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Separation, Identification, and Estimation of Human Steroid Hormones and Their Metabolites: Applications to Adrenocortical Steroids*

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Summary

The study of human metabolic networks requires the development of analytical methods capable of providing qualitative and quantitative information for relatively large numbers of compounds present in complex mixtures. Gas-phase analytical methods, using gas chromatography and mass spectrometry, provide the best approach to this problem. Separation and quantification steps may be carried out by gas chromatography, and identifications or structural determinations may be made by gas chromatography–mass spectrometry.

The specific problem described in this paper involves the separation, identification, and estimation of human urinary adrenocortical hormone metabolites. The compounds were separated as MO-TMSi and TMSi derivatives in a temperature-programmed procedure. A study was made of the quantitative aspects of an automated system for steroid analyses. The method is satisfactory for reference compounds; it is not yet entirely suitable for use with authentic urinary samples.

Enzymatic transformations of cholesterol by cells of the adrenal cortex lead to a group of C_{21} steroids of characteristic structure. Hydroxylation reactions at C-17α, C-11β, and C-21 do not occur, as far as is known, in any other human tissue, and the conversion

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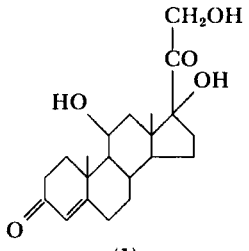
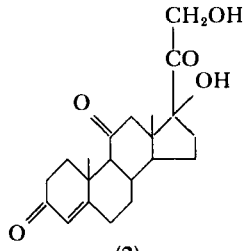
of 3β-ol-5-ene steroids to 3-one-4-ene steroids also occurs in human adrenal tissue. The best known hormones of this group are cortisol (**1**) and cortisone (**2**). Oxidation may also occur at C-18, leading



(1)



(2)

to aldosterone. When these steroids are released into the circulation they undergo structural changes, primarily in the liver, to form a large number of compounds oxygenated (hydroxyl or keto groups) at positions which include C-3 and C-20 and additional combinations of substitution involving C-6, C-11, C-17, C-18, and C-21. The adrenocortical hormones are important because they control, directly or indirectly, many aspects of human metabolism.

The analytical problem involved here, in a broad definition, is that of separating, identifying, and estimating individual adrenocortical hormones and their metabolites. The specific problem described in this paper is that relating to human urinary steroids.

SEPARATION PROCEDURE

The first study of the behavior of human adrenocortical hormones in a gas-liquid chromatographic (GLC) system was carried out by Van den Heuvel and Horning (*1*) in 1960. The conditions were those developed for the separation of cholesterol and related steroids (2). A thermostable liquid phase (SE-30, methylsiloxane polymer) was used in a thin-film packing, and small samples and a highly sensitive ionization-detection system were employed. Steroids of the 17α-hydroxyl group with a typical side chain (20-one-21-ol), including cortisone and cortisol, were found to undergo a thermal cleavage reaction with loss of the side chain to yield the corresponding 17-keto steroids.

Indirect methods for the study of adrenocortical hormone metabolites were later developed in several laboratories. A bismuthate

oxidation method, leading to 17-keto steroids, was used by Bailey (3) and by Sparagana (3), and a periodate oxidation method, leading to 17α-hydroxy-17β-carboxylic acids, was developed by Kittinger (4). Direct methods were also sought. Brooks (5) studied the GLC behavior of acetyl derivatives, Kirschner and Fales (6) prepared and studied *bis*-methylenedioxy derivatives, and Fales and Luukkainen (7) described methoxime (MO) derivatives. Luetscher and Gould (8) used the thermal cleavage reaction in an analytical procedure.

Although the methoxime derivatives of cortisone and cortisol are not thermally stable (7), the methoxime-trimethylsilyl ether (MO-TMSi) derivatives are stable under GLC separation conditions. Figure 1 shows a separation of several human steroid hor-

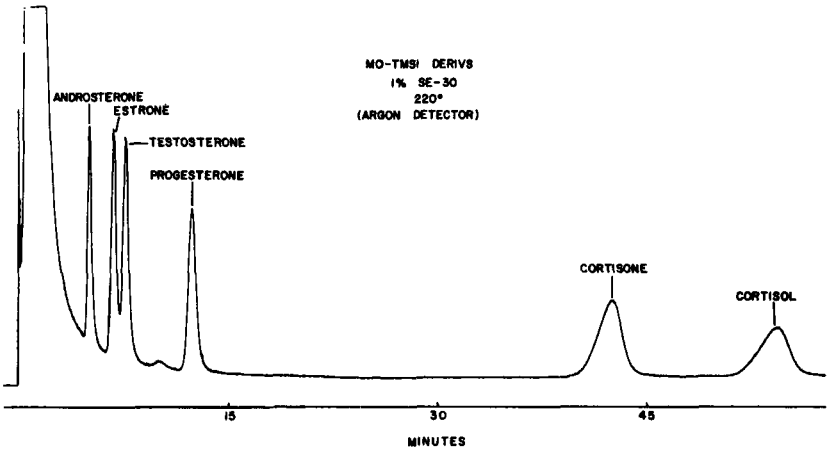


FIG. 1. Isothermal GLC separation of MO-TMSi derivatives of several human steroids with a 6-ft 1% SE-30 column at 220°. The steroids are androsterone, estrone, testosterone, progesterone, cortisone, and cortisol. The separation was carried out for qualitative purposes only with an argon detection system. Methoxime isomers are not separated under these conditions.

mones, including cortisone and cortisol, as MO-TMSi derivatives, carried out with a 1% SE-30 phase under isothermal conditions. Our original interest in this separation was in connection with a procedure for the estimation of urinary testosterone, and MO derivatives of testosterone and other steroid ketones were studied.

