QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF TRIMETHYLSILYL ETHERS OF HYDROXYLIC STEROIDS

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(Received July 6th, 1967)

INTRODUCTION

Thin-layer chromatography plays a major role in the analysis of steroids in biological material. For analytical separation alone, the steroids may, in many instances, be chromatographed without modification, but where they are to be recovered by elution, the use of derivatives is advantageous. Although monohydroxylic steroids (other than phenolic steroids such as estrone[1]) can be satisfactorily eluted from plates[2], steroids with further hydroxyl groups tend to be strongly adsorbed, and their quantitative elution is more difficult[3]. The polarity of phenolic or polyhydroxylic compounds is largely eliminated by preparing trimethylsilyl ethers, and the consequent value of these derivatives has long been recognised in the field of gas–liquid chromatography[4–6]. The corresponding applications of trimethylsilyl ethers in thin-layer chromatography have received relatively scant attention. ROSENFELD[7] first applied thin-layer chromatography to the purification of steroid trimethylsilyl ethers. The technique was later used[8] in a study of corticosteroid derivatives. More recently, attention has been drawn to the value of trimethylsilyl ethers in systematic analyses based on functional groups[9–11]. The technique has been applied in a procedure for the clinical determination of testosterone[12]. One limitation is that phenol trimethylsilyl ethers are susceptible to hydrolysis during thin-layer chromatography. In the case of non-phenolic hydroxysteroids, however, thin-layer chromatography of trimethylsilyl ethers in quantities of 1–10 μg can be accomplished without appreciable loss due to adsorption or hydrolysis[13]. The possibility has not hitherto been excluded that for substantially smaller quantities the losses might be severe enough to vitiate the quantitative application of the derivatives. This paper presents data for various steroid trimethylsilyl ethers in amounts ranging from 1 ng to 1 μg. Part of the work has been briefly reported elsewhere[14,15].

EXPERIMENTAL

Materials

Cholesterol-4-^{14}C (30 mC/mmole) and pregnenolone-4-^{14}C (3β-hydroxypregn-5-en-20-one-4-^{14}C) (24 mC/mmole) were obtained from the Radiochemical Centre, Amersham.

Pregn-5-ene-3β,20β-diol-4-^{14}C (24 mC/mmole) was prepared by reduction of pregnenolone-4-^{14}C with sodium borohydride as follows. Pregnenolone-4-^{14}C (5 μg)

was dissolved in methanol (5 ml) and sodium borohydride (2 mg) added. The reaction mixture was allowed to stand overnight and worked up by the usual method to yield crude pregn-5-ene-3β,20β-diol-4-^{14}C. This was purified by thin-layer chromatography of its trimethylsilyl ether using the methods described below.

5β-Pregnane-3α,17α,20α-triol-G-^{3}H (3200 mC/mmole) and 5β-pregnane-3α,11β, 17α,20β-tetrol-G-^{3}H (1200 mC/mmole) were prepared by tritium exchange over Pd catalyst at the Radiochemical Centre, Amersham, and were also purified *via* their trimethylsilyl ethers using the methods described below.

5α-Cholestane-3β,5α,6β-triol-4-^{14}C (0.3 mC/mmole) was prepared by performic acid oxidation of cholesterol-4-^{14}C following the method of FIESER AND RAJAGOPALAN[16]. An extract derived from human atheroma tissue, containing principally cholesterol and 5α-cholestane-3β,5α,6β-triol, was provided by Mr. G. STEEL (MRC Blood Pressure Research Unit, Glasgow).

Trimethylsilyl ethers were prepared by dissolving the steroid (5 μg) in pyridine (5 μl), adding hexamethyldisilazane (5 μl) and trimethylchlorosilane (1 μl) and allowing the reaction mixture to stand overnight at room temperature. Solvent and excess reagents were then removed in a stream of nitrogen, and the trimethylsilyl ethers extracted from the residue with chloroform.

