CHROM. 4037

PRODUCTION OF THE PARENT HYDROCARBONS FROM STEROID DRUGS AND THEIR SEPARATION BY GAS CHROMATOGRAPHY

P. M. ADHIKARY AND R. A. HARKNESS

Department of Paediatric Biochemistry, Royal Hospital for Sick Children and Department of Clinical Chemistry, University of Edinburgh (Great Britain)

(Received March 3rd, 1969)

SUMMARY

Carbon skeleton chromatography of many anabolic and progestational steroid drugs and related compounds has been carried out following high-temperature catalytic reduction on a microgram scale. Steroids with the same carbon skeleton were reduced to products with the same relative retention times on gas chromatography. These 'parent steranes' could generally be distinguished from the carbon skeletons of the naturally occurring steroids. About 5–20% of the starting material could be recovered as steranes. Although the yield from any one compound was reproducible, the recoveries were generally dependent on the position, nature and number of substituents in the starting material. Some applications of the present techniques to biological problems have been discussed.

INTRODUCTION

Chromatographic techniques have been extensively used for the tentative identification of microgram quantities of steroids. Paper chromatography has been developed by BUSH[1], EDWARDS AND TRAFFORD[2] and others, gas–liquid chromatography (GLC) by KNIGHTS AND THOMAS[3], VANDENHEUVEL AND HORNING[4], HARTMAN AND WOTIZ[5] and others. Thin-layer chromatography (TLC) has also been employed. The contributions of the functional groups in some steroid drugs to chromatographic mobility on thin layers have been evaluated[6]. Using such techniques, the nature and position of functional groups on the steroid skeleton can generally be determined. However, the identification of the hydrocarbon skeleton has been difficult. The parent hydrocarbons of compounds simpler than steroids have already been identified by GLC after high-temperature catalytic reduction; this method can now be applied to steroids and sterols[7].

The present work describes the application of the technique of high-temperature catalytic reduction to steroid drugs and related compounds on a microgram scale. The reduction products of steroids with the same carbon skeleton have identical retention times. However, the different hydrocarbons produced in the present studies

could be separated from each other by GLC. Since in most cases man does not degrade the carbon skeletons of the steroids[8–10], this 'label' is retained by the metabolites of a steroid. The present technique thus offers a simple method for detection of the metabolites of the many steroid drugs which have different carbon skeletons from the natural products.

MATERIALS AND METHODS

Materials

Reagents and solvents were of analytical grade where available. The steroids and sterols studied were pure as judged by TLC and in some cases also by GLC, except where otherwise noted. The steroid drugs which have been studied are shown in Table I. This table contains the systematic names, the official names listed in the 8th

TABLE I

NAMES AND CARBON SKELETONS OF STEROID DRUGS

Systematic name	*Official name*	*Hydrocarbon skeleton*
Anabolic steroids		
17α-Methyl-17β-hydroxyandrost-1,4-dien-3-one	Methandrostenolone	17α-Methylandrostane
17α-Methyl-11β,17β-dihydroxy-9α-fluoroandrost-4-en-3-one	Fluoxymesterone	17α-Methylandrostane
17α-Methyl-2-hydroxymethylene-17β-hydroxy-5α-androstan-3-one	Oxymetholone	17α-Methylandrostane
17α-Methyl-4,17β-dihydroxy-androst-4-en-3-one	Oxymesterone	17α-Methylandrostane
17α-Methyl-17β-hydroxyandrost-4-en-3-one	17-Methyltestosterone	17α-Methylandrostane
1β-Methyl-17β-hydroxy-5α-androst-1-en-3-one	Methenolone	1β-Methylandrostane
17α-Ethyl-17β-hydroxyoestr-4-en-3-one	Norethandrolone	17α-Ethyl-19-norandrostane
17α-Methyl-17β-hydroxy-5α-androstane-3,2c-pyrazole	Stanozolol	17α-Methylandrostane-3,2c-pyrazolidine
Progestational and related steroids		
17α-Ethynyloestr-4-ene-3β,17β-diol	Ethynodiol	19-Norpregnane[a]
17α-Ethynyloestr-5(10)-en-17β-ol-3-one	Norethynodrel	19-Norpregnane[a]
17α-Ethynyl-17β-hydroxyoestr-4-en-3-one	Norethindrone	19-Norpregnane[a]
17α-Ethynyloestra-1,3,5(10)-triene-3,17β-diol	Ethynyloestradiol	19-Norpregnane[a]
17α-Ethynyl-3-methoxyoestra-1,3,5(10)-trien-17β-ol	Mestranol	19-Norpregnane[a]
6α-Methyl-17α-acetoxypregn-4-ene-3,20-dione	Medroxyprogesterone acetate	6α-Methylpregnane
6-Methyl-17α-acetoxypregna-4,6-diene-3,20-dione	Megesterol acetate	6-Methylpregnane
16α-Methylpregn-4-ene-3,20-dione	16-Methylprogesterone	16α-Methylpregnane
6-Chloro-17α-acetoxypregna-4,6-diene-3,20-dione	Chlormadinone acetate	Pregnane