It was found that 3-one and 3-one-4-ene steroids gave two isomeric MO derivatives, separable by TLC and GLC methods with a selective phase (NGS, neopentylglycol succinate), but that the derivatives usually did not separate when a nonselective phase was used. These observations, by M. G. Horning et al. (*9*) are currently being used in the study of methods for estimating human urinary steroids present at levels below about 100 μg/24 hr.

The direct separation of adrenocortical hormone metabolites as MO-TMSi derivatives was also studied. A procedure was developed by Gardiner and Horning (*10*) for the separation of human steroids; this method is illustrated in Fig. 2. The steroid sample

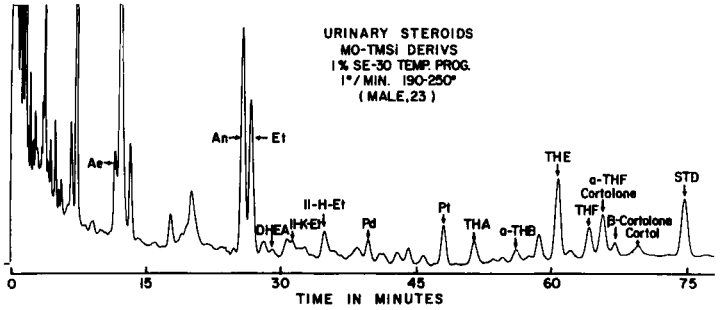


FIG. 2. Separation of human urinary steroids (24-hr collection) as MO-TMSi and TMSi derivatives with a 12-ft 1% SE-30 column with temperature programming from 190 to 250° at 1°/min. The procedure for enzymatic hydrolysis, preparation of derivatives, and GLC separation was carried out as described by Gardiner and Horning (*10*). The steroids are 16-androsten-3α-ol (Ae), androsterone (An), etiocholanolone (Et), dehydroisoandrosterone (DHEA), 11-ketoetiocholanolone (11-K-Et), 11-hydroxyetiocholanolone (11-H-Et), pregnanediol (Pd), pregnanetriol (Pt), tetrahydro-11-dehydrocorticosterone (THA), allo-tetrahydrocorticosterone (a-THB), tetrahydrocortisone (THE), tetrahydrocortisol (THF), β-cortolone, and cortol. Cortolone and allo-tetrahydrocortisol (a-THF) are not separated.

was treated with methoxylamine hydrochloride in pyridine solution, followed by reaction with hexamethyldisilazane. Reactive ketone groups were converted to methoxime groups, as indicated by Fales and Luukkainen (*7*), and reactive hydroxyl groups were converted to TMSi ether groups. Steroids with reactive hydroxyl groups, but without ketone groups, were converted to the usual TMSi derivatives.

Many methods for the GLC separation and determination of

individual steroids, or groups of steroids, have been published, but this is the first practical procedure for separating the major human urinary steroids in a single GLC operation. Its success rests upon the thermal stability of the MO-TMSi derivatives of the adrenocortical steroid hormone metabolites, and upon the use of column conditions that provide adequate resolution for a relatively large number of closely related compounds. However, there are several limitations in the separation procedure. allo-Tetrahydrocortisol (a-THF) is not separated from cortolone, and 11-ketoandrosterone and 11-ketoetiocholanolone are not separated sufficiently for measurement of one in the presence of the other. Modifications of the separation condition are now under study.

When steroids of the adrenocortical group are under study, it is usually desirable to carry out a preliminary column chromatographic (CC) or thin-layer-chromatographic (TLC) separation of the urinary steroid sample. Although current papers generally recommend a TLC separation because of the superior resolution attainable when compared with CC methods, it is our experience that a CC separation is best for the initial fractionation of a urinary sample. After the first CC separation, additional CC or TLC separations may be used for further fractionation. A combination of one or more relatively high-capacity and low-resolution CC or TLC separations with a final low-capacity, high-resolution GLC separation is useful in many biological problems. Figure 3 shows the

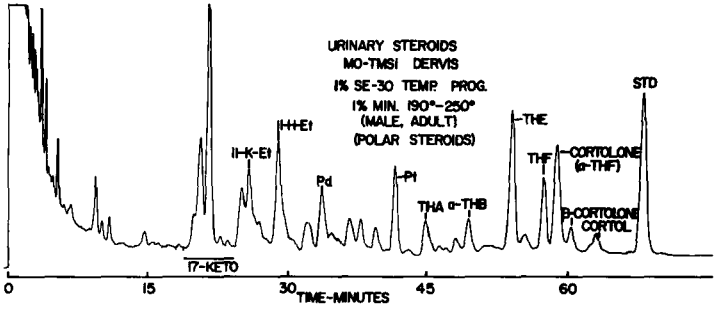


FIG. 3. Separation of "polar" urinary steroids (24 hr collection) as MO-TMSi and TMSi derivatives. The conditions were the same as for Fig. 2. The "polar" fraction was obtained by a single CC separation with silicic acid; some 17-ketosteroids remain, but the separation was used to yield a fraction containing steroids with three or more functional groups (*18*).

separation of the "polar" urinary steroids of an adult male; small amounts of the major 17-ketosteroids remain in the sample, which was obtained by a single CC fractionation.

