CHROM. 3770

REFERENCE HIGH-EFFICIENCY NONPOLAR PACKED COLUMNS FOR THE GAS–LIQUID CHROMATOGRAPHY OF NANOGRAM AMOUNTS OF STEROIDS

PART I. RETENTION TIME DATA*

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SUMMARY

The preparation and conditioning of reproducible, long-lasting, 6000 theoretical plate nonpolar columns are described. Retention data of TMS derivatives at 215, 230 and 240° are given for 140 standard steroids of the androstane, pregnane and cholestane series. Additive incremental factors permitting a precise prediction of retention times are given for many specific structural features and functional groups. Operational conditions that permit highly reproducible results with nanogram amounts of steroids are described and discussed.

INTRODUCTION

The analysis of total steroids extracted from most biological materials offer difficult problems which arise from the number, structural variety, similarity in physical properties and very low concentrations of many components. Such problems can be solved by a combined thin-layer and gas–liquid chromatography (TLC–GLC) method[1–8]; by this method, total steroids are first separated in several fractions which are characterized by molecules containing specific numbers of hydroxyl and carbonyl groups. Each of these fractions is then resolved by GLC.

Routine examination of TLC fractions by GLC, whether for the purpose of component identification of quantification, is particularly efficient and reliable with columns of high resolving power when these columns show unaltered characteristics over a long period of time.

The present paper describes the preparation and detailed properties of readily reproduced nonpolar columns which in three years of almost continuous use have

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consistently displayed identical characteristics. Their resolving power, obtained from the Keulemans expression[9] corresponds, for example, to 5500 and 6150 theoretical plates for the trimethylsilyl ether (TMS) derivatives of cholesterol and stigmasterol, respectively.

With such columns, retention times are reproducible enough to permit unequivocal discrimination between compounds which differ very little in this respect; a precise prediction of retention times from structure-dependent increments[1,2] can be made for the entire life-span of the columns.

Furthermore, reliable quantitative work can be made on the basis of calibration curves which remain valid over long periods of time.

These important advantages are obtained under simple operational conditions provided that the conditions are used consistently.

EXPERIMENTAL

Equipment

Perkin Elmer 800 gas chromatograph equipped with dual flame ionization detector fitted with ceramic jets.

Perkin Elmer 900 gas chromatograph.

Philips PR 2500 recorder, 1 mV full scale.

Perkin Elmer No. 194 Printing Integrator.

Maximum signal-to-noise ratio was obtained as follows: Chromatograph and recorder were connected to separate electrical power lines, that used for the recorder being free of noise-generating equipment. Recorder gain was adjusted for incipient "hunting" by pen-drive motor, and the chromatograph chassis was earthed by connecting to a water main. Under full operational conditions at attenuation $\times 1$, the base line was consistently straight with occasional transient deflections not exceeding 0.5 % of the chart span.

The oven of the P.E. 800 instrument was fitted with a 200–260° Anschütz thermometer (Fisher 15-165E) calibrated *in situ* and under operating conditions against a precision resistance thermometer.

Materials

JXR, 3 % dimethylpolysiloxane on 100/120 Gas Chrom Q, prepared by, and received from Dr. W. SUPINA, now with Supelco, Inc., Bellefonte, Pa. 16823, U.S.A. Hexamethyldisilazane and trimethylchlorosilane, redistilled.

CS_2, Fisher No. C 184 reagent.

Standard steroids from Steraloids Inc., P.O. Box 127, Pawling, N.Y. 12564.

Standard steroids from the Steroid Reference Collection (*cf.* Acknowledgements) indicated by S.R.C. in Tables VIII and IX.

Steroids obtained by reduction of standard steroids (*cf.* Discussion and Tables VIII and IX).

Methods

Preparing columns. Two 9 ft. straight lengths of 1/8 in. O.D. stainless steel tubing cut from the same stock were cleaned internally by connecting one end with polyethylene tubing to a partially evacuated flask and slowly aspirating chloroform–

methanol (2:1) through a polyethylene tubing connected to the other end. The tubes were dried with a stream of N_2 and clamped vertically side by side at about 2 in. from each other. Bottom ends of the tubes were plugged; each of the upper ends were connected by a short section of polyethylene tubing to one of twin funnels described in Fig. 1A.

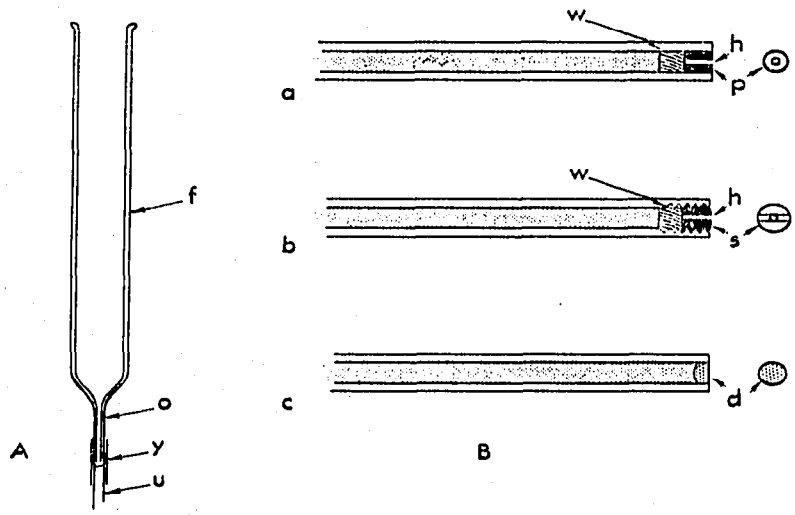

Fig. 1. (A) Glass funnel for the filling of columns. f = Graduated body, 14.3 mm I.D.; o = outlet, 1.5 mm I.D., 1/8 in. O.D., 20 mm long; y = polyethylene connective tubing; u = column. Both funnel and column are clamped on the same support. (B) Three methods for column plugging: (a) Solid plug method (w = fine Pyrex glass wool, Corning No. 3950, silanized; h = 1-mm diam. hole; p = stainless steel plug). (b) Screw method (w, as above; s = 3-48 NC screw; h = 1-mm diam. hole; a size 3-48 NC tap is used to thread the column). (c) Porous disk method (d = stainless steel porous disk, Perkin Elmer Cat. No. 008-1216).

Four grams of 3% JXR on 100–120 mesh Gas Chrom Q were placed in each of the funnels and the columns were tapped with a pencil about their middle section. Rapid, alternated tapping between the two columns caused just enough vibration to make the powder flow evenly and at the same speed, from both funnels. Packing was completed when levels of excess powder in the funnels remained constant after sustained tapping progressing from the bottom to the top of the columns. The funnels were disconnected carefully and the weights of excess powder were compared to detect a possible discrepancy. On the average, each column contained 3.60 g of packing material of density 0.3 g/ml.

