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THE USE OF STEROID PROFILING IN THE RESOLUTION OF PREGNENOLONE METABOLITES FROM PORCINE TESTICULAR PREPARATIONS

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SUMMARY

The behaviour of 22 steroids has been examined by capillary gas chromatographic profiling, and the identities of the steriods confirmed by mass spectrometry and their relative retention times. Five pairs of steroids have been separated: 5α-androstane-3α,17β- and -3β,17β-diols; 5-androstene-3β,17α- and -3β,17β-diols; pregnenolone and 3β-hydroxy-5,16-pregnadien-20-one; progesterone and 4,16-pregnadiene-3,20-dione; 17-hydroxypregnenolone and 5-pregnene-3β,20β-diol. 5α-Androst-16-en-3β-ol and 5,16-androstadien-3β-ol (as trimethylsilyl-, *tert.*-butyldimethylsilyl- and chloromethyldimethylsilyl ethers) were only partially resolved but could be well separated on thin-layer plates of Kieselgel that had previously been dipped in $AgNO_3$ solution. The profiling method was successfully applied to the separation of pregnenolone and its metabolites in porcine testicular incubation extracts.

INTRODUCTION

In studies of the biosynthesis of androgens* and 16-androstenes in porcine testis, several metabolites have been encountered that have proved difficult to separate. Earlier studies, using porcine testicular homogenates, showed that 5,16-an-

* Trivial names and abbreviations used: pregnenolone, 3β-hydroxy-5-pregnen-20-one; 16-dehydropregnenolone, 3β-hydroxy-5,16-pregnadien-20-one; progesterone, 4-pregnene-3,20-dione; 16-dehydroprogesterone, 4,16-pregnadiene-3,20-dione; testosterone, 17β-hydroxy-4-androsten-3-one; 5α-dihydrotestosterone (5α-DHT), 17β-hydroxy-5α-androstan-3-one; dehydroepiandrosterone (DHA), 3β-hydroxy-5-androsten-17-one; 17-hydroxypregnenolone, 3β,17-dihydroxy-5-pregnen-20-one; 16α-hydroxypregnenolone, 3β,16α-dihydroxy-5-pregnen-20-one; 17-hydroxyprogesterone, 17-hydroxy-4-pregnene-3,20-dione; 16α-hydroxyprogesterone, 16α-hydroxy-4-pregnene-3,20-dione.

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drostadien-3β-ol was formed in high yield from pregnenolone[1] and that this was further metabolized to other 16-androstenes, including 5α-androst-16-en-3β-ol. This compound is difficult to resolve from 5,16-androstadien-3β-ol by thin-layer chromatography (TLC)[2] although some separation may be achieved on $AgNO_3$-impregnated layers of Kieselgel G[1,3] or by column chromatography on $AgNO_3$-impregnated silicic acid[4]. Unfortunately, the steroids are difficult to elute quantitatively from $AgNO_3$-impregnated layers.

Resolution of these steroids as the chloromethyldimethylsilyl ethers has been achieved by gas chromatography (GC) on packed columns, but only when using a high proportion of polar stationary phase[5,6]. Greater difficulties have been encountered with the separation of pregnenolone from several of its possible metabolites such as 16-dehydropregnenolone[7,8] and of 17-hydroxypregnenolone from 5-pregnene-3β,20β-diol[9]. We have been unable to resolve pregnenolone from 16-dehydropregnenolone by multiple runs on thin layers of Kieselgel, either untreated or $AgNO_3$-impregnated or by column chromatography on $AgNO_3$-impregnated silicic acid[10].

In this communication, we describe the use of capillary GC to resolve a number of C_{21} and C_{19} steroids that may be encountered as metabolites of pregnenolone in porcine testicular incubations. Such profiling of the derivatized steroids, together with mass spectrometry (MS) to characterize them, has proved to be a useful and rapid technique in metabolic studies.