[a] 17α-side chain.

edition of the Merck Index, and the carbon skeleton upon which the drug is based. It should be noted that many drugs possess the same carbon skeleton, *e.g.* 17α-methylandrostane for the anabolic stercids and 19-norpregnane for the progestational agents. It is also of interest that only one of the 17 compounds listed, chlormadinone acetate, has the same carbon skeleton as naturally occurring steroids, in this example pregnane. Some compounds were obtained by chloroform extraction from tablets. The tablets were made into a suspension in 2 ml of distilled water and extracted with 5 vol. of chloroform. The extract was washed repeatedly with distilled water until clear, dried by filtration through a small quantity of anhydrous sodium sulphate, and evaporated to dryness. The extract was dissolved in absolute ethanol at a steroid concentration of approximately 1 mg/1 ml. In such cases no precise quantitative results could be obtained; where relevant, this is noted.

High-temperature catalytic reduction and gas–liquid chromatography

The steroids were subjected to high-temperature catalytic reduction by the method of ADHIKARY AND HARKNESS[7], in which samples in solution are injected into a stream of hydrogen flowing through a heated catalyst bed. The reduction products are then trapped. The apparatus consisted of a siliconised glass tube surrounded by a heated aluminium block. An injection head was fitted at one end and a narrow tube for trapping the reduction products at the other end. Using ice and water as a coolant about 77% of injected steranes could be recovered. The combination of 1–3% w/w platinum on siliconised glass beads was found superior to a number of other metal catalysts and diatomaceous earth supports. The temperature for reduction was 170–200° unless otherwise stated. It has been found that an increase in the quantity of platinum to 5% was associated with an increase in the products with short retention times, possibly due to extensive hydrogenolysis. Therefore, the stated conditions should be adhered to as closely as possible. The apparatus, its method of use and its reliability have been described in detail[7].

The products of reduction were studied by GLC on columns coated with 1% w/w SE-30 and with 1% w/w NGA. Since the hydrocarbon products were more volatile than steroids, heating of the samples on their stainless steel gauzes, before injection by the method of MENINI AND NORYMBERSKI[11], caused significant losses; these were reduced by placing insulation on the top of the flash heater. In a later modification, single solid samples were injected through a gas-tight chamber formed by two 'Rotaflo' stopcocks (Quickfit & Quartz Ltd., Great Britain) in series. The major products, generally two in number, have retention times and yields listed in the tables. Retention time data are given relative to 5α-androstane or 5α-cholestane. The coefficient of variation for a single relative retention time was about 1%. Since the means quoted are generally from four or more determinations, errors would be correspondingly reduced. For quantitation a minimum of three observations was used throughout the work.

The amounts of products were estimated by planimetry of the peak areas from the GLC tracings and expressed in terms of weight relative to the most closely related available pure sterane. For example, androstane was used as a standard for the methyl- or norandrostanes. The yields given in the tables are the amounts of major products expressed as a percentage of the starting material. The major product is identified in the tables by the letter "M" after its relative retention time; when the peak areas were approximately equal, no comment has been added.

