# Precise Ultramicro Determination of Steroid Hormones by Combined TLC and GLC Analysis[1]

**F. A. VANDENHEUVEL, Animal Research Institute, Research Branch, Department of Agriculture, Ottawa, Ontario, and G. J. HINDERKS and J. C. NIXON, National Defence Medical Center, Ottawa, Ontario, with the technical assistance of W. G. LAYNG**

## Abstract

The migration of steroid hormones on TLC plates is predictable on the basis of the number of double bonds and carbonyl and hydroxyl groups they contain. Developed plates can therefore be divided into zones or bands corresponding to well-defined classes of steroids. Steroids can be eluted from the adsorbent quantitatively and without contamination. The eluate from each zone contains a mixture which is much simpler than the original one.

The conversion of OH-containing steroids to trimethylsilyl ether (TMS) derivatives is quantitative; it is complete after 3 hr by the described technic. All TMS derivatives are very stable in $CS_2$ solution. Whether or not they contain OH groups, steroids other than corticosteroids are stable under the present GLC conditions after treatment with TMS reagents. With amts of injected sample increasing from 0 to 0.2 $\mu$g, a small apparent increase in specific response is observed with some steroids. This effect is reproducible. The use of calibration curves obtained with an internal standard (5$\alpha$-pregnane) leads to accurate analyses of mixtures with injections containing 25 nanograms (0.025 $\mu$g) of individual steroid. Values obtained from single trials fall 95% of the time within 2% of the mean.

Peak elution time of each compound is accurately predictable on the basis of number, position, and stereoconfiguration of functional groups.

An integrated TLC-GLC method of analysis based on these findings is outlined.

## Introduction

SPEED AND RELIABILITY of analyses by gas liquid chromatography (GLC) with the flame ionization detector can be appreciably increased by the use of technics which have been applied successfully with either volatile (1) or high boiling compounds (2). By lowering the quantities of injected compounds to the submicrogram level, a given resolution can be accomplished with shorter columns, lower temp and shorter elution times. Under sustained operation at low sample level and lower temp, a higher consistency in detector response and in column characteristics is obtained over prolonged periods. Because analytical time is short, the data required for calibration and for an analysis of variance can be obtained routinely. Accurate analyses are more readily accessible through these simple technics, particularly with the use of internal standards (1,2).

An extension of these advantages to the analysis of very complex mixtures requires a preliminary separation into mixtures which are simple enough to be resolved by relatively short columns. Thin-layer chromatography (TLC) has been suggested (2) for this purpose. The simplicity, the rapidity, the low sample requirements of TLC, and its adaptibility to serial determination match corresponding features of the above GLC technic. It also provides an additional cleanup step for material of biological origin.

The analysis of complex mixtures of steroid hormones extracted from animal tissues and body fluids typify the problem to which a combination of TLC and GLC opens a new and promising approach. The present communication describes detailed procedures whereby the value of this combination can be tested with mixtures of pure steroids. In addition, results on the separation of a large number of hormones and metabolites of the estran (E), androstran (A), and pregnan (P) series are offered as evidence of the workability of an integrated TLC-GLC method. Advantages in the use of two TLC solvent systems and two GLC columns differing in polarity are exemplified and the principles guiding in a selection of such pairs are briefly discussed.

Our comparative evaluation of TLC and GLC systems in relation to particular cases and specific analytical requirements is still incomplete. Hence, the systems used in the present demonstration may not be the most effective in connection with a TLC-GLC analytical method. However, their merits in this respect appear considerable enough to warrant their use as described in the following outline of the proposed method.

The sample which is a mixture of free steroids such as obtained from biological material through known extractive procedures (3), is submitted to TLC (solvent system I) and the developed plate divided into zones (Fig. 1). The adsorbent located in each of the zones of interest is removed and extracted. Band extracts are evaporated to dryness and the residue mixed with TMS reagents. $CS_2$ solutions of the products are injected into the gas chromatograph equipped with one of each (or two of either) described column type. Corticosteroids do not produce any peak under these conditions. Their estimation by GLC is made on TMS derivatives obtained after reduction of the extract from a specific TLC zone (solvent system II).

The present account comprises three sections followed by a general discussion. Section 1 describes the TLC technics including the removal and elution of bands. Section 2 gives the preparation of derivatives and the GLC procedure for all compounds except corticosteroids (to be described in a separate article). In Section 3 the use of the TLC and GLC data for the purpose of identification is demonstrated.

## Thin-Layer Chromatography

### Equipment

Desaga adjustable spreader, template, and developing tanks, and 200/200/3.8 mm glass plates.

Chromatofilm racks (No. 200–9a, Research Specialities, Richmond, Calif.).

The spreader slit width was adjusted to 0.25 mm. The tanks were fitted with a four-side, full-height inside lining made of folded chromatographic paper (Whatman No. 3MM). Narrow slits were cut out from

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the wider sides of the lining to observe plates under development. The tank lids were ground smooth with very fine emery powder and the ground surfaces used dry. Several tanks were kept under a single hood made of light transparent plastic material as a protection against draft.

#### Materials and Reagents

Adsorbosil—with added binder—, (Applied Science Laboratories, Inc., Penn State, Pa.). Methanol [Analar, B.D.H. (British Drug House)], chloroform (Fisher C-574), ethyl acetate (Analar, B.D.H.), steroids (Steraloids, Inc., Flushing 52, N.Y.), Sudan blue (No. 3788, Harleco), Phosphomolybdic Acid (Analar, British Drug House).

#### Solutions

Solvent system I, chloroform:methanol:water 200:12:1, (V/V/V); solvent system II, ethyl acetate:chloroform:water 180:20:2, (V/V/V). Sudan blue, saturated solution in ethyl ether. Phosphomolybdic acid, 10% (W/V) in 95% ethyl alcohol. Steroids, stock solutions in methanol containing 2.5 $\mu g/\mu l$. These were kept at 5C in the dark when not in use.

#### Procedure

*Preparation of Plates.* For five plates, a mixture of 30 g Adsorbosil in 45 ml water was shaken vigorously for 2 min under vacuum in a 250 ml round-bottom flask. The slurry was spread immediately, and the template tapped on the side to promote evenness. After a 30 min hardening period the plates were dried in an oven at 108C for 2 hr, cooled for 15 min and placed in a dessicator cabinet overnight.

*Tank conditioning* included an initial soaking of the paper lining. The solvent remaining from a previous operation (or 100 ml fresh solvent when starting from a clean dry tank) was used for this purpose. Excess solvent was poured out in either case and 200 ml fresh solvent were added to the tank. The latter was immediately covered and left to equilibrate for 2 hr.