EXPERIMENTAL

Materials and reagents

5-Androstene-3β,17α-diol and 16α-hydroxypregnenolone were kindly supplied by Professor D. N. Kirk from the M.R.C. Steroid Reference Collection (Westfield College, London, U.K.) and 5,16-androstadien-3β-ol by Dr. G. F. Woods, Organon Laboratories (Lanarkshire, U.K.). All other steroids and nicotinamide adenine dinucleotide phosphate (reduced form) were obtained from Sigma (Poole, U.K.). Other chemicals required for incubation studies were purchased from either Hopkin & Williams (Chadwell Heath, U.K.) or BDH (Poole, U.K.).

Analar solvents were obtained from BDH; pyridine was redistilled and stored over sodium hydroxide pellets; cyclohexane was purified by passing through an activated charcoal column and then by double-redistillation.

tert.-Butyldimethylchlorosilane–imidazole reagent and chloromethyldimethylsilane were purchased from Applied Science Labs. (State College, PA, U.S.A.); N-trimethylsilylimidazole from Phase Separations (Queensferry, U.K.); methoxyamine hydrochloride from Eastman Kodak (Rochester, NY, U.S.A.); *n*-alkanes from Analabs (North Haven, CT, U.S.A.) and Supelco (Bellefonte, PA, U.S.A.); Lipidex 5000 from Packard (Downers Grove, IL, U.S.A.) and Sephadex LH-20 from Pharmacia (Uppsala, Sweden). Pre-coated TLC plates (20 × 20 cm, 0.25 mm thickness) were obtained from Merck (silica gel 60; Anderman, Surrey, U.K.). Silica gel 80A (K5) pre-coated plates were a gift from Whatman (Maidstone, U.K.).

Gas chromatography

This was performed using a Becker (Delft, The Netherlands) 409 gas chro-

matograph equipped with a flame ionization detector. The steroid derivatives were analysed on an OV-1 coated glass capillary column (20 m × 0.32 mm I.D.; Jaeggi, Trogen, Switzerland), temperature programming from 170 to 270°C at 2.5°C/min, with helium carrier gas (1 ml/min); injector and detector were kept at 250°C.

Gas chromatography–mass spectrometry

Two GC–MS systems were used:

(a) A quadrupole Finnigan (Sunnyvale, CA, U.S.A.) MAT 4500 instrument with automatic gas chromatograph equipped with Incos Series 2000 data system. A silica column (SE 54, 30 m × 0.25 mm I.D.; Jones Chromatography, Llanbradach, U.K.) was threaded directly into the ion source. Operating conditions were as follows: temperature programme, 70–220°C at 20°C/min and then 220–325°C at 4°C/min; head pressure, 20 p.s.i.; splitless period for Grob-type injection, 36 sec; sample injection volume, 1 μl; injector temperature, 250°C; transfer line temperature, 260°C; ionizer temperature, 200°C; the electron energy, 70 eV; scan speed, 1.0 sec/scan from 50–700 atomic mass units (a.m.u.); emission current, 0.32 mA; resolution (10% valley), 1000.

(b) A Varian (Palo Alto, CA, U.S.A.) MAT 112 double focussing mass spectrometer interfaced with a Varian 1400 gas chromatograph and equipped with Finnigan MAT SS 200 data system. A silica column (CP Sil 5 CB, 25 m × 0.32 mm I.D.; Chrompack, London, U.K.) was led directly into the ion source. Operating conditions: temperature programme, 180–250°C at 2°C/min; falling needle type injector, sample injection volume, 1–2 μl; helium flow-rate, 1.8 ml/min; injector and transfer line temperatures, 230 and 250°C, respectively; ionising voltage, 70 eV; accelerating voltage, 800 V; resolution (10% valley), 1000; scan speed 1.4 sec/decade from 50–800 a.m.u.; trap current, 1.5 mA.