Evidence has already been presented by ADHIKARY AND HARKNESS[7] that this method produces the parent sterane from a number of naturally occurring steroids and cholesterol. In order to simplify the description and discussion of the results, the reduction products with identical retention times derived from compounds with identical hydrocarbon skeletons are described as parent steranes. The use of the method does not, however, depend upon the correctness of this assumption, which seems justifiable from all the available evidence, but only upon the identical retention times.

Thin-layer chromatography

In order to check the identities of the products and provide evidence of any unreduced or partially reduced starting material, thin-layer chromatography on silica gel was performed. The R_F values of the androstanes, pregnanes and cholestanes were 0.6 and 0.7 in cyclohexane and chloroform, respectively. Surprisingly, it was found that about 2–5 μg of sterane in a 0.7 cm diameter spot could be detected with 8% w/v phosphomolybdic acid in methanol acidified with a drop of conc. HCl. This reagent was more sensitive than iodine vapour or 5% w/v iodine solution in $CHCl_3$.

TABLE II

RELATIVE RETENTION TIMES AND YIELDS OF THE REDUCTION PRODUCTS OF STEROID DRUGS

Official name	*Mean yield (%)*	*Retention times of products relative to 5α-androstane*				
		SE-30 column		*NGA column*		
(A) Anabolic steroids						
17-Methyltestosterone	20.0	1.28	1.45	1.19	1.31	
Methandrostenolone	17.8	1.28	1.45	1.19	1.31	
6β-Hydroxymethandrostenolone[a]	*ca.* 15	1.28	1.45	1.19	1.31	
Oxymetholone[a]	12.5	1.28	1.45 M	1.19	1.31 M	
Oxymesterone[b]	*ca.* 6.5	1.28	1.45 M	1.19	1.31 M	
Norethandrolone	16.8	1.46 M[c]	1.60	1.30 M	1.46	
Methenolone	4.8	1.49			1.48	
Stanozolol	4.7	1.03[d]			0.81[d]	
(B) Progestational and related steroids						
Mestranol	8.5	1.41	1.55	1.34	1.53	1.60
Ethynodiol	13.8	1.41 M	1.55	1.34 M	1.53	
Norethisterone[b]	*ca.* 10	1.41 M	1.55	1.34 M	1.53	
Norethynodrel[a]	8.5	1.41 M	1.55	1.34 M	1.53	1.60
Ethynyloestradiol	13.5	1.41 M	1.55	1.34 M	1.53	
18-Hydroxypregnenolone hemiacetal	3.5			1.40	1.56 M	
Megesterol acetate	9.5	2.50 M	2.80	2.25 M	2.61	
Medroxyprogesterone acetate	11.25	2.50 M	2.80	2.25 M	2.61	
16-Methylprogesterone	8.5	2.48	2.79	2.04	2.34	
Chlormadinone acetate	5.0	2.01	2.27	1.86	2.13	
Progesterone	15.2	2.01	2.27	1.86	2.13	
Pregnenolone	13.6		2.27		2.13	
5α-Pregnane-3,20-dione	17.8		2.27		2.13	
5β-Pregnane-3,20-dione	19.2	2.01		1.86		

[a] Impure.
[b] Tablet.
[c] M = major product.
[d] Relative to 5α-cholestane.

RESULTS

Although there are a large number of steroids and sterols, these compounds are based upon a small number of parent hydrocarbons or steranes. This is true for naturally occurring compounds and even for the synthetic steroid drugs as shown in Table I. Thus reduction to the carbon skeleton is a simplification which has been achieved by the present method. The relative retention times of the reduction products of steroid drugs with the same carbon skeletons are, as predicted, identical. This is shown for the anabolic steroids by the results in Table II(A).