*Sample spotting* was made in tiny holes printed in the adsorbent layer with a sharp point every 10 mm along a line located 15 mm from the plate bottom. A template was used for this purpose. For standardization up to 2 $\mu l$ of standard solution (5 $\mu g$) per spot was delivered with a Lambda pipette. In all cases three to four dye spots (Sudan blue) (2 $\mu l$) were included. The plates were stored in a dessicator cabinet until the tanks were equilibrated.

For *development,* plates were placed back to back on plate supports, two to a tank. The solvent level of the tank reached 5 mm below the starting line. The plates were removed from the tank when the solvent front reached 15 cm from the starting line, i.e., after about 1 hr.

#### Standardization

The dry plates were sprayed with phosphomolybdic acid solution. They were left to dry for 10 min on the bench, then placed on a hot plate kept at about 200C. The stable chromatograms consisted of dark blue spots on a yellow background. A light box was used to transfer the center and contour positions of spots onto translucent millimetric paper. The distance, d, of spots from the origin was measured and the $R_b$ value determined from $d/d_b = R_b$, where $d_b$ is the distance travelled by the dye.

*$R_b$ Data.* $R_b$ values are shown in Table I where compounds are listed by bands obtained with solvent system I, in order of appearance on (JXR) dimethylpolysiloxane chromatograms. The corresponding $R_b$ values in solvent system II are given also. In Table II, $R_b$ values for corticosteroids in both systems are given. Figure 1 shows a plot of average $R_b$ values (solvent system I) for steroids with a number, X, of hydroxyls and a number, Y, of double bonds, against the number, Z, of keto groups. Each point thus corresponds to a class of steroids characterized by the same X:Y:Z formula.

#### Zone Elution

The boundaries of bands containing specific classes of steroids were marked on the plate (Fig. 1). The areas of interest were scraped off (10 sq cm $\approx$ 100 mg) and collected separately using the aspirator described in Figure 2, I. Aspirator head H was then disconnected and body B was clamped over a collecting flask and used as a filter. Methanol was poured over the powder (0.5 ml per 100 mg) with a syringe and allowed to soak for 5 min. Adapter P, connected to an adjustable pressure source, was then used to force the liquid gently through filter M (medium porosity). This was repeated twice. The total extract was evaporated under a stream of nitrogen in a small flask held in a sand bath maintained at about 45C. The TMS derivatives were prepared in the same flask (see GLC section). GLC analyses of recovered compounds were compared with parallel analyses of known volumes of the solutions used in spotting the plates. Differences observed were no larger than the standard error ($\pm$ 1.5%) of the GLC method. Background GLC analyses made on extracts of adsorbent from undeveloped and developed unspotted plates showed no evidence of impurities derived from powder or solvents when the described extraction procedure was used.

## Gas-Liquid Chromatography

#### Equipment

Perkin Elmer 800 Chromatograph.
Phillips No. PR 2216 A/21 Recorder.
Perkin Elmer No. 194 Printing Integrator.

Installation of the above equipment was made according to manufacturers instructions except that the chromatograph was electrically dissociated from the recorder and integrator by connecting the two latter instruments to a separate, "quiet" main supply line (1,2).

Perkin Elmer No. 226-11-44 silicone rubber septa were used without pretreatment (2).

#### Materials and Reagents

JXR, 3% dimethylpolysiloxane on 100/120 silanized Gas-Chrom P, and SE 52, 2% on 80/100 Gas-Chrom Z (Applied Science Laboratories, Inc.).

Hexamethyldisilazane, and Trimethylchlorosilane (Chemical Procurements Laboratories, College Point 56, N.Y.). These reagents were redistilled.

$CS_2$ (Fisher, No. C 184).

Steroids (Steraloids Inc.).

#### Column Packing and Conditioning

Two 6 ft straight lengths of 1/8 in. o.d., stainless steel tubing were cut from the same stock, plugged at one end and clamped vertically, side by side at about 2 in. from each other. The top open end of each column was connected with a short section of 1/8 in. i.d. polyethylene tubing to the 1/8 in. outlet of a 10 cm long, 14 mm i.d., graduated glass funnel (2 cm long, 1 mm i.d. outlet). Equal amts (ca. 3 g) of the packing material were placed in the funnels and the col-

TABLE I

TLC and GLC Data for Androstan (A), Estran (E) and Pregnane (P) Compounds Excluding Corticosteroids (Table II); $R_b$ Values in Solvent System I and II: Compounds listed by Bands (System I, Fig. 1) and by Relative (5α-Pregnane) Retention Times $(Rt'_R)^{a,b}$, for JXR Columns; Comparison of Experimental with Calculated (Table IV) $Log_{10}$ $Rt'_R$ Values (JXR); $Rt'_R$ Values on SE 52 Columns