Preparation of O-methyloxime–trimethylsilyl (MO–TMS) ethers

Internal standards (5 μg of each) consisting of 5α-androstane-3α,17α-diol, stigmasterol and cholesteryl butyrate were added to the samples before derivatization. O-methyloximes were prepared as previously described[8] and then silylated by adding N-trimethylsilyl–imidazole reagent (100 μl) and heating in stoppered tubes for 3 h at 100°C. The MO–TMS ether derivatives were purified by filtration through a column of Lipidex 5000[11]. Preliminary identification of unknowns were made by co-injection of the MO–TMS derivatives of the reference steroids with a series of *n*-alkanes and the retention indices expressed as methylene units[12].

tert.-Butyldimethylsilyl (tert.-BDMS) derivative

The *tert.*-BDMS derivative of steroids (5 μg) was prepared using the method of Corey and Venkateswarlu[13].

Chloromethyldimethylsilyl (CDMS) derivative

The CDMS derivative of steroids (5 μg) was prepared according to the method of Thomas and Walton[14], except that cyclohexane was used as an extracting solvent instead of hexane.

Argentation TLC

A modification of an earlier method[3] was adopted. Instead of mixing the silica

gel G with aqueous silver nitrate solution before coating the plates, pre-coated TLC, (silica gel 60) plates were used. These were dipped in a 10% $AgNO_3$ solution in water–methanol (1:1, v/v) for 10 sec and dried at 110°C for 15 min. They were protected from light until ready for use. This method gave better separation of spots without streaking. The steroids were run twice in a solvent system consisting of benzene–ethyl acetate (1:2, v/v) and developed with Allen reagent (conc. sulphuric acid–ethanol–water, 80:18:2, v/v/v)[15] and heating at 110°C for 10 min.

Preparation of microsomes for incubation studies

Testes from 3-week old Landrace piglets were obtained by castration and microsomes prepared by differential contrifugation[8]. Microsomal suspensions (3 ml), of protein concentration 5 mg/ml, were incubated with pregnenolone (final concn. 0.17 mmol/l) in the presence of NADPH (final concn. 3.6 mmol/l) at 37°C for 30 min. In the control experiment, the microsomal fractions were boiled for 5 min before incubation. The metabolites formed were extracted with ethyl acetate and purified by passing through a Sephadex LH-20 column prior to derivatization as before[8].

RESULTS

Steroid profiling of reference C_{21} and C_{19} steroids

GC of the MO, TMS or MO–TMS derivatives of reference steroids is shown in Fig. 1. With the exception of 5,16-androstadien-3β-ol and 5α-androst-16-en-3β-ol, all were separated under the conditions used. The identities of all steroids were later confirmed by obtaining mass spectra at the appropriate relative elution times from the capillary column. These relative retention times and the molecular and fragmentation ions of the derivatized steroids are summarized in Table I. It can be seen that C_{21} 5-ene-3β-hydroxysteroids are eluted before the corresponding 4-en-3-oxosteroids, *viz.*: pregnenolone > progesterone; 17-hydroxypregnenolone > 17-hydroxyprogesterone and 16α-hydroxypregnenolone > 16α-hydroxyprogesterone. Epimers at C-3 or C-17 of the androstane series, *e.g.* 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol and the corresponding 5-ene derivatives are also resolved.

Identification of pregnenolone metabolites from microsomal incubations

Fig. 2 shows the steroid profile obtained from the microsomal incubation with pregnenolone. Mass spectral confirmation was obtained for 17-hydroxy- and 16α-hydroxypregnenolone, 5,16-androstadien-3β-ol, 5α-androst-16-en-3β-ol, DHA, 5-androstene-3β,17α-diol, 5-androstene-3β,17β-diol and 5-pregnene-3β,20β-diol.