Four anabolic steroids with a 17-methylandrostane skeleton, methyltestosterone, methandrostenolone, oxymetholone and oxymesterone, were reduced to products with the same retention times on SE-30 and on NGA coated columns (Fig. 1A). A number of progestational steroids similarly showed reduction products with identical relative retention times. For example, three commonly used progestational agents

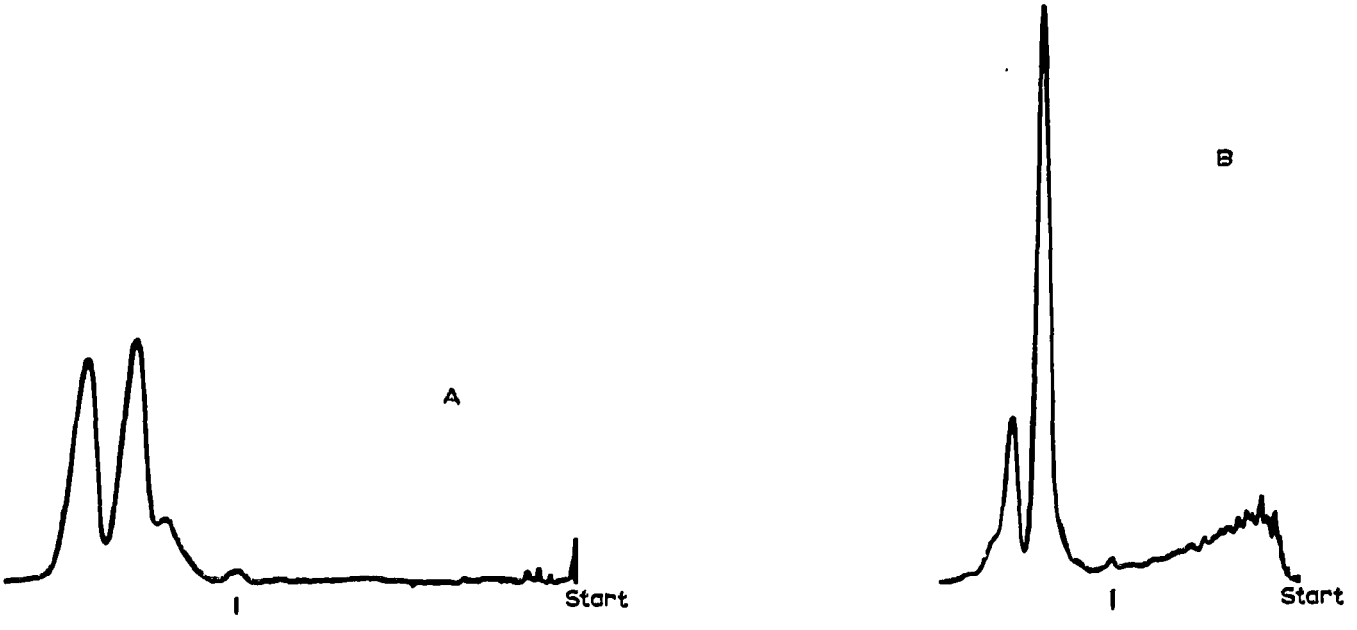

Fig. 1. (A) Reduction products from 5 μg methyltestosterone, catalyst temperature 180°, chromatographed on an SE-30 coated column at 150°; attenuation × 500. The retention time of 5α-androstane is indicated by a vertical line. (B) Reduction products from 5 μg ethynodiol, catalyst temperature 180°, chromatographed on an NGA coated column at 150°; attenuation × 200. The retention time of 5α-androstane is indicated by a vertical line.

with 19-norpregnane skeletons, ethynodiol (Fig. 1B), norethynodrel and norethisterone, as well as the oestrogens mestranol and ethynyloestradiol, were reduced to products with identical retention times (Table II(B)). It should be noted that the product with a relative retention time of 1.60 on a NGA-coated column was detectable in all cases, but relatively large amounts were obtained only from mestranol and the impure norethynodrel. Although ethynyloestradiol was reduced completely to the expected products, the reduction products from oestradiol and oestrone sometimes included products with a retention time intermediate between the androstanes and pregnanes.

It seemed justifiable to conclude from this and other evidence that the aromatic A ring was more difficult to reduce than isolated double bonds; this is consistent with studies on some naphthalene derivatives by BEROZA[12].