Methods

Plates (20 × 20 cm) for thin-layer chromatography were prepared using a BTL spreader (Baird & Tatlock Ltd., London) with a 0.25 mm layer of Kieselgel G (Merck). Before use they were washed by development with ethyl acetate, and activated by heating for 1 h in an oven at 120°. The trimethylsilyl ethers were applied to the plates as spots, using a 50 μl Hamilton syringe: in each case the concentration was such that 100 μl of solution was required. Chromatography was effected with cyclohexane–ethyl acetate (9:1, v/v) as mobile phase, and solvent development was allowed to continue until the solvent front was 15 cm from the baseline. A plate similarly prepared with inactive trimethylsilyl ethers was developed simultaneously and sprayed with 1% ceric sulphate in aqueous sulphuric acid (10% H_2SO_4 v/v) to indicate the zones on the plate to be eluted. Plates were allowed to dry and the silica gel scraped from appropriate zones was eluted with ether. Each eluate was filtered directly through a little silica gel into a scintillation counting vial and the ether removed in a stream of nitrogen. Scintillator solution [10 ml containing 0.3 % "PPO" (2,5-diphenyloxazole) and 0.01 % "dimethyl POPOP" (1,4-bis[2-(4-methyl-5-phenyloxazolyl]-benzene) in toluene] was then added to each vial. Scintillation counting was conducted using a Packard Tri-carb scintillation spectrometer, which gave an efficiency of 79 % for ^{14}C and 41% for ^{3}H. Results cited in Tables II–IV are means of five consecutive ten-minute counting periods.

Gas–liquid chromatography was carried out with a dual glass column F-11 chromatograph (Perkin-Elmer Ltd.) equipped with a flame ionisation detector. Column packings were prepared by the procedure of HORNING *et al.*[17].

RESULTS

Typical R_F values for the steroid trimethylsilyl ethers investigated in three solvent systems are given in Table I. Fig. 1 shows a representative chromatogram.

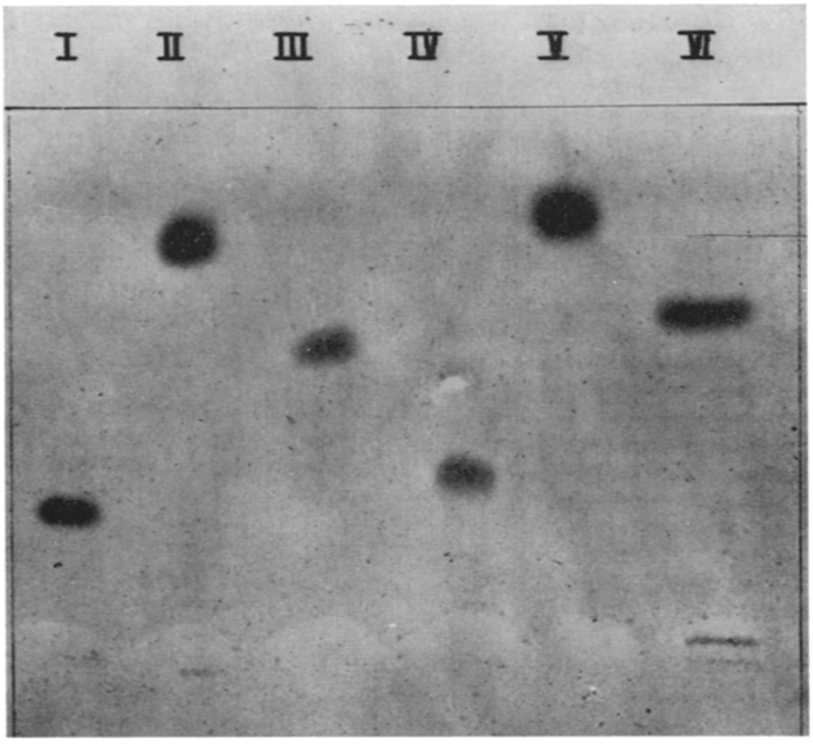

Fig. 1. Thin-layer chromatogram of six steroid trimethylsilyl ethers on Silica Gel G (Merck). Mobile phase: cyclohexane–ethyl acetate (9:1, v/v). I = Pregnenolone TMS; II = pregn-5-ene-3β,20β-diol bis(TMS); III = 5β-pregnane-3α,17α,20α-triol 3,20-bis(TMS); IV = 5β-pregnane-3α,11β,17α,-20β-tetrol 3,20-bis(TMS); V = cholesterol TMS; VI = 5α-cholestane-3β,5,6β-triol 3,6-bis(TMS).