| Structural Features | | | | TLC | | | | | | | GLC | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Class | | | | Keto(K), OH(α or β) $R_b \times 10^3$ | | | | | | | JXR[a] | | | SE 52[a] |
| | | | | | | | | | | | | $10^3 \times log_{10}$ $Rt'_R$ | | |
| Nr | 5[a] | Δ[d] | Series | 3 | 11 | 16 | 17 | 20 | I | II | $Rt'_R$[b] | Expt. | Calcul. | $Rt'_R$[b] |
| **Band I (solvent system I), $R_b$ from 0.900 upwards. Saturated and Unsaturated mono, di, and triketones.** | | | | | | | | | | | | | | |
| 1 | β | | A | | | | | | 980 | 1060 | 0.476 | —323 | —323 | 0.475 |
| 2 | α | | A | | | | | | 980 | 1030 | 0.534 | —273 | —273 | 0.534 |
| 3 | α | | P | | | | | | 990 | 1050 | 1.00 | 0 | 0 | 1.00 |
| 4 | α | | A | | | | K | | 990 | 1030 | 1.07 | 29 | 28 | 1.19 |
| 5 | α | | A | K | | | | | 990 | 1025 | 1.18 | 72 | 71 | 1.12 |
| 6 | β | | P | K | | | | | 980 | 1020 | 2.01 | 304 | 304 | 2.26 |
| 7 | β | | A | K | | | K | | 960 | 925 | 2.10 | 322 | 322 | 2.715 |
| 8 | | 4 | A | K | | | K | | 950 | 845 | 2.90 | 462 | 453 | 3.89 |
| 9 | | 1,4 | A | K | | | K | | 900 | 770 | 3.14 | 497 | 495 | 4.31 |
| 10 | | 4 | A | K | K | | K | | 920 | 715 | 3.53 | 548 | 549 | 5.14 |
| 11 | β | | P | K | | | | K | 960 | 935 | 3.69 | 567 | 565 | 4.60 |
| 12 | α | | P | K | | | | K | 965 | 945 | 4.12 | 615 | 615 | 4.83 |
| 13 | | 4 | P | K | | | | K | 950 | 905 | 4.95 | 695 | 696 | 6.68 |
| 14 | α | | P | K | K | | | K | 940 | 830 | 5.64 | 752 | 742 | 7.78 |
| 15 | | 4 | P | K | K | | | K | 930 | 710 | 6.80 | 832 | 830 | — |
| **Band II (solvent system I), $R_b$ from .725 to .900 Monohydroxy, and Monohydroxy-Monoketo Compounds.** | | | | | | | | | | | | | | |
| 16 | α | | A | α | | | | | 900 | 950 | 1.12 | 49 | 49 | 1.11 |
| 17 | α | | A | β | | | | | 865 | 910 | 1.44 | 158 | 161 | 1.46 |
| 18 | β | | A | β | | | K | | 860 | 860 | 2.17 | 326 | 333 | 2.39 |
| 19 | β | | P | α | | | | | 865 | 940 | 2.24 | 350 | 344 | 2.25 |
| 20 | α | | A | α | | | K | | 865 | 875 | 2.22 | 346 | 350 | 2.48 |
| 21 | β | | A | α | | | K | | 825 | 815 | 2.37 | 374 | 372 | 2.66 |
| 22 | α | | P | β | | | | | 835 | 910 | 2.72 | 434 | 434 | 2.75 |
| 23 | | 5 | A | β | | | K | | 800 | 850 | 2.78 | 444 | 443 | 3.19 |
| 24 | α | | A | β | | | K | | 825 | 830 | 2.90 | 462 | 462 | 3.30 |
| 25 | α | | A | K | | | β | | 840 | 860 | 3.05 | 484 | 486 | 3.56 |
| 26 | | 4 | A | K | | | α | | 830 | 795 | 3.11 | 493 | 497 | 3.84 |
| 27 | | 1,3,5 | E | OH | | | K | | 825 | 970 | 3.14 | 497 | 497 | 3.87 |
| 28 | | 4 | A | K | | | β | | 815 | 775 | 3.82 | 582 | 577 | 4.65 |
| 29 | | 1,4 | A | K | | | β | | 745 | 670 | 4.23 | 626 | 619 | 5.16 |
| 30 | | 5,16 | P | β | | | | K | 790 | 865 | 4.49 | 652 | 651 | 5.07 |
| 31 | | 5 | P | β | | | | K | 820 | 870 | 4.79 | 681 | 686 | 5.52 |
| 32 | α | | P | β | | | | K | 800 | 850 | 5.05 | 703 | 705 | 5.65 |
| 33 | α | | P | K | | | | β | 875 | 910 | 5.85 | 767 | 769 | 6.74 |
| **Band III (solvent system I), $R_b$ from .550 to .725 Diols, Monohydroxydiones, and Monoketodiols.** | | | | | | | | | | | | | | |
| 34 | α | | A | α | | | β | | 690 | 785 | 2.97 | 473 | 474 | 2.96 |
| 35 | | 5 | A | β | | | α | | 670 | 805 | 3.07 | 487 | 487 | 3.10 |
| 36 | | 4 | A | β | | | β | | 680 | 800 | 3.58 | 554 | 567 | 3.74 |
| 37 | | 5 | A | β | | | β | | 660 | 775 | 3.70 | 568 | 567 | 3.81 |
| 38 | α | | A | β | | | β | | 670 | 760 | 3.77 | 576 | 586 | 3.90 |
| 39 | | 1,3,5 | E | OH | | | β | | 625 | 900 | 4.16 | 619 | 621 | 4.61 |
| 40 | β | | P | α | | | | β | 635 | 770 | 5.84 | 766 | 769 | 5.82 |
| 41 | | 5 | A | β | | K | β | | 635 | 730 | 5.89 | 770 | 770 | 6.64 |
| 42 | β | | P | α | | | | α | 655 | 745 | 6.18 | 791 | 769 | 6.40 |
| 43 | | 4 | P | β | | | | β | 700 | 825 | 6.86 | 836 | 840 | 7.18 |
| 44 | α | | P | β | K | | | K | 730 | 670 | 7.21 | 858 | 852 | 8.74 |
| 45 | α | | P | β | | | | β | 660 | 795 | 7.24 | 860 | 859 | 8.46 |
| 46 | α | | P | β | | | α | K | 655 | 800 | 7.33 | decomposes | | 8.80 |
| 47 | α | | P | β | | | | α | 660 | 770 | 7.34 | 865 | 860 | 7.94 |
| 48 | | 4 | P | K | α | | | K | 635 | 510 | 8.59 | 934 | 934 | — |
| **Band IV (solvent system I), $R_b$ from .350 to .550 Unsaturated Monohydroxytriones, and Diol-diones.** | | | | | | | | | | | | | | |
| 49 | β | | P | α | K | | α | K | 420 | 695 | 7.74 | decomposes | | 9.60 |
| 50 | α | | P | β | K | | α | K | 496 | 670 | 10.25 | decomposes | | — |
| **Band V (solvent system I), $R_b$ from 0 to .350 Triols and Unsaturated Diol-diones.** | | | | | | | | | | | | | | |
| 51 | | 1,3,5 | E | OH | | α | β | | 174 | 475 | 8.78 | 944 | 944 | 9.94 |
| 52 | β | | P | α | | | α | α | 284 | 460 | 9.64 | 984 | 984 | 10.40 |

[a] Chromatographic conditions—Attenuation X1; Recorder, 0–5 mV.—6', ⅛" o.d. stainles steel columns. JXR column—Oven, 185C; injector, 210C; detector, 180C · He, 60 ml/min; $H_2$, 19 psi; air, 36 psi. SE 52 column—Oven, 200C; injector, 235C; detector, 190C · He, 60 ml/min; $H_2$, 19 psi; air, 32 psi.

[b] Adjusted retention times $t'_R$ were determined with stopwatch (±1/100 min) as times from the solvent front to peak maxima. The latter was taken at moment when pen just started moving downwards again. Retention time data, from compound No. 16 to No. 52, concern TMS derivatives.

[c] Stereoconfiguration of hydrogen attached to carbon No. 5.

[d] Location of double bonds.

umns lightly tapped with a pencil about their middle section. Rapidly alternated tapping between the two columns caused enough vibration to make the powder flow down evenly and at the same speed. After a final light tapping progressing from the bottom to the top of the two columns, the latter were disconnected and enough very fine, untreated borosilicate glass wool (Corning No. 3950) was firmly pressed in the open ends to form a plug no more than 5 mm long and flush with the end. Tightness was tested under 75 lb helium pressure before and after coiling the columns on a 2 in. o.d. mandrel. The columns were conditioned in the chromatograph oven while connected by their inlet end to the gas stream; connection to the detector was absolutely avoided. The helium flow to each column was adjusted (bubble meter) to give 30 ml/min under 30 lb head pressure. After about 15 min the head pressure was reduced to 2 lb and the oven heated gradually from 100C to 250C over a 2-day period. The gas flow was then cut off completely and the temp raised gradually (6 hr) to 280C where it was kept overnight. The temp was then decreased to 250C and the helium head pressure raised gradually (6 hr) to 60 lb. These conditions were maintained for 24 to 48 hr. The oven was then cooled to room temp. Only then were the columns connected to the detector and a final check of flow rates made for both columns. The system was then ready for normal operation.