The steroid profiles shown in Figs. 2 and 3 reflect the functioning of a metabolic network involving a large number of steroids. A metabolic network may be regarded as a dynamic system that is subject to change under many circumstances, and it is usually not possible to alter the concentration in the body of one compound in the group without inducing changes in other related compounds. A GLC method is well suited for the study of change in a metabolic network of this kind. There are many obvious applications of this analytical method, and the following illustrations show three potential areas of use.

An alteration of the steroid metabolic network by the ingestion of a naturally occurring steroid, or by steroidal or nonsteroidal drugs that alter cellular steroid reactions, can usually be defined by this procedure. Figure 4 shows an analysis of a 24-hr urine

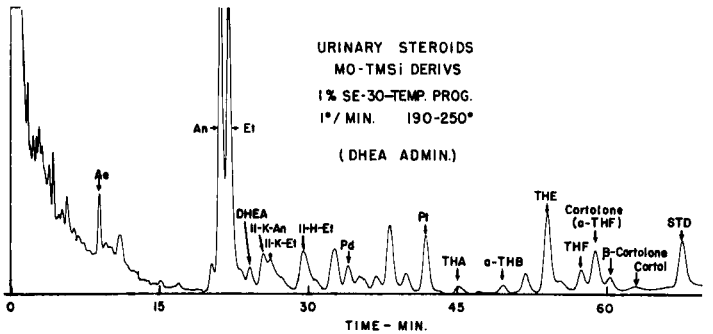


FIG. 4. Separation of urinary steroids obtained after oral administration of 100 mg of dehydroisoandrosterone (24-hr collection). The method was the same as that used for Fig. 2. The major metabolites, androsterone and etiocholanolone, gave off-chart peaks and a metabolite with an elution time of about 38 min was present in increased amount. The latter compound has been identified in preliminary studies as 5-androstene-3β,16α,17β-triol.

sample obtained after the oral ingestion of 100 mg of dehydroisoandrosterone by an adult male normally excreting only trace levels of this steroid. A large increase in the excretion of androsterone and etiocholanolone resulted, and a previously unidentified peak also increased in size. This compound was subsequently identified as 5-androsten-3β,16α,17β-triol, a steroid which is normally present

as an adult urinary steroid and which is probably formed by liver 16α-hydroxylation of dehydroisoandrosterone, followed by reduction of the 17-keto group. A small increase was also observed in the output of THE and THF, although the pattern of adrenocortical steroid metabolites was qualitatively unchanged.

Figure 5 illustrates a different type of problem. The total amounts

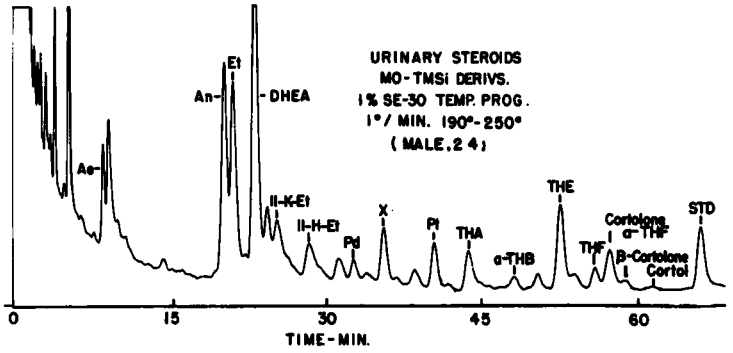


FIG. 5. Separation of urinary steroids from a young adult male (24-hr collection) with an elevated excretion of dehydroisoandrosterone (DHEA). The method was the same as that used for Fig. 2. The peak marked X is not the same as that noted in Fig. 4.

of "17-ketosteroid" and "17-ketogenic steroids" are within the normal range, but an altered metabolic network is clearly present. The circumstances that might lead to elevated dehydroisoandrosterone excretion are overproduction or underutilization of this steroid. Overproduction is known to occur as a consequence of adrenal tumors involving the cells that produce dehydroisoandrosterone, and underutilization is known to occur in some instances of testicular insufficiency (*11*). The possibility of obtaining information useful in clinical diagnostic problems involving steroids is evident.