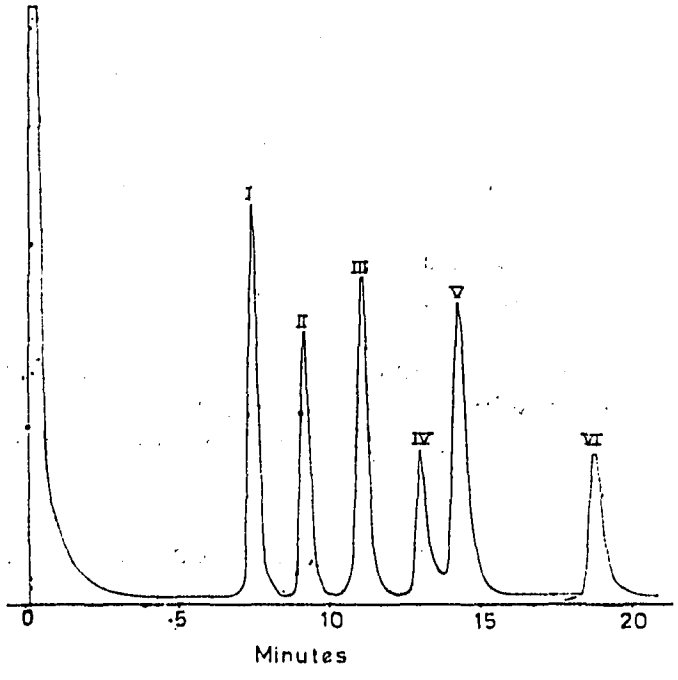

Fig. 2. Gas–liquid chromatogram of steroid trimethylsilyl ethers. Column conditions: 1% SE-30 on 100–120 mesh Gas-Chrom P prepared according to HORNING *et al.*[17]; 6 ft. × 4 mm I.D. glass coiled column; 200–280° × 3°/min; 20 p.s.i.; initial carrier gas flow rate 40 ml/min. Compounds are designated as in Fig. 1.

Fig. 2 illustrates the analytical gas chromatographic separation of the same group of six derivatives.

Initial experiments were conducted on individual trimethylsilyl ethers of cholesterol-4-^{14}C, pregnenolone-4-^{14}C and pregn-5-ene-3β,20β-diol-4-^{14}C. Chromatography was effected with approximately 1, 10 and 100 ng and with 1 μg of each steroid. The results of these experiments are summarised in Table II: they indicate that a small decrease in recovery is experienced as the quantity of steroid trimethylsilyl ether is reduced, but even at a level of 1 ng the average recovery (84 %) is still acceptable.

Further studies were then conducted on 10 ng samples of each of the five steroid trimethylsilyl ethers. Chromatography was effected with individual steroids and with the following mixtures: (1) cholesterol-4-^{14}C, pregnenolone-4-^{14}C and 5β-pregnane-3α,17α,20α-triol-G-^{3}H; (2) cholesterol-4-^{14}C and 5β-pregnane-3α,11β,17α,20β-tetrol-G-^{3}H; (3) cholesterol-4-^{14}C, pregnenolone-4-^{14}C, 5β-pregnane-3α,17α,20α-triol-G-^{3}H and 5β-pregnane-3α,11β,17α,20β-tetrol-G-^{3}H; (4 and 5) pregn-5-ene-3β,20β-diol-4-^{14}C, 5β-pregnane-3α,17α,20α-triol-G-^{3}H and 5β-pregnane-3α,11β,17α,20β-tetrol-G-^{3}H. Mixtures 1–4 were prepared from the trimethylsilyl ethers of the individual steroids. In mixture 5 the trimethylsilyl ethers were prepared from the mixture of free steroids. The results of these experiments are summarised in Table III.