Although 5α-androst-16-en-3β-ol is not well-separated from 5,16-androstadien-3β-ol (see metabolites 10 and 11 in Fig. 2) their presence as intermediates in the microsomal incubation with pregnenolone was established by plotting two sets of characteristic mass spectral ions, I and II (Fig. 3). Partial separation of the two compounds is evident. Replotting each of these sets of ions, with further characteristic ions included, gave in both cases a coincidence of ion peaks indicative of the presence of 5,16-androstadien-3β-ol and 5α-androst-16-en-3β-ol (see insets A and B of Fig. 3).

Further attempts to separate 5,16-androstadien-3β-ol from 5α-androst-16-en-3β-ol

The GC behaviour of *tert.*-BDMS and CDMS ethers of 5,16-androstadien-

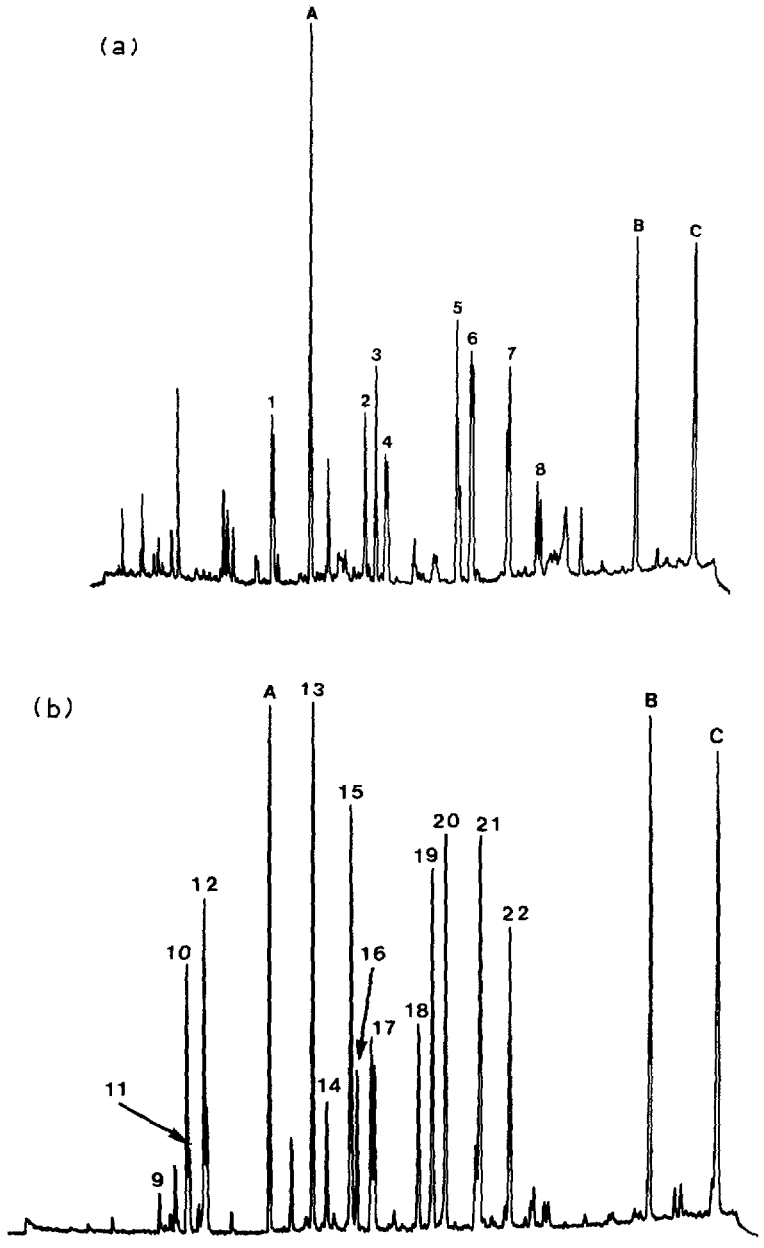

Fig. 1(a). Gas chromatographic profile of C_{21} and C_{19} steroid derivatives (as MO, TMS or MO–TMS ethers). Internal standards A, B and C are 5α-androstane-3α,17α-diol, stigmasterol and cholesteryl butyrate, respectively. The following steroids are indicated: 1, 5α-androst-16-en-3-one; 2, DHA; 3, 5-androstene-3β,17β-diol; 4, 4-androstene-3,17-dione; 5, 16-dehydroprogesterone; 6, progesterone; 7, 17-hydroxyprogesterone; 8, 16α-hydroxyprogesterone. (b) As in (a). Steroids are indicated as follows: 9, 5α-androst-16-en-3α-ol; 10, 5,16-androstadien-3β-ol; 11, 5α-androst-16-en-3β-ol; 12, 4,16-androstadien-3-one; 13, 5α-androstane-3α,17β-diol; 14, 5-androstene-3β,17α-diol; 15, 5α-androstane-3β,17β-diol; 16, 5α-DHT; 17, testosterone; 18, 16-dehydropregnenolone; 19, pregnenolone; 20, 5-pregnene-3β,20β-diol; 21, 17-hydroxypregnenolone; 22, 16α-hydroxypregnenolone.

TABLE I

RETENTION TIMES (OV-1) AND CHARACTERISTIC IONS OF REFERENCE STEROIDS OBTAINED BY GC MS

Steroid	*Derivative*	*Retention* time (MU)*	*Characteristic mass spectral ions*** m/z (%)
5α-Androst-16-en-3α-ol	TMS	21.9	346(32), 331(17), 256(39), 241(100), 217(11)