Both ends of the columns were then plugged. Three ways of effecting this operation are described in Fig. 1B and related caption. In plugging columns by methods a and b (Fig. 1B) the packing material in completely filled columns was first pushed 1/4 in. inside with a metal rod. The resulting space was then filled completely with fine silanized glass wool. Insertion of either plug, p, or screw, s, then resulted in further compression of the wool. When method b was used, the column ends were threaded over 1/2 in. before cleaning and filling the tubes. With method c, damage to the pores of disks, d, was avoided by pressing (rather than tapping) the disks in position. In all cases, damage to the terminal 1/2 in. of outer surface was avoided to permit a tight fitting of Swagelock connectors. The columns were coiled on a 2 in. O.D. mandrel.

The packed columns were connected to the gas chromatograph by their inlet end; connection to the detector was absolutely avoided. Helium flow through each column was adjusted to 60 ml/min. with a bubble meter. The gas flow was then completely cut off and the conditioning schedule shown in Table I was observed.

TABLE I

CONDITIONING[a] SCHEDULE FOR TWIN 9-FOOT, 1/8 INCH OUTER DIAMETER HIGH-EFFICIENCY JXR COLUMNS

Time (*h*)	*Temperature*[b] (°C)	*Helium flow rate* (*ml/min*)
0	Room temperature	0
8	250	0
24	250	0
32	300	0
48	300	0
49	250	0
52	300	30
72	300	30
73	300	60
76	Room temperature	60

[a] Helium flow rates through the columns must first be adjusted to the same value, *i.e.*, 60 ml/min (bubble meter). Thus both columns have been flushed with, and are full of helium when cut-off valve is turned off to begin conditioning.

[b] Changes in temperature must be gradual over the period indicated.

Setting-up conditions

Adjustment of conditions permitting duplication with any JXR column of retention times listed for 230° in Tables II–IX was made as follows. A standard CS_2 solution containing 30 ng of TMS derivative of 3β-hydroxy-5α-androstane (3β-androstanol)[4,5] and 50 ng of 5α-cholestane per μl was prepared. With the oven temperature set at 230°, standard conditions described in Table II were used. Two microliters of the standard solution were injected. Corrected retention times of both compounds were recorded with attenuation setting ×10 for the P.E. 800 instrument (×1, ×40 with P.E. 900). If the ratio of the retention time of cholestane to that of the derivative of 3β-androstanol was *higher* than 3.575 (3.575 = 680/190) the temperature was *increased* by 0.05° for every 0.001 units difference in this ratio; conversely, the temperature was *lowered* by this amount when the observed ratio was *smaller* than 3.575. The helium flow was then adjusted to bring the retention time of cholestane to 6.80 min. If the retention time ratio still differed from the correct one the operation was repeated. It should be noted that the retention time of cholestane varied by 3.7 % per °C, whereas the corresponding variation for 3β-androstane TMS was 3.2 % only.

Preparing TMS derivatives. Up to 1 mg of hydroxylated steroid were reacted in a glass-stoppered flask with 50 μl of hexamethyldisilazane and 50 μl of 10 % trimethylchlorosilane in chloroform (v/v), the reagents being added in that order. With larger amounts of steroids correspondingly larger volumes of reagents were used. Brief

mixing by stirring or vibration was applied after each addition; the top of the stopper was greased with silicone lubricant. If the steroid had been obtained by evaporating a solution, moisture and last traces of solvent were removed before adding the reagents by leaving the flask for 2 h in an evacuated dessicator over P_2O_5.

The reaction mixture was left at room temperature for at least 3 h[1,2]. Excess solvent and reagents were removed as described in ref. 2. CS_2, or a solution in CS_2 of the selected standard was then added to the flask contents. These, including residual ammonium chloride formed in the reaction, dissolved completely.

Standard solutions. It will be shown in Part II[17] of the present series of papers that the retention time of a given steroid under a given set of conditions is minimum when the quantity injected lies within a range which is specific for the structural group to which the steroid belongs.

Concentrations in the standard solutions used to produce the present data were such that 2 μl of injected solution contained amounts of individual steroids falling within the specific range for constant, minimum retention time. These concentrations were also adjusted to produce peaks of roughly comparable size at the same attenuation setting.

To begin with, solutions containing the TMS derivative of single steroids along with at least one standard steroid were prepared and the retention times were determined at 230° under adjusted operational conditions (*cf.* above). Next, several mixtures containing from 20 to 30 steroids were prepared and treated with TMS reagents. The components were selected according to the known retention times so as to produce an uninterrupted series of peaks with minimum overlap when injected together. An example of chromatograms thus obtained is given in Fig. 2. Most steroids were included in at least two of the twelve different solutions that were prepared. Most of

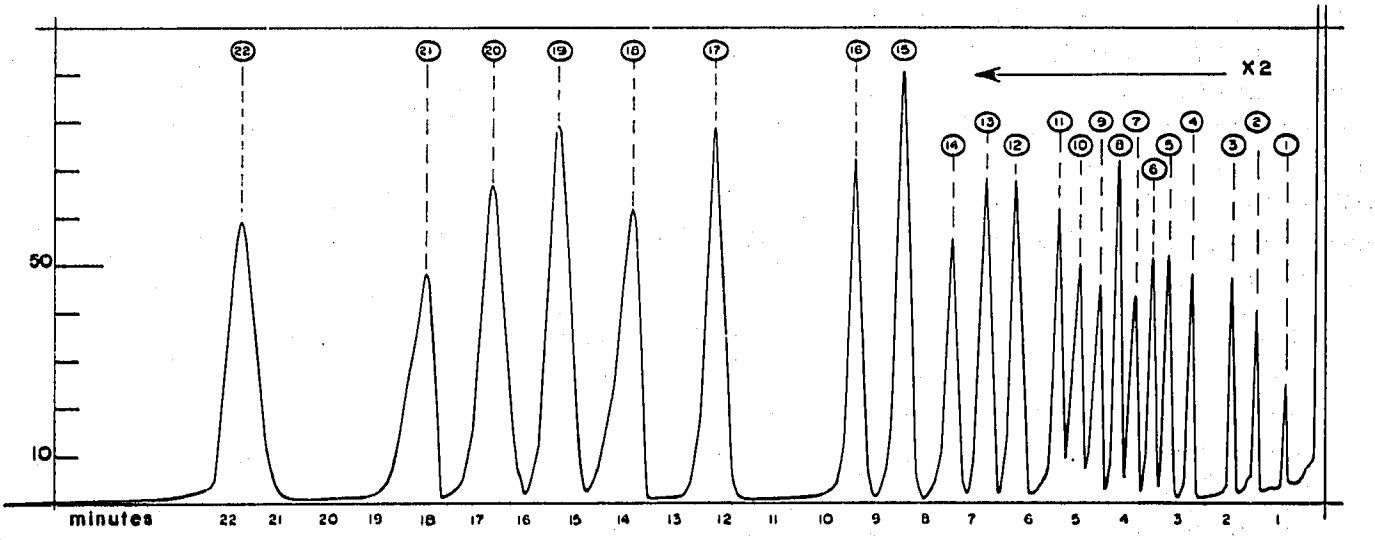

Fig. 2. Chromatogram of standard steroid mixture at 230°. Conditions: *cf.* Tables II–IX. The components, designated by peak number, group and number in group, and Table number, were as follows: 1 = A2,II; 2 = A3,II; 3 = C2,IV; 4 = D1,V; 5 = C4,IV; 6 = D5,V; 7 = D8,V; 8 = E6,VI; 9 = D11,V; 10 = D13,V; 11 = D15,V; 12 = A4,II; 13 = A7,II; 14 = A8,II; 15 = G1,VIII; 16 = G5,VIII; 17 = C5,IV; 18 = B14,III; 19 = C7,IV; 20 = C9,IV; 21 = B16,III; 22 = C16,IV. Some of the amounts injected were, in nanograms: A2, 1.0; C2, 6.0; D15, 20.0; G1, 30.5; C7, 50.1. Note separation of peak 19 (cholesterol, C7) and peak 20 (desmosterol, C9) on JXR column of 5500 theoretical plates (for cholesterol).