The above results indicated that for each compound approximately 90 % of the activity appeared at the expected R_F value. An examination of the distribution of activity along the path of the mobile phase was made for the example of 5α-cholestane-3β,5,6β-triol-4-^{14}C bis(trimethylsilyl ether). As indicated in Table IV, approximately 3 % of the activity remained at the origin and 3 % was distributed along the path of the "spot". These results are consistent with qualitative evidence from the the autoradiogram shown in Fig. 3.

Finally, the recovery observed in the presence of a natural extract was assessed. Cholesterol-4-^{14}C and 5α-cholestane-3β,5,6β-triol-4-^{14}C were added to an extract

TABLE I

CHROMATOGRAPHIC DATA FOR STEROID TRIMETHYLSILYL ETHERS

Layer: Silica Gel G (Merck).

	R_F values			*Retention index*[a]
	Cyclohexane–ethyl acetate (9:1, v/v)	*Benzene*	*Cyclohexane–chloroform (4:1, v/v)*	
Cholesterol TMS[b]	0.79	0.68	0.36	3090
Pregnenolone TMS	0.32	0.11	0.08	2700
Pregn-5-ene-3β,20β-diol bis (TMS)	0.77	0.56	0.21	2820
5β-Pregnane-3α,17α,20α-triol 3,20-bis-(TMS)	0.52	0.16	0.13	2920
5β-Pregnane-3α,11β,17α,20β-tetrol 3,20-bis(TMS)	0.33	0.13	0.08	3040
5α-Cholestane-3β,5α,6β-triol 3,6-bis(TMS)	0.62	0.24	0.18	3350

[a] Determined for a 1 % SE-30 column by programmed temperature gas chromatography 200–280° at 3°/min.

[b] TMS = trimethylsilyl ether.

TABLE II

ANALYTICAL RECOVERY OF INDIVIDUAL STEROID TRIMETHYLSILYL ETHERS (1 ng–1 μg) AFTER TLC

Compound	*1 μg*			*100 ng*			*10 ng*			*1 ng*		
	Pre-TLC (c.p.m.)	*Recovery*		*Pre-TLC (c.p.m.)*	*Recovery*		*Pre-TLC (c.p.m.)*	*Recovery*		*Pre-TLC (c.p.m.)*	*Recovery*	
		c.p.m.	%		*c.p.m.*	%		*c.p.m.*	%		*c.p.m.*	%
Cholesterol-4-^{14}C TMS	154736	151038	98	13540	12193	90	1354	1179	87	147	124	84
Pregnenolone-4-^{14}C TMS	125389	116145	93	13509	12503	93	1461	1237	85	172	154	90
Pregn-5-ene-3β,20β-diol-4-^{14}C bis(TMS)	146041	132338	91	15081	14165	94	1385	1200	87	156	121	77

TABLE III

ANALYTICAL RECOVERY OF STEROID TRIMETHYLSILYL ETHERS (each *ca.* 10 ng) AFTER TLC INDIVIDUALLY OR IN ADMIXTURE

Compound	*Individual compounds*			*Mixture 1*		*Mixture 2*		*Mixture 3*		*Mixture 4*		*Mixture 5*		
	Pre-TLC (c.p.m.)	*Recovery*		*Recovery*		*Recovery*		*Recovery*		*Recovery*		*Pre-TLC (c.p.m.)*	*Recovery*	
		c.p.m.	%	*c.p.m.*	%	*c.p.m.*	%	*c.p.m.*	%	*c.p.m.*	%		*c.p.m.*	%
Cholesterol-4-^{14}C TMS	1316	1236	94	1227	93	1200	91	1219	93					
Pregnenolone-4-^{14}C TMS	2045	1923	94	1894	93			1858	91					
Pregn-5-ene-3β,20β-diol-4-^{14}C bis(TMS)	1773	1647	93							1622	92	960	910	89
5β-Pregnane-3α,17α,20α-triol-G-^{3}H bis(TMS)	89730	81296	91	78825	88	80125	89	80965	90	82478	92	67351	61865	92
5β-Pregnane-3α,11β,17α,20β-tetrol-G-^{3}H bis(TMS)	33598	30359	90			30580	91	28665	85	30783	91	29177	26135	90