These methods are also valuable in comparative and developmental biochemical studies. It is known that the urinary steroids of the newborn infant are different from those of the child or adult. This aspect of human developmental chemistry is highly important but little studied, because of previously inadequate analytical methodology for studying microgram amounts of complex steroid mixtures. Paper-chromatographic separations have been used to demonstrate differences in newborn and adult steroids (*12*), and

several of the steroids of the newborn have been identified in the past few years. The most extensive studies have been those of Bongiovanni and his colleagues (*13*) and of Reynolds (*14*) in the United States, and of Mitchell and his colleagues in Scotland (*12,15*).

Figures 6 and 7 show steroid profiles for the "polar" steroids

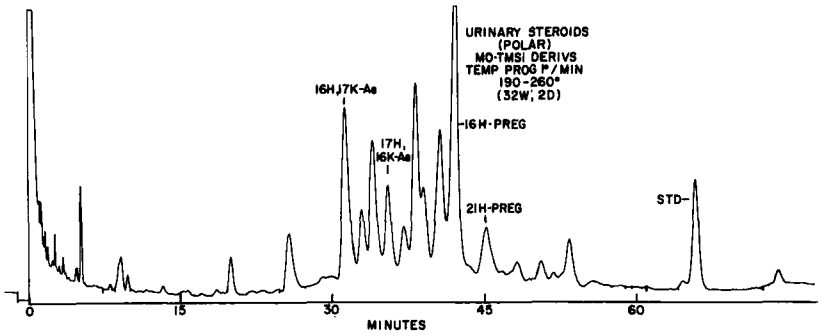


FIG. 6. Separation of "polar" urinary steroids for an infant delivered at 32 weeks; the 24-hr sample was obtained 2 days after birth. The method was the same as that used for Fig. 3. The compounds are 16α-hydroxydehydroisoandrosterone (5-androsten-3β,16α-diol-17-one) (16H,17K-Ae), 16-ketoandrostenediol (5-androsten-3β,17β-diol-16-one) (17H,16K-Ae), 16α-hydroxypregnenolone (5-pregnen-3β,16α-diol-20-one) (16H-Preg), 21-hydroxypregnenolone (5-pregnen-3β,21-diol-20-one) (21H-Preg), and cholesteryl butyrate (STD).

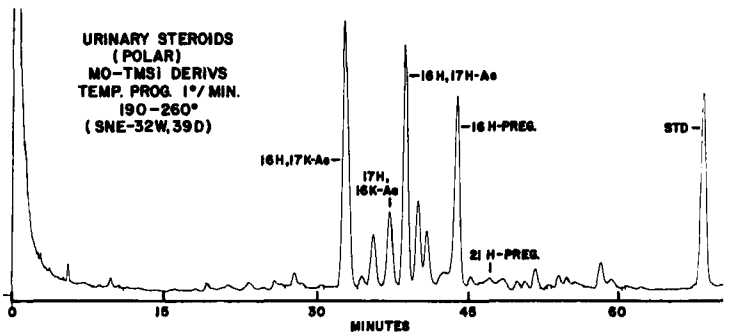


FIG. 7. Separation of "polar" urinary steroids for an infant delivered at 32 weeks; the 24-hr sample was obtained 39 days after birth. The method was the same as that used for Fig. 3. The compounds are the same as those in Fig. 6, with the added identification of 5-androsten-3β,16α,17β-triol (16H,17H-Ae).

of two infants; a single 24-hr urine sample provides sufficient material for an analytical study. These profiles show considerable variation in quantitative relationships; the origin of this effect is not known.

Methods for the identification of the steroids in separations of the kind shown in Figs. 2 to 7 and the development of procedures for the quantification of these metabolic networks are discussed in the next sections.

IDENTIFICATION METHODS

Current GLC methods for studying steroid mixtures are based on the use of nonclassical derivatives and thin-film GLC columns with about 3000 to 6000 theoretical plates. In our experience it is possible to find a separation condition for all naturally occurring steroids that have been studied, but the limitations of GLC data in providing structural information are well known. An identification cannot be made on the basis of a single GLC comparison, although considerable structural information can be gained through the study of derivatives by the "steroid number" approach (*16,17*). When complex mixtures are under study, the most effective methods of identification are those of GLC-mass spectrometry, supported by accessory GLC and other instrumental or chromatographic data. These methods have been used to identify several urinary steroids of the newborn human infant (*18*). A sample of the steroid mixture shown in Fig. 7, before derivative formation, was fractionated by thin-layer chromatography on silica gel. A major band was separated; the steroids of the zone were converted to MO-TMSi derivatives and subjected to GLC analysis with the result shown in Fig. 8. The major component, as the MO-TMSi derivative, was studied by GLC-mass spectrometry. The mass spectrum indicated that the steroid had a 3β-ol-5-ene structure (a peak at m/e 129 was observed) with one hydroxyl group (convertible to a TMSi ether group) in addition to a pregnenolone structure. Comparison of the GLC and GLC-MS data for the unknown and for an authentic sample of 16α-hydroxypregnenolone indicated that the two substances were identical in structure.