six steroids considered as potential internal standards in future work were included in all solutions. These were the TMS derivatives of 3α-hydroxy-5α-androstane, 3α,20α-hydroxy-5β-pregnane (pregnanediol), 3β-hydroxycholest-5-ene (cholesterol), and the hydrocarbons 5α-pregnane, 5α-cholestane and stigmastane.

Using these solutions it was possible to obtain readily the retention times of a large number of steroids and to correlate these retention times to those of the above six key steroids.

It was observed that standard solutions of TMS derivatives prepared in the way described were stable under the following simple conditions[1,2]: cold storage (−5°) between uses of at least 5 ml of solution in narrow-necked flasks fitted with ground-glass stoppers lubricated with silicone grease; *bringing to room temperature* before opening a flask and minimizing exposure to moisture during use.

Retention time data

A Precision-Scientific "Time It" instrument giving a digital reading to the nearest 1/100th of a minute was started (zero time) when a rapid deflection of the recorder pen following injection marked the solvent vapour surge into the detector. This occurred 23 sec. after injections at 230°, for example. For each of the successive peaks, time was read as the recorder pen just left the peak apex on its downward course. Thus "corrected" retention times were recorded within ± 1/100th of a minute.

The retention time data listed in Tables II to IX were obtained with the P.E. 800 chromatographer at 215.0, 230.0, and 240.0° with the 3-year-old columns. In these experiments the temperature, as indicated by the Anschütz thermometer, never varied more than ± 0.1° in the course of each experiment (≈ 30 min). Very slow cyclic variations within ± 0.25° were observed in the course of many hours: thus the temperature from experiment to experiment varied to some extent and the observed retention times varied accordingly.

However, when observed retention times were normalized to the nominal, or set temperature of the experiment, normalized retention times agreed within ± 0.2 %. Normalization was obtained by multiplying an observed value by the ratio $t'_{T^s_n}/t'_{T^s_e}$

ABLE II

ORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

ROUP A—HYDROCARBONS

o.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	*230°*	*240°*	*215°*	*230°*	*240°*
	5β-Androstane	Etiocholane	113	77	61	2053	1887	1784
	5α-Androstane	Androstane	124	84	66	2093	1924	1819
	5α-Pregnane	Allopregnane	218	141	108	2338	2150	2033
	5β-Cholestane	Coprostane	1089	620	435	3037	2792	2638
	5α-Cholest-2-ene		1175	667	469	3070	2824	2671
	Cholest-5-ene	Cholestene	1200	678	475	3079	2831	2676
	5α-Cholestane	Cholestane	1200	680	477	3079	2833	2678
	Cholesta-3,5-diene		1332	748	522	3124	2874	2717
	5α,24β-Ethyl-cholestane	Stigmastane	2038	1118	763	3309	3048	2882

[a] Operational conditions: Inlet temperature, 270°; detector = oven temperature. Inlet pressure t romatographer, 60 lb.; helium flow rate, 60 ml/min. Hydrogen inlet pressure, 18 lb.; air, 50 lb.

TABLE III

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP B—MONO- AND POLYKETONES

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	230°	240°	215°	230°	240°
1	5α-Androstan-17-one	Androstanone	241	155	119	2382	2190	2076
2	5α-Androstan-3-one		264	169	127	2421	2228	2104
3	5β-Pregnan-3-one		429	262	194	2632	2418	2288
4	5β-Androstan-17-one		468	288	213	2670	2459	2328
5	Androst-4-ene-3,17-dione		635	384	287	2803	2584	2458
6	Androsta-1,4-diene-3,17-dione		690	416	304	2839	2619	2483
7	5β-Pregnane-3,20-dione	Prenanedione	760	449	325	2880	2652	2512
8	Androst-4-ene-3,11,17-trione		798	475	341	2902	2676	2533
9	5α-Pregnane-3,20-dione		840	497	360	2924	2696	2556
10	5β-Pregnane-3,11,20-trione		960	572	411	2982	2757	2614
11	Pregn-4-ene-3,20-dione	Progesterone	1030	613	440	3013	2788	2643
12	5α-Pregnane-3,11,20-trione		1090	649	467	3038	2812	2669
13	5β-Cholestan-3-one	Coprostanone	2340	1255	860	3369	3098	2934
14	5α-Cholestan-3-one	Cholestanone	2605	1390	951	3415	3143	2977
15	Cholesta-3,5-dien-7-one		2875	1525	1039	3458	3183	3017
16	Cholesta-4,6-dien-3-one		3497	1905	1224	3544	3256	3087

[a] Operational conditions: *cf.* Table II.

where $t'^{S}_{T_c}$ is the retention time observed in the same experiment for an internal standard included in the mixture, and $t'^{S}_{T_n}$ its retention time at the nominal temperature of the experiment. In most experiments normalization of the data could be effected by using several internal standards. In no case were the necessary corrections larger than 1%.

From retention time logarithms listed in Tables II to IX incremental factors of these logarithms shown in Tables X and XII were computed. The values obtained for specific functional groups or combination of groups were often averages derived from data on several steroids which included the relevant structural features. Variations observed for individual values of such increments were usually less than 1%; thus retention times could be accurately predicted from the structural formulae simply by adding up appropriate increments[1,2]. Examples applying to androstane- and pregnanediols are given in Table XI. Table XII gives incremental values for many combinations of structural features concerning sterols.

Incremental values of this type were first described by KNIGHTS AND THOMAS[9]. Their use in the detection and structural identification of steroids will be discussed in detail in Part II[17] of the present series of papers. Quantification problems at the nanogram level will be discussed in Part III.

