Dorfman and Shipley[59] found greatly elevated excretions of 3β-hydroxy-17-ketosteroids in 20 of 24 cases of adrenal cortical tumors.

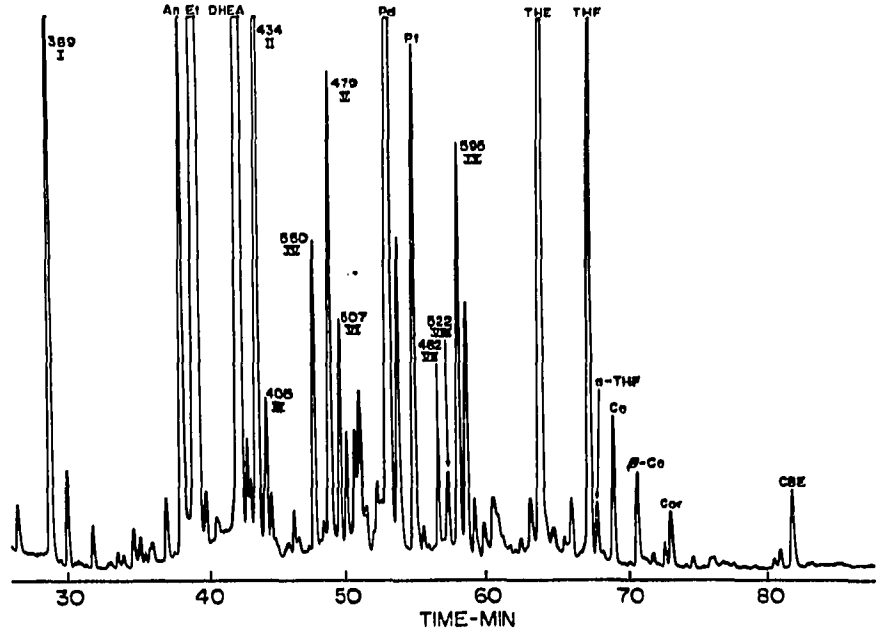

Fig. 7. Metabolic profile of a post-menopausal female with a DHEA-secreting adrenal tumor. Components I–IX, see text. Analysis conditions are similar to those in Fig. 1.

A GC–mass spectrometric analysis of this sample was carried out to identify the components numbered I–IX. Methylene unit and fragmentation data led to the following tentative identifications, MO-TMS derivatives of: I (M = 389), 9,(5β)-androstene-3α-ol-17-one; II (M = 434), 5-androstene-3β,17β-diol; III (M = 405), 11-ketoetiocholanolone; IV (M = 550), 5-pregnene-3β,17α,20α-triol; V (M = 479), 11β-hydroxyandrosterone; VI (M = 507), 5β-pregnane-3α,17α-diol-20-one; VII (M = 462), 5-pregnene-3β,20α-diol; VIII (M = 522), 5-androstene-3β,16α,17β-triol and IX (M = 595), 5β-pregnane-3α,17α,20α-triol-11-one.

Table I contains data for the six samples which correspond to Figs. 1–6. The glass capillary columns used in this work are capable of handling very large amounts of components which, when chromatographed, do not obscure minor components. Thus, as illustrated in Fig. 3, estriol (E_3) is well separated from *allo*-tetrahydrocortexolone (a-THS). In Fig. 5 the descending side of the etiocholanolone peak remains sharp with little trailing; and the tetrahydrocortisone (THE) peak does not obscure the tetrahydro-11-dehydrocorticosterone (THA) component.

TABLE I

EXCRETION OF HUMAN URINARY STEROID METABOLITES

Data from 24-h urine collections. For abbreviations of the steroids, see text.

Steroid	*MU value**	*Analysis*** *corresponding to Fig.*					
		1	*2*	*3****	*4*	*5§*	*6*
An	25.31	2.7	0.5	1.1	3.1	74	2.7
Et	25.45	5.1	1.6	0.8	1.5	50	1.8
DHEA	26.11	0.5	—§§§	—	0.3	1.0	—
11-HAn	27.12	1.0	1.4	1.1	0.8	11	—
11-HEt	27.25	0.5	—	0.6	0.3	1.9	—
Pd	27.80	2.7	2.2	8.6	0.4	0.9	—
Pt	28.12	1.2	3.6	1.8	3 6	8.5	3.1
Atr	28.51	0.5	0.5	1.5	0.3	1.2	—
THE	29.72	2.7	5.7	2.2	3.5	5.1	—
THA	29.82	0.2	0.1	0.2	0.1	0.3	—
THB	30.03	0.2	0.2	0.3	0.1	0.2	—
a-THB	30.21	0.2	0.2	0.1	0.3	0.1	—
THF	30.30	1.3	3.9	1.3	2.4	3.0	—
a-THF	30.41	0.4	0.8	0.4	2.5	1.8	—
Co	30.59	0.6	1.4	0.8	1.2	1.0	—
β-Co§§	30.90	0.3	1.6	0.4	0.8	0.6	—
Cor	31.32	0.6	0.4	0.2	0.4	0.2	—

* Determined with a 60-m SE-30/Silanox glass open tubular capillary column, temperature-programmed at 1°/min from 200°.

** Calculated on basis of equivalent response with respect to cholesteryl butyl ether (MU = 32.86) and in terms of mg/g creatinine. Response factors are needed for conversion to mg values.

*** Estriol (MU = 28.91) was a major urinary steroid.

§ Urine diluted 20× before analysis.

§§ Values for β-cortolone were not adjusted for traces of β-cortol (MU = 30.91) which co-elutes.

§§§ Not detected.

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