TABLE IV

ANALYTICAL RECOVERY OF 5α-CHOLESTANE-3β,5,6β-TRIOL-4-^{14}C 3,6-BIS(TRIMETHYLSILYL ETHER) AFTER THIN-LAYER CHROMATOGRAPHY[a]

1. Triol bis(TMS) alone[b]

Zone eluted (R_F range)	*c.p.m.*		*Recovery (%)*
	Pre-TLC	*Post-TLC*	
0.55–0.70	655	589	90
0.05–0.55	—	22	3.5
—0.05–0.05	—	19	2.9

2. Trimethylsilylated mixture of triol-4-^{14}C, cholesterol-4-^{14}C and an extract (derived from human atheroma plaques) containing these two (unlabelled) steroids

Zone eluted (R_F range)	*c.p.m.*		*Recovery (%)*
	Pre-TLC	*Post-TLC*	
0.55–0.70	655	575	87.5
0.70–0.85	15182	14574	96

[a] The mobile phase (cyclohexane–ethyl acetate, 9:1) traversed 15 cm.
[b] Approximately 5 μg (0.3 mC/mmole).

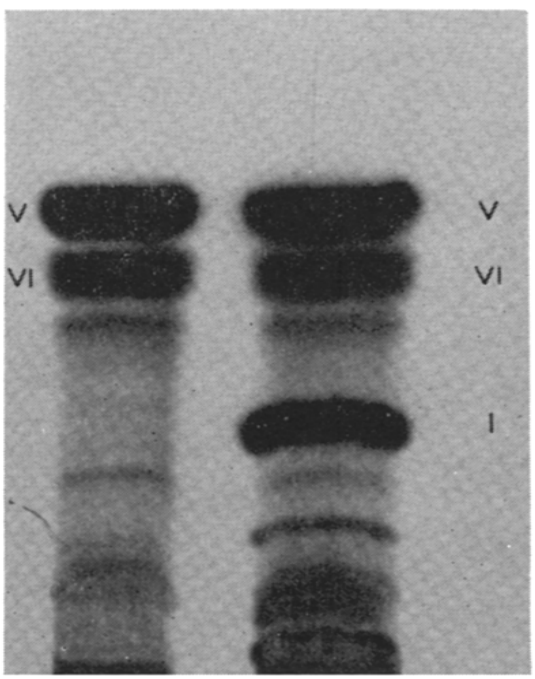

Fig. 3. Autoradiogram depicting three 4-^{14}C-steroid trimethylsilyl ethers after thin-layer chromatography. Conditions and designations as in Fig. 1. Quantities applied were: I = 0.76 μC (~ 10 μg); V = 0.78 μC (~ 10 μg); VI = 0.78 μC (~ 1 mg). The left-hand chromatogram includes only compounds V and VI.

derived from human atheroma tissue and converted to their respective trimethylsilyl ethers in the extract. The conditions were such as to produce the 3,6-bis(trimethylsilyl ether) of the triol, rather than the tris(trimethylsilyl ether), which can be formed under more powerful acid catalysis[18]. Recoveries were assessed as before and the results are summarised in Table IV.

DISCUSSION

The comparative neglect of steroid trimethylsilyl ethers, as derivatives for thin-layer chromatographic separations, appears to have been due to misapprehension as to their ease of hydrolysis. While it is true that certain trimethylsilyl ethers (*e.g.*, those of phenols and of oximes) are readily hydrolysed, the derivatives formed from alcoholic hydroxyl groups are relatively stable at neutral pH, even in aqueous media.