DISCUSSION

The importance of minimizing vibration during the packing of columns must be stressed. By tapping the columns as described the flow of packing material was slow and even; air pockets did not form, the powder packed almost maximally, and about 20 min were required to fill the columns. Further tapping usually resulted in some increase in packing density. Excess vibration, on the other hand, did not increase

TABLE IV

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP C—MONOHYDROXY COMPOUNDS

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	230°	240°	215°	230°	240°
1	3α-Hydroxy-5α-androstane	3α-Androstanol	239	150	113	2378	2175	2053
2	3β-Hydroxy-5α-androstane	3β-Androstanol	305	190	141	2484	2278	2149
3	3α-Hydroxy-5β-pregnane	3α-Pregnanol	444	266	192	2647	2424	2283
4	3β-Hydroxy-5α-pregnane	3β-Allopregnanol	540	321	232	2732	2506	2365
5	3β-Hydroxy-5β-cholestane	3β-Coprostanol	2305	1227	825	3362	3088	2916
6	3α-Hydroxy-5β-cholestane	3α-Coprostanol	2439	1268	850	3387	3103	2929
7	3β-Hydroxycholest-5-ene	Cholesterol	2936	1536	1022	3467	3186	3009
8	3β-Hydroxy-5α-cholestane	3β-Cholestanol	3005	1574	1047	3477	3197	3020
9	3β-Hydroxycholesta-5,24-diene	Desmosterol	3217	1673	1108	3507	3224	3044
10	3β-Hydroxycholesta-5,7-diene	7-Dehydrocholesterol	3250	1677	1117	3511	3225	3048
11	3β-Hydroxycholest-7-ene	Lathosterol[b]	3360	1750	1150	3526	3243	3060
12	3β-Hydroxycholesta-8,24-diene	Zymosterol[b]	3365	1754	1155	3527	3244	3063
13	3β-Hydroxycholesta-5,7,24-triene	[b]	3540	1841	1200	3549	3265	3089
14	3β-Hydroxy-24β-methylcholesta-5,7,22-triene	Ergosterol	3640	1885	1268	3561	3275	3103
15	3β-Hydroxy-24α-methylcholest-5-ene	Campesterol	3905	1998	1316	3591	3301	3119
16	3β-Hydroxy-24β-ethylcholesta-5,22-diene	Stigmasterol	4263	2170	1425	3623	3336	3153
17	3β-Hydroxy-4α-methylcholest-7-ene	Methostenol[b]	4310	2195	1435	3634	3341	3157
18	3β-Hydroxy-4,4',14α-trimethyl-5α-cholesta-8,24-diene	Lanosterol	4725	2410	1573	3674	3382	3197
19	3β-Hydroxy-24α-ethylcholest-5-ene	β-Sitosterol	5002	2511	1634	3699	3399	3213
20	3β-Hydroxy-4,4'-dimethylcholest-7-ene	[b]	6070	2568	1670	3783	3410	3223

[a] Operational conditions: *cf.* Table II.

[b] Samples obtained from FUMAGALLI, confirmed by mass spectrum by GALLI AND MARONI[10].

TABLE V

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP D—MONOHYDROXY, MONOKETO COMPOUNDS

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	*230°*	*240°*	*215°*	*230°*	*240°*
1	3β-Hydroxy-5β-androstan-17-one		458	275	201	2661	2439	2303
2	3α-Hydroxy-5α-androstan-17-one	Androsterone	462	278	202	2664	2444	2304
3	3α-Hydroxy-5β-androstan-17-one	Etiocholanolone	485	288	207	2686	2459	2316
4	3β-Hydroxyandrost-5-en-17-one	DHA	573	337	244	2758	2527	2387
5	3β-Hydroxy-5α-androstan-17-one	Epiandrosterone	593	350	253	2773	2544	2403
6	3-Hydroxyestra-1,3,5(10)-trien-3-one	Estrone	637	372	267	2804	2570	2421
7	17β-Hydroxy-5α-androstan-3-one	Allodihydrotestosterone	642	378	272	2807	2577	2434
8	17α-Hydroxyandrost-4-en-3-one	Epitestosterone	660	387	278	2819	2587	2444
9	17β-Hydroxy-19-norandrost-4-en-3-one	19-Nor-testosterone	695	399	284	2842	2600	2453
10	3β-Hydroxy-5β-pregnan-20-one	Pregnanolone	732	434	308	2864	2638	2489
11	17β-Hydroxyandrost-4-en-3-one	Testosterone	785	459	329	2895	2662	2517
12	3α-Hydroxy-5β-pregnan-20-one	Epipregnanolone	792	454	321	2898	2657	2506
13	17β-Hydroxypregna-1,4-dien-3-one		865	503	358	2937	2701	2553
14	3β-Hydroxypregna-5,16-dien-20-one		866	496	348	2937	2695	2541
15	3β-Hydroxypregn-5-en-20-one	Pregnenolone	944	538	380	2975	2730	2580
16	3β-Hydroxy-5α-pregnan-20-one	Allopregnanolone	973	553	389	2988	2742	2590
17	20β-Hydroxy-5α-pregnan-3-one		1140	644	454	3057	2809	2657
18	20β-Hydroxypregn-4-en-3-one		1441	799	552	3158	2899	2742

[a] Operational conditions: *cf.* Table II.

TABLE VI

CORRECTED RETENTION TIMES[a] t'_R and LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP E—DIHYDROXY COMPOUNDS

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (min)			$10^3 \times \log t'_R$		
			215°	230°	240°	215°	230°	240°
1	3α,17β-Dihydroxy-5α-androstane	Dihydroandrosterone	586	339	241	2768	2530	2382
2	3β-17α-Dihydroxyandrost-5-ene		586	336	240	2767	2526	2380
3	3α,6α-Dihydroxy-5β-pregnane		645	367	258	2809	2564	2410
4	3β,17β-Dihydroxyandrost-4-ene	Δ^4-Androstenediol	695	401	282	2842	2602	2450
5	3β,16α-Dihydroxy-5α-androstane	16α-Androstanediol	698	401	282	2843	2602	2450
6	3β,17β-Dihydroxyandrost-5-ene	Δ^5-Androstenediol	724	415	294	2859	2618	2468
7	3,17α-Dihydroxyestra-1,3,5(10)-triene	17α-Estradiol	728	414	289	2862	2617	2461
8	3β,17β-Dihydroxy-5α-androstane		736	426	300	2867	2628	2477
9	3,17β-Dihydroxyestra-1,3,5(10)-triene	Estradiol	809	460	322	2908	2663	2507
10	3β,20β-Dihydroxy-5β-pregnane		1006	564	393	3002	2751	2594
11	3α,20β-Dihydroxy-5β-pregnane		1058	585	402	3024	2767	2604
12	3α,20α-Dihydroxy-5β-pregnane	Pregnanediol	1154	634	437	3062	2802	2640
13	3β,20α-Dihydroxypregna-5,16-diene		1080	599	414	3033	2777	2617
14	3β,20β-Dihydroxypregn-4-ene	Pregnenediol	1238	687	470	3092	2837	2762
15	3β,20β-Dihydroxy-5α-pregnane		1315	728	503	3118	2862	2701
16	3β,20α-Dihydroxy-5α-pregnane		1403	770	529	3147	2886	2723

[a] Operational conditions: *cf.* Table II.