JXR and SE 52 columns conditioned in this way displayed very low bleeding rate. Bleed deflection (flame on—flame off) for any single column at 200C, under 50 ml/min helium flow and at max sensitivity (attenuator X1, 0 to 5 mv) was never higher than 40 chart divisions. Background deflection (both flames

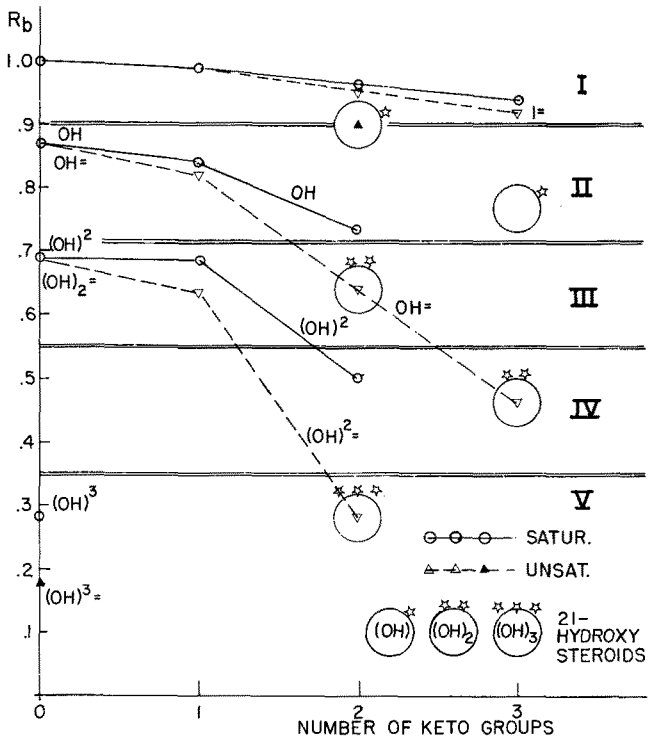


FIG. 1. Migration of steroids on TLC plates developed with solvent system I. $R_b$ values plotted against number of keto groups. Points corresponding to equal number of OH and double bonds have been interconnected to show interrelationships. The 21-hydroxy steroids (large circles), fit in the system as if they contained 1 OH less, i.e., as if the No. 21-OH does not influence their migration.

on—flames off) was never higher than 15 chart divisions under the same conditions; drift from the initial base line never exceeded one chart division in 8 hr. This excellent performance could be maintained indefinitely when low levels of injected sample were used. With the above packing materials the number of theoretical plates, measured for a large number of compounds by using Keulemans' expression (4) varied between 2000 and 3000 for 6 ft columns.

### TMS Derivatives

From 0.1 to 10 mg of hydroxylated steroid were reacted in a 10 ml glass stoppered flask with 50 $\mu$l hexamethyldisilazane and 50 $\mu$l of 10% trimethylchlorosilane in chloroform (v/v). With from 10 to 100 $\mu$g steroid, 1/5 of these reagents and a 1 ml flask were used. The mixture was allowed to react for 3 hr. The solvent and excess reagent were then removed under vacuum using the adapter shown in Figure 2, *II*. The dry residue was diluted with a known volume of $CS_2$ solution of 5$\alpha$-pregnane (cf. below, Internal Standard) and the solution was injected into the chromatograph.

TABLE II

$R_b$ Values in Solvent Systems I and II for 21-Hydroxy Compounds[a] Including the Major Corticosteroid Hormones

| Structural features Keto (K), OH ($\alpha$ or $\beta$) | | | | | | | | Common name | $R_b$ (X $10^3$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Nr | 5[b] | Δ[c] | 3 | 11 | 17 | 20 | 21 | | I | II |
| 53 | | 4 | K | | | K | OH | Desoxycorticosterone | 900 | 780 |
| 54 | | 4 | K | K | | K | OH | Dehydrocorticosterone | 800 | 540 |
| 55 | $\beta$ | | K | | | K | OH | | 650 | 775 |
| 56 | | 4 | K | | $\alpha$ | K | OH | Cortexolone | 640 | 745 |
| 57 | | 4 | K | $\beta$ | | K | OH | Corticosterone | 600 | 575 |
| 58 | | 4 | K | K | $\alpha$ | K | OH | Cortisone | 460 | 610 |
| 59 | $\beta$ | | K | K | $\alpha$ | K | OH | | 60 | 650 |
| 60 | | 4 | K | $\beta$ | $\alpha$ | K | OH | Hydrocortisone | 80 | 575 |
| 61 | | 4 | K | | $\alpha$ | K | OH | (18 al) Aldosterone | 290 | 290 |
| 62 | $\alpha$ | | $\beta$ | K | $\alpha$ | K | OH | | 260 | 510 |
| 63 | $\beta$ | | $\alpha$ | K | $\alpha$ | K | OH | | 210 | 400 |

[a] All are of the pregnane series.
[b] Indicate stereoconfiguration at carbon No. 5.
[c] Position of double bond.

TABLE III

Nanogram Quantity Q of Compound Injected[a]; the Ratio R of Peak Height to 5$\alpha$-Pregnane Peak Height for Reaction Time t (Hours); Average $R_a$ of Ratio for the Plateau Value, and Maximum % Deviation Δ of $R_a$ over 24 Hours

| Q | Compound Nr (see Table I) | Time, hrs 0.5 | 1 | 2 | 3 | 5 | 8 | 24 | $R_a$ | Δ |
|---|---|---|---|---|---|---|---|---|---|---|
| 19.1 | 2 | 2.72 | 2.20 | 2.18 | 2.21<br>2.18 | 2.18<br>2.19 | 2.21 | 2.16<br>2.20 | 2.19 | 1.5 |
| 33.2 | 16 | 1.70 | 1.66 | 1.87 | 2.12<br>2.12 | 2.14<br>2.10 | 2.14 | 2.13<br>2.09 | 2.12 | 1.5 |
| 66.2 | 20 | 1.47 | 1.62 | 1.76 | 1.91<br>1.88 | 1.92<br>1.89 | 1.92 | 1.91<br>1.90 | 1.90 | 1.0 |
| 101.0 | 27 | 2.98 | 1.99 | 1.96 | 1.93<br>1.94 | 1.96<br>1.94 | 1.97 | 1.94<br>1.93 | 1.94 | 1.5 |
| 101.0 | 39 | 1.08 | 1.56 | 1.71 | 1.75<br>1.75 | 1.76<br>1.74 | 1.75 | 1.74<br>1.75 | 1.75 | 0.5 |

[a] Conditions as described in Table I for JXR column.