Using the present method, products with the retention times of the parent steranes were always detectable and were generally the major products. The different steranes from the compounds studied were separated by GLC and these separations were in most cases good. Products which had different numbers of carbon atoms were clearly distinguished; as expected, the retention times were approximately linearly related to the carbon number[4]. High-temperature catalytic reduction was performed on steroids and sterols with from 18 to 30 carbon atoms. Two major products which behaved like the parent hydrocarbons were obtained from a C_{30} compound, lanosterol, 4α,4β,14α-trimethylcholesta-8,24-dien-3β-ol. It should also be noted that different structures with the same number of carbon atoms were also separable. The 18-nor-androstanes were separated from the 19-norandrostanes (Table III). 17-Methyl-

TABLE III

RELATIVE RETENTION TIMES AND YIELDS OF THE REDUCTION PRODUCTS OF C-18 AND C-19 HYDROXYLATED AND NORSTEROIDS

Compound	*Mean yield (%)*	*Retention times of products relative to 5α-androstane*			
		SE-30 column		*NGA column*	
19-Nortestosterone	9.3	0.69		0.60	
19-Hydroxyandrost-4-ene-3,17-dione	8.7	0.69		0.60	
Oestrone	6.2	0.69		0.60	
Oestradiol-17β	6.6	0.69		0.60	
18-Nordehydroepiandrosterone	11.1	0.76	0.83	0.73	0.80
18-Hydroxytestosterone	2.8	0.76	0.83	0.73	0.80

androstanes were separable from 1-methylandrostanes and 6-methylpregnanes from 16-methylpregnanes (Table II).

The amount recovered as steranes varied with the structure of the starting material. Recoveries were reduced by extra substituents, for example, hydroxyl groups. This is illustrated by the yield of 20% from 17-methyltestosterone compared to 6.5% from oxymesterone with an extra hydroxyl group at position 4.

The position of a methyl group could have an important effect on the yield. The recovery of steranes from 17-methyltestosterone was similar to that from testosterone whereas from methenolone with a 1-methyl group only 4.8% was recovered. In this compound the 1-methyl group may sterically hinder the reduction of the double bond at C-1; this double bond was readily reduced when relatively unhindered as in methandrostenolone (Table II).

Using the present method, various groups could be reduced or removed from the carbon skeleton. Hydroxyl or ketone groups were readily reduced; fluoro and chloro substituents, at C-9 and C-6 respectively, could be removed, although fluoxymesterone only yielded small amounts of 17-methylandrostanes, about 3% of the starting material, with significant quantities of other products with shorter retention times which were the major products. However, it must be emphasised that 17-methyl-

androstanes were produced from fluoxymesterone as from the other anabolic steroids with a 17-methylandrostane skeleton (Table II(A)). The carboxyl group in the bile acids was reduced in low yield. Reduction products with relative retention times identical with those of 5α-cholane were always detected after reduction of bile acids. However, it was expected from the work of BEROZA[12] that the major products would have lost the C-24 carbon atom. Ester and ether linkages were readily split. Double bonds at various positions, 1, 3, 5 and 5(10) were readily reduced. After reduction of hydroxyl groups at various positions, the parent hydrocarbon was always detectable; steroids with hydroxyl groups at positions 1, 2, 3, 4, 6, 7, 11, 12, 15, 16, 17, 18, 19 and 21 were successfully reduced. The position of the hydroxyl groups did not generally affect the production of a single major product corresponding to the parent hydrocarbon except where the hydroxyl group was in a terminal position, for example, C-18, C-19 or C-21.

Fig. 2. (A) Reduction products from 5 μg 16α-hydroxydehydroepiandrosterone, catalyst temperature 200°, chromatographed on an NGA coated column at 150°; attenuation × 100. The retention time of 5α-androstane is indicated by a vertical line. (B) Reduction products from 10 μg cortisol, catalyst temperature 190°, chromatographed on an SE-30 coated column at 160°; attenuation × 100. The retention time of 5α-androstane is indicated by a vertical line.

The 18- and 19-hydroxysteroids were reduced, as predicted from the work of BEROZA[12] and that of THOMPSON *et al.*[13] on simpler compounds, largely to corresponding 18- or 19-norsteroids with smaller amounts of the parent hydrocarbons. The 2-hydroxymethylene group was removed from oxymetholone.