TABLE VII

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 215, 230 AND 240°

GROUP F—MONOHYDROXYDIKETONES AND DIHYDROXYMONO- AND DIKETONES[b]

No.	*Compound*	*Trivial name*	$10^2 \times t'_R$ (*min*)			$10^3 \times \log t'_R$		
			215°	*230°*	*240°*	*215°*	*230°*	*240°*
1	3α-Hydroxy-5α-androstane-11,17-dione	11-Ketoandrosterone	583	346	250	2765	2539	2398
2	3α-Hydroxy-5β-androstane-11,17-dione	11-Ketoetiocholanolone	591	350	252	2771	2544	2402
3	12α-Hydroxy-5β-pregnane-3,20-dione		934	534	381	2970	2727	2581
4	3α-Hydroxy-5β-pregnane-11,20-dione		1070	599	419	3029	2777	2622
5	3β-Hydroxy-5α-pregnane-11,20-dione		1325	746	510	3122	2873	2708
6	11α-Hydroxypregn-4-ene-3,20-dione	11α-Hydroxyprogesterone	1720	932	634	3235	2970	2802
7	11β-Hydroxypregn-4-ene-3,20-dione		1995	1041	705	3299	3017	2848
8	3α,6α-Dihydroxy-5α-androstan-17-one		692	390	274	2840	2591	2436
9	3α,11β-Dihydroxy-5α-androstan-17-one	11β-Hydroxyandrosterone	753	437	311	2877	2640	2492
10	3α,11β-Dihydroxy-5β-androstan-17-one	11β-Hydroxyetiocholanone	784	445	317	2894	2648	2502
11	3β,17α-Dihydroxy-5β-pregnan-20-one		1090	609	425	3037	2785	2628
12	3α,17α-Dihydroxy-5β-pregnan-20-one		1097	614	430	3040	2788	2633
13	3α,6α-Dihydroxy-5β-pregnan-20-one		1135	617	430	3055	2790	2633
14	3β,17β-Dihydroxypregn-5-en-16-one		1128	628	435	3052	2798	2638
15	3β,16α-Dihydroxypregn-4-en-20-one		1376	769	533	3138	2886	2727
16	3β,17α-Dihydroxy-5α-pregnan-20-one				[c]		[c]	
17	17α,20β-Dihydroxypregn-4-en-3-one			1337			3126	
18	17α,20α-Dihydroxypregn-4-en-3-one			1391			3143	
19	3α,5α-Dihydroxy-5α-cholestan-6-one		7175	3560	2294	3855	3551	3360
20	3β,17α-Dihydroxy-5α-pregnane-11,20-dione				[c]		[c]	
21	3α,17α-Dihydroxy-5β-pregnane-11,20-dione				[c]		[c]	
22	11α,17α-Dihydroxypregn-4-ene-3,20-dione			1416			3151	

[a] Operational conditions: *cf.* Table II.

[b] More of these compounds are listed wiht 21-hydroxy corticosteroids, Table IX.

[c] Some decomposition; the reduced compounds chromatograph very well: *cf.* Table VIII, G16, G18.

TABLE VIII

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 230°

GROUP G—TRI- AND TETRAHYDROXY COMPOUNDS[b]

No.	*Compound*	*Trivial name and source*[c]	$10^2 \times t'_R$ (*min*)	$10^3 \times \log t'_R$
			230°	*230°*
1	3,16α,17β-Trihydroxyestra-1,3,5(10)-triene	Estriol	846	2926
2	3β,17α,20β-Trihydroxy-5β-pregnane	Red. F11, Table VII	845	2926
3	3α,17α,20β-Trihydroxy-5β-pregnane	S.R.C.: also Red. F12	857	2933
4	3β,11β,20β-Trihydroxy-5β-pregnane	Red. B10, Table III	865	2936
5	3α,11β,20β-Trihydroxy-5β-pregnane	Red. F4	869	2939
6	3α,17α,20α-Trihydroxy-5β-pregnane	Pregnanetriol	942	2974
7	3β,11α,20β-Trihydroxypregn-4-ene	Red. F6	973	2988
8	3α,11β,20α-Trihydroxy-5β-pregnane	Red. B10, G4 = main product	984	2992
9	3β,17α,20β-Trihydroxypregn-4-ene	Red. F17	1038	3016
10	3β,11β,20β-Trihydroxypregn-4-ene	Red. F7	1066	3028
11	3α,11α,20β-Trihydroxypregn-4-ene	Red. F6, G7 = main product	1076	3032
12	3β,17α,20α-Trihydroxypregn-4-ene	Red. F19	1088	3037
13	3β,17α,20β-Trihydroxy-5α-pregnane	Red. F16	1117	3048
14	3β,11β,20β-Trihydroxy-5α-pregnane	Red. F5; also Red. B12	1165	3066
15	3α,11β,20β-Trihydroxypregn-4-ene	Red. F7, G10 = main product	1175	3070
16	3β,11β,20α-Trihydroxy-5α-pregnane	Red. B12, G14 = main product	1287	3110
17	3α,11β,17α,20β-Tetrahydroxy-5β-pregnane	also Red. F21	1248	3096
18	3α,11β,17α,20α-Tetrahydroxy-5β-pregnane	S.R.C.	1416	3151
19	3α,11α,17α,20β-Tetrahydroxypregn-4-ene	Red. F22	1638	3214

[a] Operational conditions: *cf.* Table II.

[b] More of these compounds and also pentahydroxysteroids are listed among 21-hydroxycorticosteroids in Table IX.

[c] Estriol and pregnanetriol obtained from Steraloids; S.R.C. = Steroid Reference Collection. Compounds the source of which is indicated as Red. followed by a letter and number, have been obtained by reduction of the steroid thus designated. Example: F2 is compound No. 2 in Group F (Table VII).

TABLE IX

CORRECTED RETENTION TIMES[a] t'_R AND LOG t'_R OF TRIMETHYLSILYL DERIVATIVES OF HYDROXYLATED STEROIDS ON JXR COLUMNS AT 230°

GROUP H—21-HYDROXYCORTICOSTEROIDS

No.	*Compound*	*Trivial name and source*[b]	$10^2 \times t'_R$ (*min*)	$10^3 \times \log t'_R$
1	21-Hydroxy-5β-pregnane-3,20-dione		957	2981
2	21-Hydroxypregn-4-ene-3,20-dione	Cortexone	1340[c]	3127
3	11β,21-Dihydroxypregn-4-ene-3,20-dione	Corticosterone	2250[c]	3352
4	17α,21-Dihydroxypregn-4-ene-3,20-dione	Cortexolone	[c]	[c]
5	17α,21-Dihydroxy-5β-pregnane-3,20-dione		[c]	[c]
6	21-Hydroxypregn-4-ene-3,11-20-trione	11-Dehydrocortisone	1647[c]	3214
7	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	Cortisol	[c]	[c]
8	17α,21-Dihydroxypregn-4-ene-3,11,20-trione	Cortisone	[c]	[c]
9	3β,17α,21-Trihydroxy-5α-pregnane-11,20-dione		[c]	[c]
10	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione		1074[c]	3031
11	17α,21-Dihydroxy-5β-pregnane-3,11-20-trione		[c]	[c]
12	3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one	S.R.C.	2090	3320
13	3β,20β,21-Trihydroxy-5β-pregnane	Red. H1	1204	3080
14	3β,20β,21-Trihydroxypregn-4-ene	Red. H2	1462	3165
15	3β,17α,20β,21-Tetrahydroxy-5β-pregnane		1728	3238
16	3β,17α,20β,21-Tetrahydroxypregn-4-ene	S.R.C. also Red. H4	2100	3320
17	3β,11β,20β,21-Tetrahydroxypregn-4-ene	Red. H3, also Red. H6	2254	3354
18	3α,11β,17α,20α,21-Pentahydroxy-5β-pregnane	S.R.C. also Red. H11	2436	3387
19	3α,11β,17α,20β,21-Pentahydroxy-5β-pregnane	also Red. H10	2528	3403
20	3β,11β,17α,20β,21-Pentahydroxy-5β-pregnane	also Red. H11	2544	3404
21	3β,11β,17α,20β,21-Pentahydroxypregn-4-ene	Red. H7, also Red. H8	3180	3502
22	3β,11β,17α,20β,21-Pentahydroxy-5α-pregnane	S.R.C. also Red. H9	3520	3547

[a] Operational conditions: *cf.* Table II.