5,16-Androstadien-3β-ol	TMS	22.7	344(26), 254(37), 239(27), 215(44), 129(100), 93(52)
5α-Androst-16-en-3β-ol	TMS	22.7	346(29), 331(8), 256(51), 241(37), 217(100), 129(92)
4,16-Androstadien-3-one	MO	22.9/23.0	299(24), 153(29), 137(12), 129(100), 125(30), 93(26)
5α-Androst-16-en-3-one	MO	23.3/23.4	301(84), 270(38), 153(84), 137(89), 125(100), 79(51)
5α-Androstane-3α,17β-diol	TMS	25.3	436(17), 346(22), 256(47), 241(59), 129(73), 73(100)
5-Androstene-3β,17α-diol	TMS	25.7	434(15), 254(41), 239(45), 215(47), 129(65), 73(100)
DHA*	MO–TMS	25.9	389(5), 358(19), 299(9), 268(30), 260(35), 129(100)
5α-Androstane-3β,17β-diol	TMS	26.0	436(13), 346(25), 256(22), 241(36), 129(100), 73(97)
5-Androstene-3β,17β-diol	TMS	26.1	434(11), 344(27), 254(26), 239(34), 215(29), 73(100)
5α-DHT	MO–TMS	26.2/26.3	391(39), 376(13), 360(12), 270(13), 129(100), 73(70)
4-Androstene-3,17-dione	MO	26.4/26.5	344(97), 313(63), 281(19), 137(73), 125(100)
Testosterone	MO–TMS	26.6/26.7	389(83), 358(16), 268(27), 153(71), 125(67), 73(100)
16-Dehydropregnenolone	MO–TMS	27.5	415(51), 310(29), 294(31), 286(62), 129(93), 69(100)
Pregnenolone*	MO–TMS	27.8	417(10), 402(40), 386(45), 312(47), 296(35), 129(100)
16-Dehydroprogesterone	MO	27.8/27.9	370(100), 339(47), 153(40), 137(38), 125(50)
5-Pregnene-3β,20β-diol$^+$	TMS	28.0	462(31), 372(26), 357(20), 169(100), 129 & 117 (very intense)
Progesterone	MO	28.2/28.3	372(100), 341(64), 273(40), 153(57), 125(57), 100(71)
17-Hydroxypregnenolone	MO–TMS	28.8	505(10), 474(40), 188(33), 158(23), 156(26), 73(100)
17-Hydroxyprogesterone	MO–TMS	29.2	460(19), 429(65), 339(18), 188(17), 158(20), 73(100)
16α-Hydroxypregnenolone	MO–TMS	29.4	505(2), 474(72), 384(14), 188(42), 156(32), 73(100)
16α-Hydroxyprogesterone	MO–TMS	29.9/30.0	460(3), 429(81), 339(13), 188(100), 156(46), 114(29)

* Paired values arose from *syn-* and *anti-* forms of the MO derivative of a single compound, in methylene units (MU).