Evidence now available shows that trimethylsilyl ethers can be usefully applied to separations of steroids in quantities ranging from 1 ng to 100 mg with no serious loss either by hydrolysis or adsorption. Thus preparative "thick-layer" chromatographic separation of trimethylsilyl ethers of cholesterol and triterpenoid alcohols has been accomplished using 80 mg of sample[19]. Quantitative recovery of steroid trimethylsilyl ethers after column or thin-layer chromatography on the scale of 10–100 μg can be readily demonstrated by gas–liquid chromatographic estimation[13]. Finally, in the present work it has been shown that thin-layer chromatography can be applied to quantities as low as 1 ng without any appreciable increase in the proportion lost by adsorption or hydrolysis.

It is particularly notable that the recovery of the trimethylsilyl ethers of pregnanetriol and pregnanetetrol (retaining, under the conditions used, respectively one and two unreactive hydroxyl groups)* is not materially different from that of the other compounds examined. Factors such as steric compression (11β-OH), tertiary character (17α-OH) and hydrogen bonding (17α-OH,20-OR) which render certain groups less reactive, also reduce their degree of adsorption so that the mobility and ease of elution are not greatly affected.

Among many important applications of trimethylsilyl ethers in the gas chromatography of steroids, their use as derivatives of polyhydroxylic steroids is notable. Thus trimethylsilyl ethers of cortols and cortolones are stable towards gas–liquid chromatography[7], while the combined use of O-methyloximes and trimethylsilyl ethers permits the majority of corticosteroid metabolites to be succesfully analysed[21]. It is accordingly very satisfactory that trimethylsilyl derivatives prove suitable for preliminary separation by thin-layer chromatography on a scale compatible with gas chromatographic procedures based on sensitive ionisation detectors. As pointed out elsewhere[10], thin-layer or column chromatography of trimethylsilyl ethers is particularly effective in group separations, *e.g.* for isolating purely hydroxylic steroids from those possessing ketonic groups (*cf.* Fig. 1 and Table I), but it can also be adapted to the separation of individual steroids.

The selection of trimethylsilyl ethers, rather than the generally more polar acyl derivatives, for thin-layer chromatography is frequently advantageous in other respects:

* We have not observed, under our experimental conditions, the trimethylsilylation of the 17α-OH reported by HARA *et al.*[20].

(i) As is well known, the gas chromatographic behaviour of compounds containing several trimethylsilyl ether groups is relatively insensitive to the number of such groups because of the low contribution of the trimethylsilyloxy group to the retention: this permits convenient study of trimethylsilyl ethers where the analogous acetates would have inordinately long retention times[18].

(ii) In many instances the parent hydroxysteroids can be recovered by hydrolysis under very mild conditions, somewhat akin to those suitable for hydrolysis of formates. Catalysts such as ammonia or acetic acid may be effective. Quantitative hydrolysis of cholesterol trimethylsilyl ether has been demonstrated[19], using 0.01 *N* hydrogen chloride in propan-2-ol/water (9:1, w/w). There appears to have been no systematic study of structural influences on the hydrolysis of steroid trimethylsilyl ethers. As it is evident from qualitative observations that the rates of hydrolysis of differently constituted derivatives may be quite dissimilar, further work in this area is clearly needed.

(iii) The transformation of trimethylsilyl ethers to acyl derivatives (*e.g.* trifluoroacetates[22]) may be accomplished by direct reaction with appropriate acylating reagents.

(iv) Trimethylsilyl ethers are particularly amenable to vacuum sublimation[13], which affords an excellent means of isolating them from extraneous materials of lower volatility.

It is concluded that trimethylsilyl ethers, by virtue of their very low polarity, can play a particularly effective role in the isolation and separation of polyhydroxylic steroids by thin-layer chromatography, which complements their well-established application in analyses based on gas–liquid chromatography. The present results indicate that the derivatives should be suitable for the micro-scale separations frequently required in the analysis of steroids in biological extracts.

ACKNOWLEDGEMENTS

We are greatly indebted to the Medical Research Council for a research grant. We also thank Dr. A. F. LEVER (M.R.C. Blood Pressure Research Unit) for placing radioactive counting equipment at our disposal. Dr. A. M. M. BERRIE (Botany Department, University of Glasgow) kindly provided the autoradiogram shown in Fig. 3. We thank Perkin-Elmer Ltd. (Beaconsfield) for supplying a gas chromatograph for evaluation.