[b] Compounds 7 to 11, 16, 19 and 20 were obtained from Steraloids, Inc.; S.R.C. = Steroid Reference Collection. Compounds the source of which is indicated as Red. followed by letter and number, have been obtained by reduction of the steroid thus designated. Example: H2 = steroid number 2 in Group H of this table.

[c] Decomposition—All compounds from 12 to 22 are polyhydroxysteroids whose TMS derivatives are very stable.

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TABLE X

VALUES OF $10^3 \times \Delta \log t'_R$ AT 230° ON JXR COLUMNS UNDER STANDARD CONDITIONS (TABLES II–IX) FOR FUNCTIONAL GROUPS IN THE ANDROSTANE (A) AND PREGNANE (P) SERIES

Group	*With*	*A-Ring features*											
		5α[a]	*5β*[a]	*5β,3β*	*5α,3α*	*5β,3K*	*5β,3α*	*5α,3K*	*5α,3β*	*Δ*[4]*,3β*	*Δ*[4]*,3K*	*Δ*[5]*,3β*	*Δ*[1,4]*,3K*
A		1924	1887	2175	2178	2190	2194	2227	2280	2255	2322	2264	2359
P		2150	2113	2401	2404	2416	2420	2453	2506	2481	2548	2490	2585
11α	P									153	178		
11β	P			170			170		203	193	226		
16α	A[b]								322				
17α	A[b] or P[b]	260	260	260	260	260	260	260	260	260	260	260	260
17α	A[b] or P[b]	350	350	350	350	350	350	350	350	350	350	350	350
20α	P[b]	380	380	380	380	380	380	380	380	380	380	380	380
20β	P[b]	354	354	354	354	354	354	354	354	354	354	354	354
17α,20α	P						554			556	595		
17α,20β	P			526			512		539	535	578		
17α,20K	P			384			368		391		425		
11K	A				90		90						
11K	P						118		118				
17K	A or P[b]	266	266	266	266	266	266	266	266	266	266	266	266
20K	P[b]	243	243	243	243	243	243	243	243	243	243	243	243
21-OH	20K[b]	335	335	335	335	335	335	335	335	335	335	335	
21-OH	20β[b]	315	315	315	315	315	315	315	315	315	315	315	
21-OH	17α,20β	305	305	305	305	305	305	305	305	305	305	305	
21-OH	17α,20α	215	215	215	215	215	215	215	215	215	215	215	

[a] No other feature in A-ring.

[b] No substitution in D-ring.

TABLE XI

CALCULATED AND EXPERIMENTAL CORRECTED RETENTION TIMES[a] t'_R AT 230° FOR DIOLS[b] OF THE ANDROSTANE (A) AND PREGNANE (P) SERIES

Diol (TMS)	t'_R (10^{-2} *min*)		*Diol (TMS)*	t'_R (10^{-2} *min*)	
	Calculated	*Experimental*		*Calculated*	*Experimental*
5β-A-3β,17α-	272		5β-P-3β,20β-	567	564
5α-A-3α,17α-	274		5α-P-3α,20β-	573	
5β-A-3α,17α-	284		5β-P-3α,20β-	593	585
Δ⁴-A-3β,17α-	327		5β-P-3β,20α	603	
5β-A-3β,17β-	335		5α-P-3α,20α-	608	
Δ⁵-A-3β,17α-	335	336	5β-P-3α,20α-	631	630
5α-A-3α,17β-	338	339	Δ⁴-P-3β,20β-	684	686
5α-A-3β,17α-	349		Δ⁵-P-3β,20β-	692	
5β-A-3α,17β-	350		5α-P-3β,20β-	730	731
Δ⁴-A-3β,17β-	403	401	Δ⁴-P-3β,20α-	726	
Δ⁵-A-3β-17β-	412	415	Δ⁵-P-3β,20α-	742	
5α-A-3β,17β-	430	426	5α-P-3β,20α-	774	770

[a] Operational conditions: *cf.* Table II.
[b] *Cf.* Table VI.

packing density, yet caused breakdown of particles and exposure of unsilanized material detrimental to the performance of the columns.

The absence of gas flow during the first stages of conditioning (Table I) presumably allowed condensation of vaporized stationary phase on the column wall and prevented movement of vaporized phase toward the column exit. Heating under these conditions would at first promote a more even distribution of phase within particles, then further polymerization of JXR on both support and column wall. Eventually, fully polymerized stationary phase could no longer be displaced even under high gas flow at high temperature.

In routinely obtained chromatograms examplified in Fig. 2, peaks were generally symmetrical. Response to small amounts of steroid injected was excellent; decrease in response was appreciable at the low nanogram level only. This effect was reproducible and amenable to precise calibration.

Several duplicates of the columns prepared and conditioned in the manner described have been prepared in this and other laboratories. All showed properties identical to those of the 3-year old column pair in effecting separations demonstrated in Tables II to IX: relative retention times were the same and incremental factors listed in Tables X and XII were applicable in all cases. The only variations observed were small; they concerned the number of theoretical plates (from 5400 to 5700 for cholesterol) and the temperatures at which the listed values of relative retention times were obtained. These temperatures varied from column to column within a range of about 2° and must be regarded as life-time characteristics of each column.

Curtailing the conditioning schedule shown in Table I did not seem to modify retention time characteristics appreciably; it did, however, reduce the separating power in two ways: the number of theoretical plates was decreased and some tailing of peaks became apparent particularly when initial heating in the absence of gas flow was shortened.

TABLE XII

CALCULATED VALUES OF $10^3 \times \log t'_R$ ON JXR COLUMNS UNDER STANDARD CONDITIONS (TABLE IV) FOR STRUCTURAL FEATURES OF STEROLS

$10^3 \times \log t'_R = 3197^a + 10^3 \times \Delta \log t'_R{}^b$.