These methods were used to identify 21-hydroxypregnenolone, 16α-hydroxydehydroisoandrosterone, and 16-ketoandrostendiol, in addition to 16α-hydroxypregnenolone. These steroids have been identified previously as compounds that may be found as excretion

products of the newborn [21-hydroxypregnenolone (*15*); 16α-hydroxydehydroisoandrosterone (*14,19*); 5-androsten-3β,17β-diol-16-one (*14*); 16α-hydroxypregnenolone (*14*)]. In addition, 5-androsten-3β,16α,17β-triol has been identified recently as another steroid of the newborn (*20*); this compound is known to be a steroid of fetal blood (*21*), and it has also been identified as an adult urinary steroid. Several still unidentified steroids may also have a 3β-ol-5-ene structure.

The study of mass spectra of nonclassical derivatives of steroids is a specialized field that is best discussed separately. However, in addition to use in structural studies, MS methods are also of key

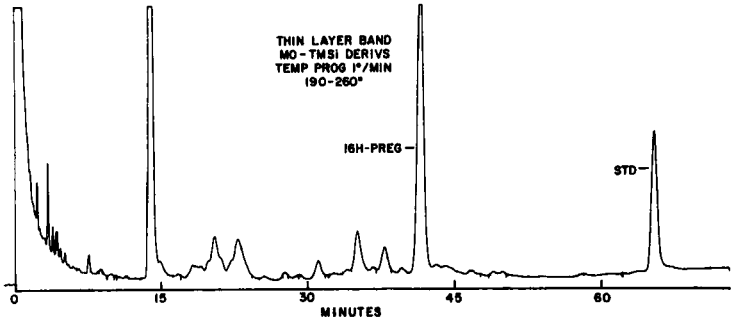


FIG. 8. Separation of MO-TMSi and TMSi derivatives of steroids present in a TLC zone. The original steroid sample was identical with that used for Fig. 6. The method was described by Gardiner et al. (*18*); the major component was identified as 16α-hydroxypregnenolone by GLC-mass spectrometry.

importance in determining homogeneity of a GLC peak that is being measured for quantitative purposes in a biological study. When a large increase in a peak occurs in a typical GLC separation representing a metabolic network, the conclusion is usually drawn that the large peak has the same composition as the smaller peak found previously in the same position. This may not be true, and a GLC-MS identification should be carried out to determine the identity of the substance or substances present in the major peak before quantification is attempted.

QUANTIFICATION

The usefulness of hydrogen flame ionization detection systems in quantitative studies is now well established, and it is known

that quantification may be achieved in the temperature-programmed separation of fatty acid methyl esters (22). However, very little information has been published with respect to the quantitative aspects of temperature-programmed steroid separations. We have studied this problem for the purpose of developing an automated GLC steroid analysis system for separations of the kind shown in Figs. 2 to 7.

The apparatus was a gas chromatograph (Barber-Colman Model 5000) modified for use with a special sample introduction device. The samples were introduced on a stainless steel or platinum gauze by gravity drop from a Teflon turntable into a slotted glass thimble extending into the vaporization zone. The temperature cycling was automated with a nominal 1°/min temperature rise over the separation period. The entire cycle, including cool-down and equilibration before sample addition, required about 1.5 hr. A special adapter was also constructed for experimental studies; this permitted side-arm addition of gauze (stainless steel) samples (by magnet manipulation) and ordinary liquid addition by use of a long (5″) needle Hamilton syringe.

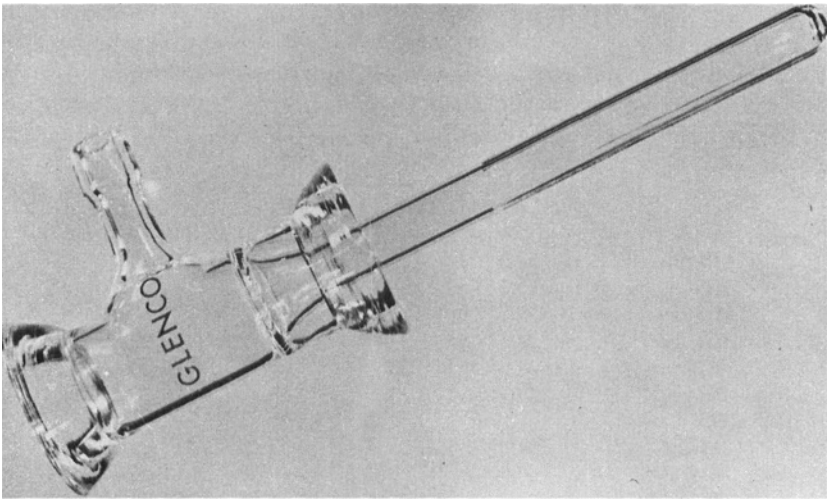


FIG. 9. Adapter used to connect the chromatographic tube to the automatic turntable for sample addition. The lower end of the adapter tube is slotted, and serves as a receptacle for the gauze supports. The tube may be of varying dimensions; in this instance the tube was 8 mm OD with a length arranged so that the bottom was near the lower end of the flash heater zone.

The technique of sample addition on a metal gauze or plate has been described several times. Our method was modified from that of Menini and Sommerville (23). The amount of solvent evaporated on a single stainless-steel or platinum gauze was arbitrarily set at 100 μl. A polished Teflon plate with 2-cm-diameter depressions was used for the evaporation stage.