The time for complete reaction (3 hr) was determined as follows. Mixtures of steroids were weighed out. Stock solutions were obtained by diluting the weighed mixtures in known volumes of methanol. One milliliter volumes of these solutions were then placed in series of 10 ml volumetric flasks and the solvent was removed under vacuum. To the dry residues, equal quantities of the TMS reagents (see above) were added and the stoppered flasks were left for varying lengths of time before the contents were processed as described above. One microliter injections of each of the resulting solutions were made. The ratio R of peak height to 5$\alpha$-pregnane peak height was determined for all compounds. Table III shows typical results obtained with one of the mixtures.

### Internal Standard

Instead of $CS_2$, a solution in $CS_2$ of 5$\alpha$-pregnane was used to dissolve all samples. A large batch of this solution was prepared and stored at 5C in tightly stoppered, tared bottles. These were brought to room temp before use, the solvent loss was determined by weighing and made up with pure $CS_2$. The concn of 5$\alpha$-pregnane was such as to produce a peak height from 50 to 75 chart divisoins with the selected volume, V, of solution injected, under the conditions used in calibration and in all subsequent analyses (1,2).

5$\alpha$-Pregnane was used as an internal standard in preference to 5$\alpha$-androstane or cholestane because the 5$\alpha$-androstane peak, occuring on the trailing end of the solvent surge (Fig. 4), gave rise to occasional erratic values. On the other hand, the cholestane peak occurs in a generally crowded area of the chromatograms. The 5$\alpha$-pregnane peak is suitably located (Fig. 4, compound No. 3); in the present work a concn of about 40 $\mu$g per ml was used for an injected volume of 1 $\mu$l.

The $Rt'_R$ value of cholestane under conditions used with JXR columns (Table I) was 6.18 (11.17 min). The 5$\alpha$-pregnane peak occurred at 1.81 min.

The $Rt'_R$ value of cholestane for SE 52 columns was 6.70 (17.08 min) and the 5$\alpha$-pregnane peak occurred at 2.55 min.)

### Calibration

In precise quantitative work, the nature of the compounds of interest and the range of concn encountered in the particular type of material investigated are usually known. It is thus possible to weigh out quantities of these compounds which constitute a mixture of typical composition. Calibration techniques starting from a representative mixture of this kind were applied as follows. The mixture was converted to TMS derivatives and a master standard solution in $CS_2$ containing 5$\alpha$-pregnane was prepared from them. Aliquots of this solution were then used to make four di-

lutions in the same solvent, using accurately calibrated syringes and small calibrated volumetric flasks. The highest concn of these dilutions was such that the fixed injected volume, V, (from 1 to 2 $\mu$l) produced at least one peak of about 80 to 90 chart divisions. None of the quantities thus injected exceeded 0.15 $\mu$g (150 nanograms) for any compound. The higher dilutions represented 75%, 50%, and 25%, respectively, of the highest concn. The four calibration solutions were stored between uses at low temp in glass stoppered volumetric flasks. These were brought to room temp and the level was adjusted to the mark with pure $CS_2$ before use. Since very small volumes of these solutions were used in calibration work, volume losses were for all practical purposes due to evaporation only, and the relative amts of steroids to 5$\alpha$-pregnane never varied. However, since differences in specific response with absolute amts injected do occur with some steroids (Fig. 3), the original concn (volume) had to be preserved.

Calibration curves were obtained by injecting the same volume, V, of each calibration solution and plotting the ratio R of peak height to 5$\alpha$-pregnane peak height vs. amts of compound injected or vs. amts per $\mu$l. An example is given in Figure 3. For most purposes a single injection for each dilution was sufficient since values of relative heights can be duplicated within $\pm$ 1.5%. Duplicate injections usually gave averages falling within $\pm$ 1% of the mean of a series of 20 trials. As would be expected (1,2) the standard error increased when the peak height fell below 10 chart divisions. Figure 3 shows that straight lines passing through the origin were not obtained in all cases. From this diagram, it is seen that 20 to 25 nanograms of injected steroid should suffice for an analysis. With 2 $\mu$l injections, 1 $\mu$g of steroid in 100 $\mu$l of test solution represents an acceptable sample. This volume can be handled efficiently by using the small flask previously described (2). A microflask designed for the efficient handling of 20 $\mu$l of solution is presently under study.

#### Retention Data

The "adjusted" rentention times used to calculate the relative retention times $Rt'_R$ (to 5$\alpha$-pregnane) listed in Table I were determined to the nearest 1/100 min starting from the sharp deflection due to the solvent surge into the detector, and ending at the moment when the peak observed reached its max. It was found that this moment could be more reproducibly determined when the recorder needle just started its downward move. The $Rt'_R$ values are listed for compounds arranged in bands corresponding to specific TLC zones, and in each band in order of emergence from the JXR column. The corresponding $Rt'_R$ values for the SE 52 column are given in the last column of this Table. Conditions used are given in footnotes. This Table gives $10^3 \times \log_{10} Rt'_R$ for the JXR column only. A set of factors representing structurally related increments of $10^3 \times \log Rt'_R$ is given in Table IV. The use of these factors for identification purposes is discussed in the following section.

## Identification

The points of the plot in Figure 1 indicate averages of $R_b$ values (System I) for the class they represent. Variations around these averages are no doubt due to differences in stereoconfigurations and to some extent can be used for further identification. From the values listed in Table I the following rules are suggested. For monohydroxymonoketones (band II), the unsaturated,

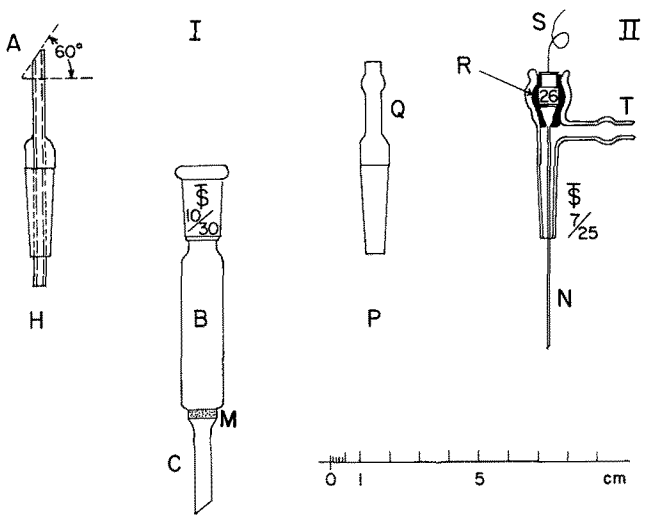


FIG. 2. *I.* Aspirator-filter combination. Collecting fractions of TLC adsorbent powder gathered on plate: Aspirator head *H* fitted to body *B* connected by *C* to water pump. Bevelled mouth A contact powder and system used as vacuum cleaner. For elution: connect *Q* to 5 cc syringe by long tubing. Fit pressure head *P* to *B* and gently force extract through medium porosity filter *M* into receiver.