In the compounds with adjacent hydroxyl and ketone groups at C-16 and C-17, the parent hydrocarbons were the major products; in addition, two other products with short retention times were regularly produced, possibly by hydrogenolysis[13] (Fig. 2A and Table IV). These two products could be more easily lost during solvent evaporation than the parent steranes. This pattern of products proved useful in the identification of 16β-hydroxydehydroepiandrosterone from infant urine[14].

Reduction of the corticosteroids, cortisol, corticosterone and deoxycorticoste-

TABLE IV

RELATIVE RETENTION TIMES AND YIELDS OF THE REDUCTION PRODUCTS FROM 16,17-DIOXYANDROSTANE DERIVATIVES

Compound	*Mean yield of 5α- and 5β-androstanes (%)*	*Retention times of products relative to 5α-androstane (NGA column)*	
16α-Hydroxydehydroepiandrosterone	5.8	0.88	1.00 M
		0.33	0.34
16β-Hydroxydehydroepiandrosterone[a]	6.5	0.88	1.00 M
		0.33	0.34 M
3β, 16α- 17β-Trihydroxyandrost-5-ene	5.7	0.88	1.00 M
		0.33	0.34
3β, 17β-Dihydroxyandrost-5-en-16-one	6.0	0.88	1.00 M
		0.33	0.34

[a] Impure.

rone, with highly oxygenated side chains produced about 1–3% of the parent pregnanes and about 5–10% as androstanes. However, peak areas of about 34–95% of the major peak area appeared as products with retention times relative to 5α-androstane of 1.40 and 1.56. In contrast to the products from the majority of steroids and sterols, there was a complex pattern of reduction products from corticosteroids (Fig. 2B). The yield of reduction products from the corticosteroids was more variable than from compounds like progesterone and the 17-oxosteroids; the coefficient of variation for a single observation was about 5–10% for 17-oxosteroids in contrast to 30–50% for corticosteroids. However, the major reduction products were generally the androstanes and the parent hydrocarbon could always be detected.

The main reduction products of the heterocyclic anabolic steroid stanozolol had a retention time somewhat similar to that of cholestane (Table IIA); this product did not correspond to a methylandrostane. Although BEROZA[12] found that high-temperature catalytic reduction removed amino groups, THOMSON *et al.*[13] found that high temperatures and a special catalyst were necessary to remove nitrogen from the carbazole ring. It, therefore, seems probable that the pyrazole ring in stanozolol was reduced to pyrazolidine and retained as part of the skeleton.

DISCUSSION

Using the present technique, crude fractions can be shown to contain steroids. Thus at a very early stage in work on the isolation and identification of unknown natural products evidence of a sterane hydrocarbon skeleton can be obtained. It is difficult to assess the importance of this from the literature because mistakes are rarely acknowledged; although caffeine has been recognised as a source of confusion[15]. Carbon skeleton chromatography can be supplemented by existing microchemical identification of functional groups to suggest a limited number of possible structures and thus ease the problem of selection of compounds for more detailed comparison. These possibilities can then be investigated by other methods, generally using highly purified samples.

The simplicity of the present technique has been compared and contrasted to the more complex processes of pyrolysis and mass spectrometry[16]. The chromatographic pattern of the reduction products was generally simple and often characteristic of the starting material. For example, the ratio of the 5α:5β steranes was affected by the C-4 or C-5 position of a double bond in naturally occurring compounds[7]. However, in the present studies some 19-nor compounds gave predominantly one product (Table III). The more detailed interpretation of patterns of products from mixtures may be difficult. For example, a mixture of an androstane derivative with small quantities of a compound with a stable pregnane skeleton would give a somewhat similar pattern of reduction products to an unstable corticosteroid, that is, androstanes with small amounts of pregnanes.

The hydrocarbon skeletons of many steroids are not broken down in man[8–10]. Although O-methylation is a significant pathway of drug catabolism[17] it is quantitatively unimportant for steroids. In addition methoxy groups are removed by the present method, as are additional hydroxyl groups. The metabolites of a steroid drug should, therefore, be reduced, by the present method, to the same products as the steroid drug itself (see 6β-hydroxymethandrostenolone in Table II(A)). The steranes produced are not difficult to separate (Table II), and even better results have been obtained on columns coated with OV-17. Thus many steroid drugs and their metabolites, with carbon skeletons which do not occur in nature, should be detectable. Many steroid drugs possess the same carbon skeleton; for example, the metabolites of several different anabolic steroids would all be reduced to 17-methylandrostanes. The data presented in this paper should thus simplify the detection of these drugs.