It is generally believed that a relatively large volume should be avoided for the vaporization chamber. However, to provide a holding volume for the gauze samples it was necessary to employ a gas-chromatographic tube with an enlarged vaporization chamber; the adapter holding the samples after injection is shown in Fig. 9. The initial studies were with four-component hydrocarbon samples of known composition, and the objective was to compare analyses obtained with gauze injection and with the ordinary solution technique. Table 1 shows the results. In this instance the gauze samples were added through a side arm rather than by an automated system.

Table 2 contains the results of a similar experiment with a four-component hydrocarbon mixture in which all samples were added by the metal gauze procedure.

Table 3 contains the results of a series of steroid analyses using

TABLE 1

Replicate Analyses of Four-Component Hydrocarbon Mixture ($C_{22}H_{46}$ to C_{28} to H_{58})[a]

	Hydrocarbon			
	$C_{22}H_{46}$	$C_{24}H_{50}$	$C_{26}H_{54}$	$C_{28}H_{58}$
Liquid	24.2	26.2	25.8	23.7
Gauze	25.1	25.3	25.8	23.9
Gauze	25.7	25.2	26.1	23.0
Gauze	24.6	26.0	26.6	22.8
Liquid	24.9	25.1	26.9	23.1
Liquid	25.1	25.1	26.6	23.1
Mean	24.9	25.5	26.3	23.3
Std. dev.	0.5	0.4	0.6	0.6
Weight %	24.9	25.4	25.4	24.3
Deviation	0.0	0.1	0.9	−1.0

[a] These analyses were carried out in the sequence shown, with both liquid sample injection in isooctane solution and by gauze injection of sample from the same solution.

TABLE 2

Replicate Analyses of Four-Component Hydrocarbon Mixture ($C_{22}H_{46}$ to $C_{28}H_{58}$) with Gauze Injection[a]

	Hydrocarbon			
	$C_{22}H_{46}$	$C_{24}H_{50}$	$C_{26}H_{54}$	$C_{28}H_{58}$
	24.5	25.3	26.5	23.6
	25.2	25.9	25.4	23.5
	24.3	25.3	26.5	23.9
	24.1	25.5	26.7	23.6
	24.3	25.1	26.8	23.8
	24.5	25.5	26.4	23.6
	24.6	25.3	26.0	24.0
	25.1	25.7	26.2	23.0
Mean	24.6	25.5	26.3	23.6
Std. dev.	0.4	0.3	0.4	0.3
Weight %	24.9	25.4	25.4	24.3
Deviation	−0.3	0.1	0.9	−0.7

[a] All samples were added by gauze injection over a 3-day period with daily resetting of gas-flow valves.

reference compounds. The results were calculated by normalization to determine the precision of the gauze procedure.

Table 4 contains analytical data for repetitive automated analyses of a reference mixture containing androsterone, dehydroisoandrosterone, pregnanediol, pregnanetriol, tetrahydrocortisone, tetrahydrocortisol, and an internal standard (cholesteryl butyrate). The steroids were converted to MO-TMSi and TMSi derivatives by our usual procedure (*10*). The calculations are based on an arbitrary assignment of cholesteryl butyrate area = 10.0.

No systematic errors were evident. Figure 10 shows a typical analytical chart obtained in an automated separation. A gradual broadening of the peaks with time may be observed; this is believed to be due to differences in rate of vaporization of the compounds in the vaporizing chamber. Retention times were highly reproducible. The measurements of areas were made by taking peak height × width at half-height. A faster chart speed, or electronic integration of the areas, may be used to increase the precision of the measurements.

The values in Table 4 represent area relationships, and for actual

analyses these values must be multiplied by experimentally determined response factors. There is at present no theoretical basis for the calculation of response factors for MO-TMSi and TMSi derivatives of steroids of differing structure; these values should be determined locally with reference samples of high purity.

These results indicate that temperature-programmed separations of steroids may be carried out in an automated system, with metal gauze injection, in a fashion suitable for quantitative analytical work. However, the analysis of samples of biologic origin is a more complicated matter than the analysis of mixtures of reference compounds. Impurities or additional substances of biologic origin are normally present, although this will depend on the origin and method of preparation of the sample, and it is necessary to determine the extent to which these impurities or associated compounds will affect the results. Two aspects of this problem have been studied. Figure 11 shows the adrenocortical region of a chart

TABLE 3

Replicate Analyses of Four-Component Mixture of MO-TMSi and TMSi Derivatives of Steroids[a]

	Steroid			
	An[b]	DHEA[c]	Pd[d]	Pt[e]
	24.8	20.2	38.3	16.7
	24.9	20.1	37.4	17.6
	24.7	19.6	38.2	17.5
	24.9	19.6	38.1	17.5
	24.6	20.0	38.2	17.2
	24.6	20.0	37.5	17.9
	24.1	20.0	37.8	17.7
	24.8	20.0	37.7	17.3
	25.1	19.9	37.5	17.5
	24.2	20.0	38.2	17.5
Mean	24.7	19.9	37.9	17.4
Std. dev.	0.3	0.2	0.3	0.3