*II.* Evaporator head. Side tube *T* connected to vacuum (10–15 mm Hg—Drierite tube if water pump used). Stylus *S* in hypodermic needle *N* for leak adjustment. Seal *R* is polyethylene tubing pressed in after heating glass. Note: Use polyethylene or Teflon connections only, in both *I* and *II* systems.

5$\alpha$-3$\beta$-ol, and 5$\beta$-3$\alpha$-ol compounds correspond to the lowest $R_b$ values (ca. 0.820), whereas the highest (ca. 0.865) are found with saturated 5$\beta$-3$\beta$-ol or 5$\alpha$-3$\alpha$-ol compounds. For diols, (band III) the lowest $R_b$ values (ca 0.635) correspond to saturated 5$\beta$- compounds while for 5$\alpha$- or unsaturated species, $R_b$ values fall between 0.660 and 0.700. However, if normal fluctuations in $R_b$ values are taken into account, identification based on these differences should be considered as tentative only.

Much closer identificatoin can be made by using $R_b$ values in conjunction with GLC retention data. Table IV lists a number of factors found by computation of the $Rt'_R$ values (JXR column) given in Table I. Each factor corresponds to some configurational feature. Compound No. 38 (see Table I), i.e., 5$\alpha$-androstane-3$\beta$, 17$\beta$-diol is considered in the following example: the factor for androstane (A) is $-273$ (Table IV, general

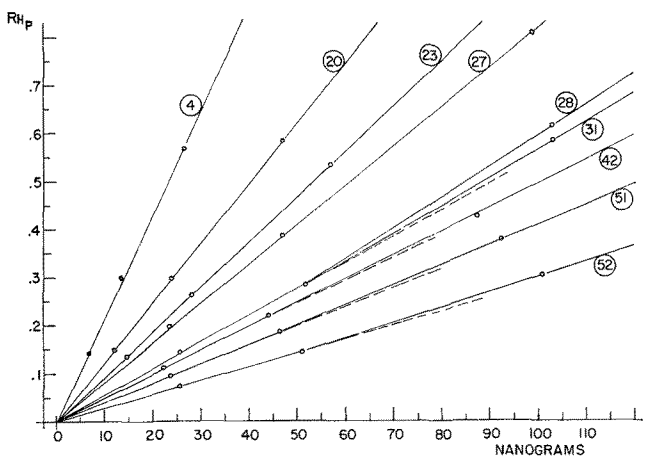


FIG. 3. Calibration plots obtained with mixture of steroids after treatment for TMS conversion of OH groups. Conditions for JXR column and formulae by numbers (see Table I). Nonlinearity of five plots indicated by dashed lines extending straight lower section. Relative peak height, $RH_P$ (5$\alpha$-pregnane used as internal standard), are plotted vs. amts injected in 1 $\mu$l.

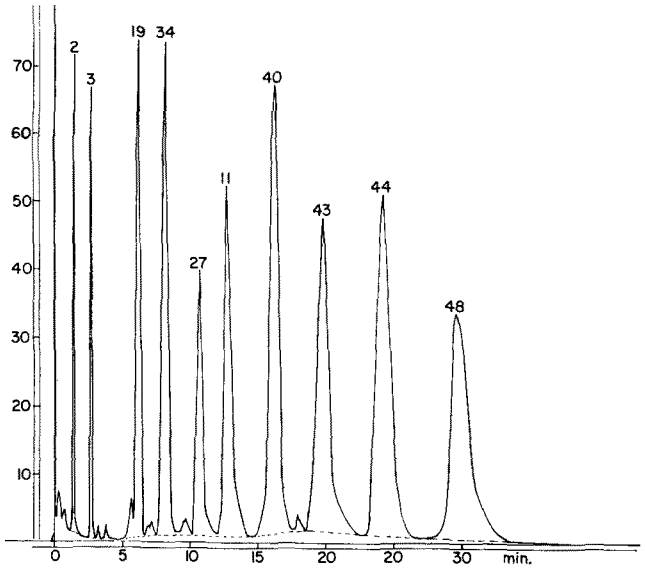


FIG. 4. Chromatogram obtained with mixture of steroids after treatment for TMS conversion of OH groups. Conditions for SE 52 column and formulae by numbers (see Table I). Dashed lines from trough to trough used as zero for peak height measurement. Peaks No. 2 and No. 3 are 5α-androstane and 5α-pregnane, respectively. Background is largely caused by small amts of impurities in steroid sample used. Impurities, which show up in some cases as small peaks, also appear on TLC plates. Most of them have been identified. Amts (nanograms) injected: No. 2, 18.8; No. 3, 37.5; No. 19, 87; No. 34, 101; No. 27, 56.2; No. 11, 102; No. 40, 100; No. 43, 150; No. 44, 150; No. 48, 150.

factors); the factor for 5α-3β-ol is 434 (column 3, Factors relative to position No. 3); the factor for 17β-ol is 425 (both for A and P compounds). The sum $-273 + 434 + 425 = 586$, corresponds to $\log_{10} Rt'_R = 576$ found for compound No. 38.

It can be seen from a comparison of experimental to calculated values that the correspondence is generally excellent, discrepencies being no greater than 2% in most cases. Aside from compounds No. 46, No. 49, and No. 50, which clearly decompose (see Discussion), the only exception is No. 42 for which the discrepency is 2.5%. Elucidation of this case will require examination of other 17α-hydroxy compounds, since it is possible that the factor corresponding to this functional group was somewhat underestimated.

An unknown steroid could only belong to one of the few classes which correspond to its $R_b$ value in solvent system I (Fig. 1, Table I). Assuming that the compound gives a GLC peak, i.e., is not a corticosteroid, the value of $10^3 \times \log_{10} Rt'_R$ can be determined and compared to $10^3 \times \log Rt'_R$ values calculated by using the factors found in Table IV for all possible combinations of positions and stereoconfigurations of the known functional groups in the classes concerned.

## Discussion

The multiplicity of steroid hormones and metabolites in mixtures of natural origin makes a quantitative analysis by GLC alone a problem of considerable complexity. This difficulty is met when any single chromatographic method is employed (3). In general cases, the proposed preliminary TLC separation offers decided advantages over other methods suited to simplifying the GLC problem. On the other hand, it is possible that solvent systems other than those described may prove more adequate in specific problems. Resolution by TLC is limited by random variations in spot position. With the systems used, the average maximum fluctuation of $R_b$ values around the mean of at least 20 observations was $\pm$ 2.5%. For a few multifunctional steroids, this variation reached about 5%. On the other hand, the relative position of spots, groups, and band boundaries were much less affected by fluctuation since, to an appreciable degree, variations did occur in the same direction for directly related spots, and to some extent for all spots on the same plate.