The stability of the carbon skeleton will be important in determining the sensitivity of the method. However, even the highly unstable corticosteroids yield about 1% as parent pregnanes. Since the flame ionisation detector is highly sensitive and the losses of hydrocarbons even on present columns are small, corticosteroids should be detected in quantities down to 10 μg; correspondingly smaller amounts of more stable steroids should be detectable. Thus normal therapeutic quantities of some steroid drugs can be administered and the metabolites detected, in contrast to the large doses previously necessary[18,19]. The metabolism of normal therapeutic quantities of some steroid drugs can be studied without the difficulties of obtaining pure radioactively labelled compounds and the potential dangers of their use in man[20].

ACKNOWLEDGEMENTS

We are grateful to Professor L. G. WHITBY and Dr. P. J. SYKES for their help, to Mr. A. M. TORRANCE for his technical assistance, to Dr. A. SEGALOFF for a supply of 6β-hydroxymethandrostenolone, and to the pharmaceutical firms, Bayer, BDH, Ciba, Eli Lilly, ICI, Schering and G.D. Searle, for the majority of compounds used in this work.

P. M. ADHIKARY is a recipient of a Nepal/U.K. Colombo Plan scholarship.

REFERENCES

1 I. E. BUSH, *The Chromatography of Steroids*, Pergamon, Oxford, 1961, pp. 256–310.
2 R. W. H. EDWARDS AND D. J. H. TRAFFORD, *Biochem. J.*, 108 (1968) 185.
3 B. A. KNIGHTS AND G. H. THOMAS, *Nature*, 194 (1962) 833.

4 W. J. A. VANDENHEUVEL AND E. C. HORNING, *Biochim. Biophys. Acta*, 64 (1962) 416.
5 I. S. HARTMAN AND H. H. WOTIZ, *Biochim. Biophys. Acta*, 90 (1964) 334.
6 S. HARA AND K. MIBE, *Anal. Chem.*, 40 (1968) 1605.
7 P. M. ADHIKARY AND R. A. HARKNESS, *Anal. Chem.*, 41 (1969) 470.
8 G. E. TURFITT, *Biochem. J.*, 42 (1948) 376.
9 J. M. COOPER AND A. E. KELLE, *Steroids*, 11 (1968) 133.
10 P. TALALAY, *Physiol. Rev.*, 37 (1957) 362.
11 E. MENINI AND J. K. NORYMBERSKI, *Biochem. J.*, 95 (1965) 1.
12 M. BEROZA, *Anal. Chem.*, 34 (1962) 1801.
13 C. J. THOMPSON, H. J. COLEMAN, R. L. HOPKINS AND H. T. RALL, *J. Gas Chromatog.*, 5 (1967) 1.
14 C. H. L. SHACKLETON, R. W. KELLY, P. M. ADHIKARY, C. J. W. BROOKS, R. A. HARKNESS, P. J. SYKES AND F. L. MITCHELL, *Steroids*, 12 (1968) 705.
15 E. E. NISHIZAWA AND K. B. EIK NES, *J. Chromatog.*, 10 (1963) 478.
16 M. BEROZA AND F. ACREE, JR., *J. Assoc. Offic. Agr. Chemists*, 47 (1964) 1.
17 D. V. PARKE, *The Biochemistry of Foreign Compounds*, Pergamon, Oxford, 1968, p. 83.
18 E. L. RONGONE, A. SEGALOFF, J. FRIED AND E. F. SABO, *J. Biol. Chem.*, 236 (1961) 2624.
19 L. L. ENGEL, J. ALEXANDER AND M. WHEELER, *J. Biol. Chem.*, 231 (1958) 159.
20 P. M. ADHIKARY AND R. A. HARKNESS, *Biochem. J.*, (1969) in press.