	C 27	28	29	29	28	28	30
	—	14αMe	14αMe 4αMe	14αMe 24Me[c]	4αMe	24Me[c]	4,4'Me 14αMe
	1	2	3	4	5	6	7
(a) 5:22	−74.3	−103	− 7	12	22	40	64
(b) 5:8:22	−64	− 92	4	22	32	51	74
(c) 22	−64	− 92	4	22	32	51	74
(d) 8:22	−53.6	− 82	14	32	42	61	85
(e) 5:7:22	−33.3	− 61	35	53	63	80	105
(f) 7:22	−17.7	− 46	50	68	78	96	121
(g) 5	−10.4	− 39	57	76	86	104	128
(h) —	0	− 28.3	+ 67.7	86.4	96.0	114.7	138.3
(i) 5:8	0	− 28	68	86	96	115	138
(j) 8	+10.3	− 18	78	96	106	125	149
(k) 5:24	26.7	− 2	94	112	123	141	165
(l) 5:7	30.8	+ 2	98	116	127	146	169
(m) 24	37.0	9	105	123	133	152	175
(n) 5:8:24	37.0	9	105	123	133	152	175
(o) 7	46.3	18	114	133	142	161	185
(p) 8:24	47.3	19	115	134	143	162	186
(q) 5:7:24	67.8	40	136	154	164	183	206
(r) 7:24	83.4	55	151	170	179	198	222

[a] 3197 = $10^3 \times \log t'_R$ of 3β-hydroxy-5α-cholestane (cholestanol) at 230° under the same conditions.
[b] Example: Compound a11 is 3β-hydroxy-24β-ethylcholesta-5,22-diene (stigmasterol): $10^3 \times \log t'_R$ = 3197 + 140 = 3337. The experimental value for stigmasterol (Table IV, C16) is 3336.
[c] α or β.

The longevity of columns undoubtedly stems also from factors other than preparation and conditioning. Among these the proportion of JXR to support (3 % by weight) is high by comparison with columns generally used for the GLC of steroids. In addition, consistent operation at a very low level of injected material would tend to minimize interactions with the liquid phase of steroids and their thermal decomposition products which tend to affect the stability and separation characteristics of the packing material. Observations at low attenuation following injections clearly showed a considerable increase in the persistence of residual background with increased quantity injected: both residence time and amounts of decomposition products were thereby very much increased. Hence the rate and extent of physical modification of the stationary phase by thermal breakdown products and by polymeric material arising from these products were greatly minimized at the level of injected material used in the present work.

As shown in Fig. 2, low background and stability were obtained by injecting nanogram amounts at very low attenuation. As shown in Part III of this series, precise quantifications were routinely achieved at these levels. The method is therefore entirely suitable for the analysis of very low concentrations of steroids found in most biological materials.

Samples of biological origin invariably contain amounts of extraneous material

29	*30*	*29*	*29*	*31*	*30*	*30*	*32*	*31*
4,4'Me	*14αMe 24Et*[c]	*4αMe 24Me*[c]	*24Et*[c]	*4,4'Me 14αMe 24Me*[c]	*4,4'Me 24Me*[c]	*4αMe 24Et*[c]	*4,4'Me 14αMe 24Et*[c]	*4,4'Me 24Et*[c]
8	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
92	111	136	140	179	207	236	278	306
103	122	147	150	189	217	246	288	317
103	122	147	150	189	217	246	288	317
113	132	157	160	199	227	256	298	327
133	153	178	181	220	248	277	319	348
149	168	193	196	235	263	292	334	363
156	175	201	204	243	271	300	342	370
166.6	185.7	210.7	214	253	281.3	310.0	352.3	380.6
167	186	211	214	253	281	310	352	381
177	196	221	224	263	291	320	362	391
193	212	237	241	280	308	337	378	407
197	217	242	245	284	312	340	383	411
204	223	248	251	290	318	347	389	417
204	223	248	251	290	318	347	389	417
213	232	257	260	299	328	356	399	426
214	233	258	261	300	329	357	400	427
234	254	280	282	321	349	382	420	448
250	269	294	297	336	365	393	436	463

often far in excess of the complex steroid mixtures to be analyzed. Quantification thus generally requires preliminary clean-up processes as well as preliminary fractionation procedures. The systematic use of TLC[1–8] allows both these requirements to be met. Injection of "clean" samples obtained from TLC plates undoubtedly contributed to the longevity of the present columns by minimizing possible interactions of the stationary phase with extraneous materials, their thermal breakdown and polymeric products. In four years of uninterrupted use neither the injection port, nor the detector of the P.E. 800 chromatograph has required cleaning.

The use of CS_2 as a solvent for TMS derivatives may have contributed to the maintenance of column characteristics. Among advantages derived from the use of CS_2 which have been discussed by one of us[11,12], the abolition of solvent trailing (Fig. 2) and the ability of CS_2 to dissolve TMS derivatives and reaction products (*cf.* above) are noteworthy. In addition, this solvent shows little affinity for most stationary phases and therefore, will not cause much physical alteration of the packing material.

TMS derivatives[13] in contrast to others[1,2] are of general application in the GLC of steroids. With the present method, complete conversion of most steroids was obtained in 3 h at room temperature[1,2]. Very few steroids required longer reaction times; among these, estriol required 10 h. It should be noted that some steroids are

sensitive to direct contact with dimethylsilylchloride. With these, reversing the order of addition of reagents (*cf.* above) will lead to abnormal products.

Trace amounts of unreacted trimethyldisilazane in the final product could have beneficial effects in eliminating "active sites" within the column[1,2]. The injection of large amounts of this compound does, however, undesirably modify column characteristics. The high separating power of the present columns partly resulted from the use of 100–120 mesh Gas Chrom Q[14]. With another silanized support the number of theoretical plates per foot was 500 at best[1,2] although mesh size, percentage JXR and conditioning were identical.

JXR, a dimethylsilane polymer is more stable thermally than the analogous phase SE 30. Our experience with OV1 is too limited to warrant an estimation in this respect. Separation characteristics of the three phases were similar yet appreciably different. Differences observed with OV1 did not confer advantages to this phase over JXR in the present type of work.

Although longer, more efficient columns can undoubtedly be prepared by the present procedure, their use is restricted by present instrumental limitations in permitting required high carrier-gas fore-pressures. Internal flow control systems would have to be changed or by-passed. Longer columns have a significant damping effect on pressure changes which could obviate the need for an internal control system. With 20-ft. columns, the number of theoretical plates could be at least 10,000. However, residence time of steroids in such columns would be twice that in 9-ft. columns for the same flow rate: thermal destruction at low level of steroids may then become significant.

The stability of TMS derivatives in CS_2 solutions has been discussed[1,2]. Under the conditions described, retention times of TMS derivatives obtained with standard solutions prepared three years ago and used repeatedly over this period did not vary; hence the columns characteristics remained constant in spite of extensive use involving many thousands of injections during this period. The thermal stability of these derivatives under present GLC conditions was generally excellent. Among the few that were unstable (Tables VII and IX), some produced well defined peaks; however, the retention times clearly corresponded to compounds of smaller molecular size produced by decomposition. In other cases, several overlapping peaks emerged in close succession. The instability of unsaturated 21-corticosteroids including the steroid hormones is well known[15]. Treatment of all these steroids with sodium borohydride, under conditions to be described in a forthcoming publication, resulted in their quantitative conversion to polyhydroxysteroids whose TMS derivatives could be readily chromatographed (Tables VIII and IX). The retention times of the reduced steroids (TMS) were distinct and specific. Polyhydroxylated steroids thus obtained could also be converted to halogenated derivatives suitable for quantification by electron capture.