Spots were more uniformly distributed on plates developed with solvent system I (Table I), leading to less crowded bands I and II. On the other hand, the use of solvent system II should simplify problems involving compounds with $R_b$ values between 0.4 and 0.75 in this system since the corresponding zone is less crowded. As this is the region where most corticosteroids migrate in solvent system II (Table II), this system can be used to isolate these compounds from a large number of other steroids. While a choice between the two systems is dependent on the problem at hand, elucidation of some cases could require parallel runs in both systems.

Of several derivatives proposed for the GLC analysis of steroids (5) few seem to be of general interest in the field of steroid hormones. The preparation of fluoroacetates, for instance, leads to the partial or total destruction of some of the most important compounds. Testosterone is completely destroyed under conditions leading to max yields of androsterone derivative. Dilution of the reaction mixture with chloroform, a decrease in reagent quantities (pyridine, trifluoroacetic anhydride) or any other means of promoting milder conditions only succeeds in decreasing the extent of this effect. The amt of testosterone (or progesterone) destroyed is then found to be time-dependent. It is evident that the method cannot be used when all steroid hormones in a mixture are of interest; it could otherwise offer some advantage in simplifying a problem. Other methods such as the preparation of eneamines and bis-methylenedioxy derivatives (5) lead to the formation of two or even three peaks with some compounds, thus introducing considerable complication.

The preparation of TMS derivatives (6), is entirely free of undesirable effects. In fact, compounds in a treated mixture, which do not form derivatives (i.e., progesterone) appear to be protected in subsequent GLC analysis and give rise to much more symmetrical peaks than when chromatographed alone. This effect may be due to traces of the reagent remaining in the product. The effect of hexamethyldisilazane or trimethylchlorosilane in removing "active sites" from a column packing or metal surface is well known.

In experiments made to determine the time for completion of TMS conversion (cf. GLC section and Table III), the chromatograms corresponding to reaction times of less than 2 hr usually showed some peaks due to unreacted compounds as well as those due to derivatives; a few of the latter occasionally overlapped with some of the former. Consequently some peak heights, i.e., R values were abnormally increased. However, the constancy of R values after 3 hr is clearly shown in Table III. Prolonged contact with the reagents did not affect either derivatives or unreactive steroids present in the mixtures. It also was clearly apparent that the TMS conversion is a highly reproducible method since deviation from $R_a$ values never exceeded 2%. In the light of these results, the Q data in Table III clearly demonstrate the accuracy attainable in estimation of very small amts of steroids. It should be emphasized that this cannot be achieved unless procedures already described are

strictly observed: the system must be made electronically stable, the columns must be carefully conditioned, and low levels of injected sample must be used throughout. Equally important is the use of an internal standard. Under the best conditions, injected volumes vary from 5 to 7% about a mean value (1,2). When the plot of quantity vs. detector response is a straight line passing through the origin for *all compounds* of a mixture, the detector responses can be used to calculate an accurate relative composition by internal normalization (2), provided that responses for a unit quantity are known for all compounds. This situation is seldom observed. Hence the need of calibration in quantitative work. The use of an internal standard will reduce the uncertainty due to injection volume error by a factor of at least 4 (1,2) and the number of trials necessary to obtain a given accuracy by a factor of at least $4^2 = 16$. Calibration with an internal standard, which does not involve additional time or effort, is therefore much faster and more reliable.

For many steroids, GLC calibration curves obtained over the 0 to 100 nanograms range (Fig. 3) approximated straight lines passing through the origin. For these, calibration should only require the determination of a single point. Calibration at three to four different levels is necessitated however, by the presence of some compounds for which actual curves are obtained. The max departure from linearity, observed in a few cases, was 15%. This phenomenon, which could be related to partial destruction or retention of these compounds in the system, was however, quite reproducible. Curves corresponding to exclusively ketonic steroids, (unable to form TMS derivatives) did not necessarily show this effect; the curve for compound No. 11 (5$\beta$-pregnane-3, 20-dione, Fig. 4) was rectilinear either with JXR or SE 52 columns. When instead of peak heights, peak areas (determined with the Perkin Elmer printing integrator) were used, the following was observed. Calibration plots which were linear with relative peak heights, were also linear with relative peak areas. In the other cases, the curvature of calibration curves was occasionally either increased or decreased depending on the compound involved. In any case the specific responses (area for 100 nanograms) were very similar to those of the presumably very stable androstane, pregnane, and cholestane, suggesting that destruction, if it occured, never exceeded 15% at the lowest level (0 to 10 nanograms). These facts are in definite contrast with limited observations made elsewhere in the same range (7). In the present study, sharp decreases in specific responses with decreases in injected samples were not observed although, interestingly, an all metal system was used.

Partial breakdown of corticosteroids to simpler GL-chromatographically stable parent $C_{19}$ steroids was observed in other systems (5). Attendant complication in $C_{19}$ steroid analysis could only be avoided by the preliminary separation of corticosteroids. For similar reasons, this separation is also necessary if corticosteroids are converted by oxydation to the parent 17-carboxy compounds (8). On the other hand, the complete retention of corticosteroids under present conditions permits a direct analysis of all other steroids.

The three compounds giving evidence of decomposition (Table I) correspond to broader peaks of normal areas; for two of these (No. 46 and No. 49) the retention time indicated the possible loss of one carbonyl group.

It was observed that a 10% difference in relative retention time, or a 40 unit difference between the $10^3 \times \log_{10} Rt'_R$ of any steroid pair, indicated sufficient resolution for precise quantification with either of the two columns described. While most peaks were remarkably symmetrical, those of a few compounds (Fig. 4) were affected by trailing. A 15% difference in $Rt'_R$ value was then required for adequate resolution. This information allows predictions, based on the data in Table I to be made as to the feasibility of specific separations. The main estrogens (No. 27, No. 39, and No. 51) for example, which can be obtained free of most steroids in a separate extract (acidic fraction), are cleanly resolved by GLC. Both progesterone (No. 13), and testosterone (No. 28) are easily resolved by GLC from many other steroids after preliminary TLC separation. A GLC analysis of the major 17-ketosteroids (No. 18, No. 20, No. 23) is made much easier by their isolation in band II (solvent system I). In predictions of this sort, one must keep in mind that several of the compounds listed do not appear in all mixtures of natural origin; some are not naturally occuring. Data on these steroids is of interest however, in analyses of synthetic hormones or in investigations of the plasma and excreta of patients and animals treated with these hormones.