[a] All samples were added by gauze injection over a 3-day period with daily resetting of gas-flow valves.
[b] Androsterone as MO-TMSi derivative.
[c] Dehydroepiandrosterone as MO-TMSi derivative.
[d] Pregnanediol as TMSi derivative.
[e] Pregnanetriol as TMSi derivative.

obtained during a temperature-programmed analysis of an authentic urinary steroid sample, prepared in the form of MO-TMSi and TMSi derivatives according to Gardiner and Horning (*10*), with the use of a metal gauze for sample addition through a side-arm adapter. The same result was obtained by liquid sampling of another portion of the mixture, indicating that no alteration of composition occurred when a gauze support was used. However, when metal gauze supports carrying urinary samples were allowed to remain in the automated device for several hours, a deterioration of the sample, with loss of adrenocortical metabolites, was observed. Liquid sampling of the original mixtures (containing

TABLE 4

Replicate Analyses of Six-Component Mixture of Steroids as MO-TMSi and TMSi Derivatives, with Cholesteryl Butyrate as an Internal Standard[a]

	Steroid					
	An	DHIA	Pd	Pt	THE	THF
	7.6	5.9	10.0	4.6	5.6	3.3
	7.7	5.9	9.7	4.8	5.8	3.3
	7.3	5.8	9.9	4.7	5.7	3.4
	7.1	5.7	9.6	4.6	5.6	3.2
	7.5	5.7	9.7	4.6	5.6	3.1
	7.2	5.8	9.8	4.6	5.8	3.1
	7.3	5.8	10.1	4.6	5.4	3.1
	7.7	5.9	10.0	4.8	5.1	3.0
	7.5	5.6	9.8	4.6	5.0	2.8
	7.2	5.4	9.4	4.3	5.0	2.8
	7.2	5.6	10.0	4.9	5.8	3.4
	7.4	5.8	10.4	4.8	6.0	3.5
	7.4	5.9	9.9	4.9	5.8	3.4
	7.3	5.6	9.8	4.7	5.5	3.3
	7.3	5.7	9.8	4.8	5.8	3.3
	7.9	6.5	11.0	5.2	5.9	3.5
	7.8	6.1	10.5	4.7	6.2	3.7
	7.5	6.3	10.3	4.9	6.2	3.9
	7.7	5.9	10.1	4.6	5.9	3.4
	7.3	5.9	9.7	4.8	5.9	3.4
Mean	7.5	5.8	10.0	4.7	5.7	3.3
Std. dev.	0.2	0.2	0.4	0.2	0.3	0.3

[a] The area measurements were calculated with the arbitrary value of 10.0 for cholesteryl butyrate. All samples were added by automated gauze injection.

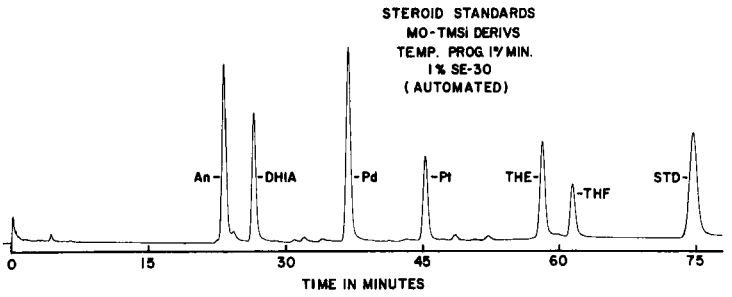


FIG. 10. Separation of reference compounds as MO-TMSi and TMSi derivatives with an automated GLC system. The steroids are androsterone (An), dehydroisoandrosterone (DHIA), pregnanediol (Pd), pregnanetriol (Pt), tetrahydrocortisone (THE), tetrahydrocortisol (THF), and cholesteryl butyrate (STD). The column was a glass 12 ft × 4 mm diameter W-tube with 1% SE-30 on 100–120 mesh Gas Chrom P; the packing was prepared according to the usual methods of this laboratory, and the procedure for derivative formation was that used for Figs. 1 to 7. Small stainless-steel or platinum gauze rolls were used as the support.

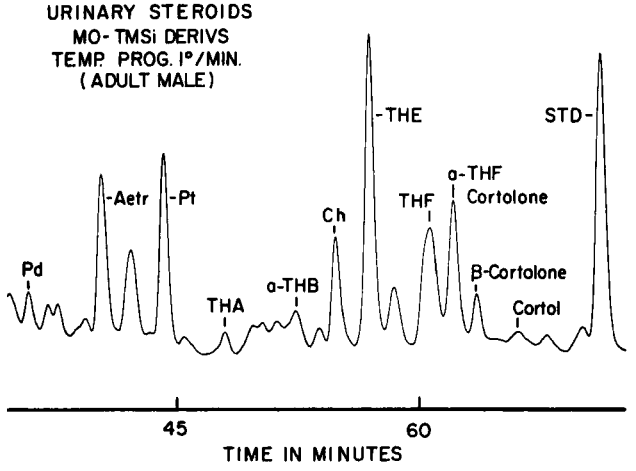


FIG. 11. Terminal section of chart from an analytical separation of MO-TMSi and TMSi derivatives of human urinary steroids. The sample was not identical with that used for Fig. 3, but it was from a similar experiment. The analysis was carried out in the same way as for Fig. 3, but the sample was added on a stainless-steel gauze roll introduced through a side-arm adapter. The same results were obtained when a liquid sample of the reaction mixture was introduced with the aid of a long (5-in.) needle Hamilton syringe.