The choice of standard temperatures used in the present study was governed by the following consideration. Mixed steroids generally obtained from biological material could be adequately separated at 230°; at this temperature a complete chromatogram was usually obtained in 30 min. With urinary steroid hormones and metabolites a somewhat better separation resulted at 215° in 30 min. On the other hand, the GLC of high molecular weight sterols from molds, bacteria or algae, and also reduced 21-corticosteroids could be achieved more readily at 240°. Operation at 230° was adequate in all cases.

The sensitivity of retention times to temperature changes explains the necessity for precisely controlled oven temperature. Under the described set of conditions, very little change could be observed in retention times. That of cholestane, for example, never varied by more than ± 0.02 min (± 0.3 %) in several hours.

From the data in Tables II to VII the accuracy of the following relation can be demonstrated for all steroids:

$$10^3 \times \log t'_{Ri} = A_i + 10^3 \times B_i T^{-1} \quad (1)$$

where A_i and B_i are constants independent of T, the absolute temperature.

A similar relation can be found for standard steroids included in a mixture, hence:

$$10^3 \times \log t'_{Rs} = A_s + 10^3 \times B_s T^{-1} \quad (2)$$

From eqns. (1) and (2) the following expression

$$10^3 \times \log t'_{Ri}/t'_{Rs} = A_i - A_s + (B_i - B_s)T^{-1} \quad (3)$$

is obtained for the relative retention time t'_{Ri}/t'_{Rs} of a given steroid. Since this is a constant for a given temperature within a relatively wide range of carrier gas flow rates,

$$\bar{A}(i,s) = A_i - A_s \text{ and } \bar{B}(i,s) = B_i - B_s \quad (4)$$

are constants independent of both temperature and carrier gas flow rate. Hence accurate determinations of relative retention times at two temperatures give access to specific constants $\bar{A}(i,s)$ and $\bar{B}(i,s)$ by which unknown steroids can be identified with considerable certainty. Use of these factors, for which we suggest the name of Retention Constants (R.C.), will be described in detail in Part II of the present series.

Incremental factors listed in Tables X and XII are simply additive. When corresponding functional groups or features are sufficiently separated in the molecule to prevent a crowding effect, the values apply in all cases; thus values for 17α-hydroxy, 17β-hydroxy, and all other values which are repeated in every column of Table X indicate independence of the corresponding features on the presence of other groups. Values for 11α- and 11β-hydroxy do not show this independence since they vary with different A-ring features; the value for 21-hydroxy depends on the nature of neighboring C20 or D-ring substitution. A crowding effect is indicated by aggregate values for 17α,20α; 17α,20β; 17α,10K, etc. being smaller than the sum of values for component functional groups taken singly.

With the values listed in Tables X and XII, most retention times can be predicted within ± 1 %. Discrepancies higher than 2 % are exceptional. Table XI lists calculated and observed values for the TMS derivatives of androstane- and pregnanediols. Discrepancies observed with sterols (Table XII) are even lower.

No values corresponding to Δ^4,3α are given in Table X since a determination for this group in the absence of crowding effect awaits the availability of suitable compounds. However, at least three compounds which include this A-ring feature are listed as G11, G15 and G19 in Table VIII. If incremental values listed under

$\Delta^4,3\beta$ in Table X for other groups included in these compounds are used, a value of 2525 for $\Delta^4,3\alpha$ (pregnane) is found in all cases. Hence it is probable that values listed in Table X for $\Delta^4,3\beta$ apply to $\Delta^4,3\alpha$ also, and that 2525 is the value for $\Delta^4,3\alpha$ (pregnane).

It should be noted that in the two first lines of Table X, aggregate values have been entered for convenience. Each of the values listed in these lines corresponds to a sum, including either 1924 (5α-androstane, *cf.* column 3) or 2150 (5α-pregnane, *cf.* column 3) with the specific value for each A-ring feature; for example, in column 5, 2175 = 1924 + 251 and 2401 = 2150 + 251; in column 6, 2178 = 1924 + 254 and 2404 = 2150 + 254; in column 7, 2190 = 1924 + 266 and 2416 = 2150 + 266; etc. Hence the specific increment corresponding to each A-ring feature is the same for compounds of both androstane and pregnane series.

These and other increments also apply to compounds of the cholestane, or C series. A comparison of incremental values found for the C series with corresponding values in the androstane (A) and pregnane (P) series shows the following correspondences: 5αC,3K = 310 (5αA,3K = 5αP,3K = 307); 5αC,3β = 363 (5αA,3β = 5αP,3β = 355); 5βC,3K = 265 (5βA,3K = 5βP,3K = 269); 5βC,3α = 270 (5βA,3α = 270); 5βC,3β = 255 (5βA,3β = 5βP,3β = 248). Note that 114.7 being the value for methyl in the chain (Table XII, 24Me), 6 × 114.7 = 688. Hence the increment for 5αC should be 2150 (pregnane) + 688 = 2838. The value found for 5αC is 2833.

Independent incremental factors of log t'_R were observed with nonpolar stationary phases only. Polar phases induce phase–steroid interactions which vary in extent with each different configuration. Since values of incremental factors vary with each structural situation, accurate prediction of retention times from structural features is impossible with polar phases.

It is generally believed that separations on nonpolar phases are induced mainly by differences in molecular weight. Furthermore, the name selective is applied to polar columns to indicate a greater sensitivity to differences in structural features. This appellation is misleading. The data presented in Tables II to XII undoubtedly show considerable discrimination by nonpolar JXR columns of subtle structural differences (*cf.* Table XI, for example). The common belief that polar phases have generally useful selective properties is likewise unfounded since such polar phases do not allow many separations which are readily achieved with nonpolar phases.

Use of TLC as a preliminary step in the separation of steroids affords a means of effecting a complementary separation on the basis of polarity. Zones containing steroids differing as to the number of carbonyl and hydroxy groups, and subzones containing steroids of different stereoconfiguration are sharply separated on TLC plates[1–5]. The problem of locating the center of any given zone or subzone within ± 1 mm has been solved in this laboratory by including several pilot dyes in the mixture analyzed and relating the position of interest to that of the nearest dye pair. A forthcoming paper[18] on this procedure will demonstrate that steroids which are poorly separated on JXR columns migrate in separate TLC subzones which can be independently removed from the plates and quantified by GLC. Such is the case, for example with several pairs of compounds listed in Table V: etiocholanolone (D2) and androsterone (D3); testosterone (D11) and epipregnanolone (D12); pregnenolone (D15) and allopregnanolone (D16).

In addition, silver nitrate TLC[16] permits preliminary separations on the basis of differences in unsaturation. Hence separation problems generally believed to

require the use of polar columns can be solved by the combination of TLC with GLC on high-efficiency JXR columns.

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