SUMMARY

Trimethylsilyl ethers derived from isotope-labelled steroids containing up to four hydroxyl groups have been subjected to thin-layer chromatography. Elution of the appropriate zones gives practically quantitative recovery of material (as assessed by radioactivity) for quantities ranging from 1 ng to 1 μg. For the steroids examined, losses due to hydrolysis or adsorption during thin-layer chromatography are slight and of constant proportions over this range. This result confirms the suitability of trimethylsilyl ethers for thin-layer chromatography of polyhydroxylic steroids.

REFERENCES

1 T. FEHÉR, *Mikrochim. Acta*, 1 (1965) 105.
2 H. S. KROMAN, M. L. MOSKOVITZ, S. R. BENDER AND A. N. BREST, *J. Gas Chromatog.*, 4 (1966) 147.
3 D. R. IDLER, N. R. KIMBALL AND B. TRUSCOTT, *Steroids*, 8 (1966) 865.
4 S. H. LANGER, P. PANTAGES AND I. WENDER, *Chem. Ind. (London)*, (1958) 1664.
5 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2497.
6 T. LUUKKAINEN, W. J. A. VANDENHEUVEL, E. O. A. HAAHTI AND E. C. HORNING, *Biochim. Biophys. Acta*, 52 (1961) 599.
7 R. S. ROSENFELD, *Steroids*, 4 (1964) 147.
8 H. GOTTFRIED, in M. B. LIPSETT (Editor), *Gas Chromatography of Steroids in Biological Fluids*, Plenum Press, New York, 1965.
9 C. J. W. BROOKS AND J. G. CARRIE, *Biochem. J.*, 99 (1966) 47P.
10 C. J. W. BROOKS, E. M. CHAMBAZ, W. L. GARDINER AND E. C. HORNING, *Excerpta Med., Intern. Congr. Ser.*, No. 111, (1966) Abstr. No. 51.
11 C. J. W. BROOKS, E. M. CHAMBAZ AND E. C. HORNING, *Anal. Biochem.*, 19(1967) 234.
12 R. V. BROOKS, in J. K. GRANT (Editor), *The Gas–Liquid Chromatography of Steroids: Mem. Soc. Endocrinol.*, No. 16 (1967) 143.
13 C. J. W. BROOKS, E. M. CHAMBAZ, W. L. GARDINER AND E. C. HORNING, *Meeting Gas Chromatog. Determination Hormonal Steroids, Rome*, 1966.
14 J. WATSON AND C. J. W. BROOKS, *Biochem. J.*, 104 (1967) 3P.
15 C. J. W. BROOKS AND J. WATSON, *Commun. Round Table Conf. on GLC, Fondation de Recherche en Hormonologie, Paris, May 1967.*
16 L. F. FIESER AND S. RAJAGOPALAN, *J. Am. Chem. Soc.*, 71 (1949) 3938.
17 E. C. HORNING, W. J. A. VANDENHEUVEL AND B. G. CREECH, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 11, Interscience, New York, 1963.
18 C. J. W. BROOKS, L. HANAINEH, A. MCCORMICK, G. STEEL AND J. S. YOUNG, *Commun. Round Table Conference on GLC, Fondation de Recherche en Hormonologie, Paris, May 1967.*
19 B. O. LINDGREN AND C. M. SVAHN, *Acta Chem. Scand.*, 20 (1966) 1763.
20 S. HARA, T. WATABE AND Y. IKE, *Chem. Pharm. Bull.*, 14 (1966) 1311.
21 W. L. GARDINER AND E. C. HORNING, *Biochim. Biophys. Acta*, 115 (1966) 524.
22 J. SJÖVALL, in J. K. GRANT (Editor), *The Gas–Liquid Chromatography of Steroids: Mem. Soc. Endocrinol.*, No. 16 (1967) p. 243.