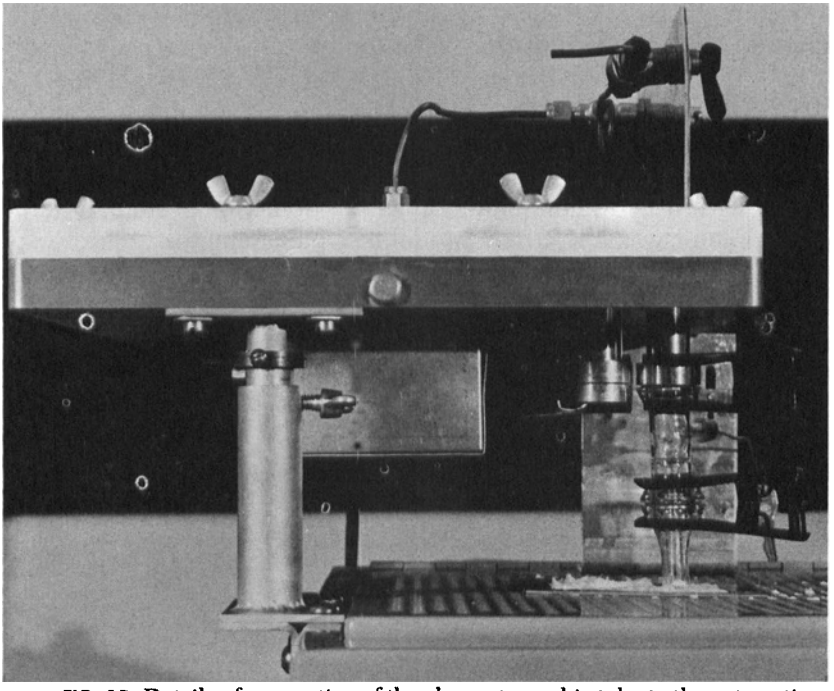


FIG. 12. Details of connection of the chromatographic tube to the automatic sample addition device with the adapter shown in Fig. 9.

excess hexamethyldisilazane) showed that no change had occurred in solution. This effect was presumably due to incomplete purging and to the presence of materials in the sample which might catalyze hydrolytic reactions in the absence of excess hexamethyldisilazane. Current work is directed to studying the effects of preliminary CC silicic acid purification of the steroid sample (in the form of free steroids or after derivative formation), and of the effect of efficient purging of the chambers of the sample addition device. The stability of the sample is a matter of key importance for an automated system of this kind.

The separation was good and there was no evidence of sample deterioration. The shape of the THF peak suggests that two components were present; the other peaks have their usual appearance. Two compounds not noted in the other figures are 5-androsten-3β,16α,17β-triol (Aetr), and cholesterol (Ch).

Figure 12 shows the details of the connection of the chromatographic column (a 12-ft glass W-tube, 4-mm diam., packed with 100–120 mesh Gas Chrom P containing 1% SE-30 polymer) to the sample addition device, by means of the adapter shown in Fig. 9, and Fig 13 shows the instrumental assembly. The oven and automated controls were modified units of the Barber-Colman 5000 series with a hydrogen flame ionization detection system, and the electrometer was a Keithley Model 417 picoammeter.

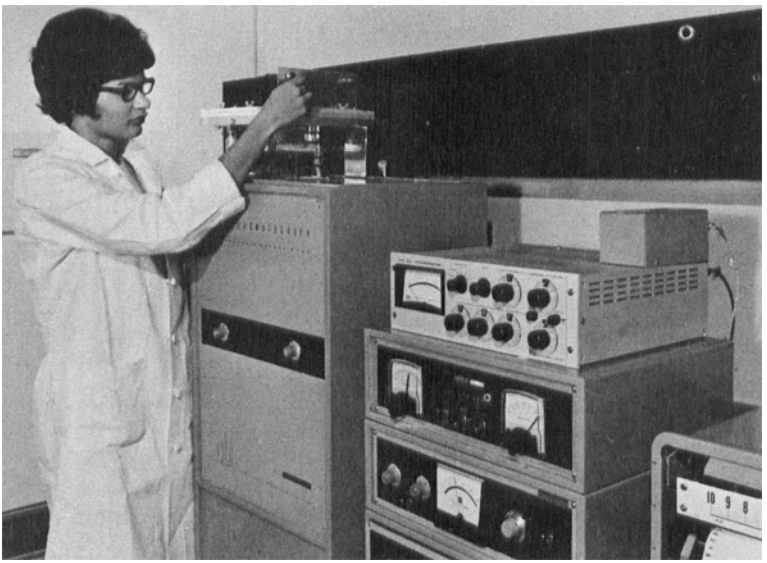


FIG. 13. Assembly of equipment for automated system.

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