Differences in $Rt'_R$ values observed between the JXR and the SE 52 columns, although not considerable, should be useful in many cases, particularly since either of these columns can be used in turn when the chromatograph is fitted with both. It is seen, for example, that the spread of ketonic steroids in band I is wider with SE 52 columns than with JXR. The SE 52 column is thus better suited to the analysis of these compounds. Among steroids in other bands (see last column in Table I) elution times do not always occur in the same order for the two columns. Comparison of chromatograms obtained with each column should help, therefore, in problems of identification and quantification.

Further investigation of other column systems will show what column pair is most adequate in solving most problems of this sort. It is probable that a non polar column, such as JXR should always be included. Stability should undoubtedly be one of the considerations in selecting the polar counterpart. On the other hand, the elution time of the more polar compounds is very much increased on highly selective polar columns. With a preliminary TLC separation of the less polar steroids (band I, solvent system I, Table I) the

TABLE IV

Additive Factors[a] Relating Structure to $10^3 \times \text{Log}_{10}$ (Table I) of Relative Retention Time $Rt'_R$ (5$\alpha$-Pregnane) on JXR Columns for Compounds of Androstane (A), Pregnane (P), and Estran (E) Series

A. General factors A, −273; 1,3,5E 3ol, 196; P, 0.00.
B. Factors related to No. 3 position

| Class | Substituent | | |
|---|---|---|---|
| | Keto (K) | $\beta$ ol | $\alpha$ ol |
| 5$\alpha$ | 344 | 434 | 322 |
| 5$\beta$ | 305 | 305 | 344 |
| $\Delta$ 4 or $\Delta$ 5 | 425 | 415 | (344) |
| $\Delta$ 1,4 | 467 | (467) | (395) |

C. Factors related to other positions

| | | Positions | | | | | |
|---|---|---|---|---|---|---|---|
| | | 11$\alpha$ | 16$\alpha$ | 17 | | 20 | |
| Ketone | A,E | 86 | 203 | 301 | | | |
| | P | 127 | | | | 271 | |
| TMS ether group | A,E | | 323 | $\alpha$ 345 | $\beta$ 425 | $\beta$ $\Delta$ 425[b] | $\alpha$ $\Delta$ 445 |
| | P | 240 | | | 425[b] | | |

a Factors apply within 175C–200C range.
Factors in brackets are extrapolated, tentative values.
b Aggregate factor for 17$\alpha$20$\alpha$ is 620.

use of such columns may no longer be necessary nor desirable. For these reasons we believe that phases of moderate to medium polarity among the stable silicone polymers, such as SE 52 and XE 60, will prove most useful in conjunction with the present method. Stability in both JXR and SE 52 was not the least contributing factor in promoting favorable conditions for analyses at high instrumental sensitivity. These phases were the most stable among all types available in the nonpolar (JXR) and the more polar (SE 52) categories. Dimethylpolysiloxane JXR is somewhat more stable than SE 30, although similar to SE 30 in general characteristics.

Newly packed columns had a tendency to become more porous with concomitant decrease in retention time. Original retention times could be maintained by the routine adjustment of helium flow using a compound of average retention time as reference. Calibration curves then remained valid for prolonged periods. The rapidity and reliability of calibrating operations by the proposed method simplified the routine checking of column characteristics and permitted the determination of analytical variance (1,2). The last feature alone is of considerable import in that it gives access to an estimation of the significance of relatively small analytical differences, i.e., to a clearer interpretation of results.

The principle at the basis of the factors listed in Table IV is identical to that applied by Knights and Thomas (9) in their determination of $\Delta R_{Mg}$ factors. Both series of factors represent specific structural contributions to the relative retention time, i.e., $Rt'_R$ in the present nomenclature and $r$ for the above authors. The factors in Table IV are dimensionally different however, since the columns, conditions, and reference compounds were different. Both types are closely related to the steroid numbers, SN, and F factors of VandenHeuvel and Horning (10) since the latter factors are also related to relative retention times by formally simple expressions. Thus

$$SN_X = 19 + \Sigma F = 19 + 8 \log Rt'_{RX}/\log Rt'_{RC}$$

where $Rt'_{RX}$ and $Rt'_{RC}$ are the relative retention times for a compound X and cholestane, respectively, and both relative to androstane, gives the value of a steroid number. It is evident from this expression that the range of validity of $SN_X$ will correspond to a range of conditions where relative retention times $Rt'_R$ are sufficiently constant, as the proponent authors have pointed out (10).

The relation

$$SN_X = 19 + 8 \frac{\log Rt'_{RX} - \log Rt'_{RA}}{\log Rt'_{RC} - \log Rt'_{RA}}$$

where all $Rt'_R$ values are relative to the same reference compound, and $Rt'_{RA}$ corresponds to androstane, allows the calculation of $SN_X$ values from $Rt_R$ data obtained with any column. It would seem that SN factors represent a more involved expression of the relative retention times than the $\Delta R_{Mg}$ factors of Knights and Thomas and those derived in the present study. The latter are valid from 175C to 200C with JXR columns.

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# Ultramicro Fatty Acid Analysis of Polar Lipids: Gas-Liquid Chromatography After Column and Thin-Layer Chromatographic Separation

**G. L. FELDMAN, Department of Ophthalmology, Baylor University College of Medicine, Houston, Texas, and GEORGE ROUSER Department of Biochemistry, City of Hope Medical Center, Duarte, California**

## Abstract

A procedure is described for the analysis of the fatty acid composition of polar lipid classes in the nanogram range. The lipids are first fractionated by column chromatography followed by further separation into pure lipid classes by thin-layer chromatography. Lipid spots scraped from the thin-layer plates are esterified directly (i.e., without prior elution) with 6% sulfuric acid in methanol. The methyl esters are then analyzed by gas-liquid chromatography with a hydrogen flame ionization detector. Samples of 200 nanograms or less give accurate results with helium as carrier gas, oxygen rather than air to support combustion, careful adjustment of the recorder and general attention to optimum electrical connections, dissociation of the column oven from the recorder and electrometer, and careful preconditioning of columns. Under proper conditions the base line is stable and a 10% of full scale deflection of the recorder can be obtained from 1 nanogram of a methyl ester, allowing highly precise analyses of fatty acid composition from the amount of lipid obtainable from one spot on a thin-layer chromatogram. Control studies demonstrated that extraneous peaks did not arise from the procedure or from the sphingosine and dihydrosphingosine of sphingolipids. The thin-layer chromatographic procedure did not influence the fatty acid composition of a pure sample of glucocerebroside isolated by column chromatography and the method was applied to lecithin and sphingomyelin or normal and pathological human brain specimens.

## Introduction

DETERMINATION OF FATTY ACID composition of specific lipid classes is a difficult problem for investigators working with very small samples. The preferred general procedure is gas-liquid chromatography