** The first ion of each list is the molecular ion. This is followed by the most intense fragment ions (max. 6). Most of the mass spectra were obtained with the Finnigan mass spectrometer; the rest with the Varian instrument. In the former case, the mass spectra were normalized to the most intense peak whereas in the latter, mass spectra were normalized above *m/z* 100* or $150^{+\cdot}$.

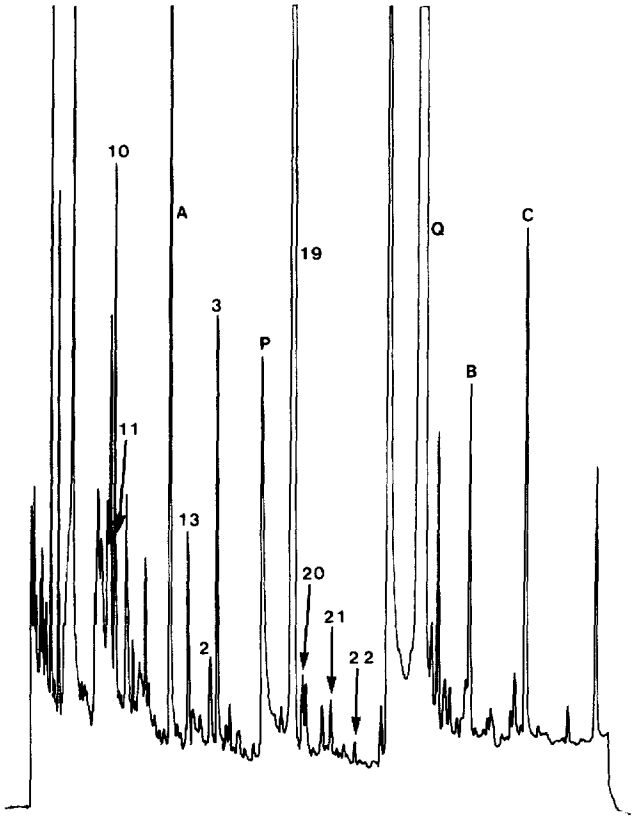

Fig. 2. Steroid profile of pregnenolone metabolites from microsomal incubation. A, B, C are as defined in Fig. 1a; P and Q indicate sucrose (from Tris–sucrose incubation buffer) and cholesterol, respectively. The steroids identified are numbered according to the scheme given in Fig. 1a and b.

3β-ol and 5α-androst-16-en-3β-ol was compared on the OV-1 capillary column, but good resolution of neither derivative was obtained (Table II). Using argentation TLC (pre-coated silica gel 60 plates), however, a good separation of these two compounds

TABLE II

COMPARISON OF CAPILLARY GC AND ARGENTATION TLC IN THE SEPARATION OF 5,16-ANDROSTADIEN-3β-OL FROM 5α-ANDROST-16-EN-3β-OL

	*GC**		*TLC***	
	tert.-BDMS	*CDMS*	*Silica gel 60*	*Silica gel 80A*
5,16-Androstadien-3β-ol	0.60	0.39	83	55
5α-Androst-16-en-3β-ol	0.61	0.40	107	111

* Retention times relative to those of the *tert.*-BDMS (21.3 min) and the CDMS derivatives (35.3 min) of 5α-androstane-3α,17α-diol (= 1).

** Distances in mm were measured from the origin to the mid-point of each spot; solvent system used was benzene–ethyl acetate (1:2, v/v), run twice.

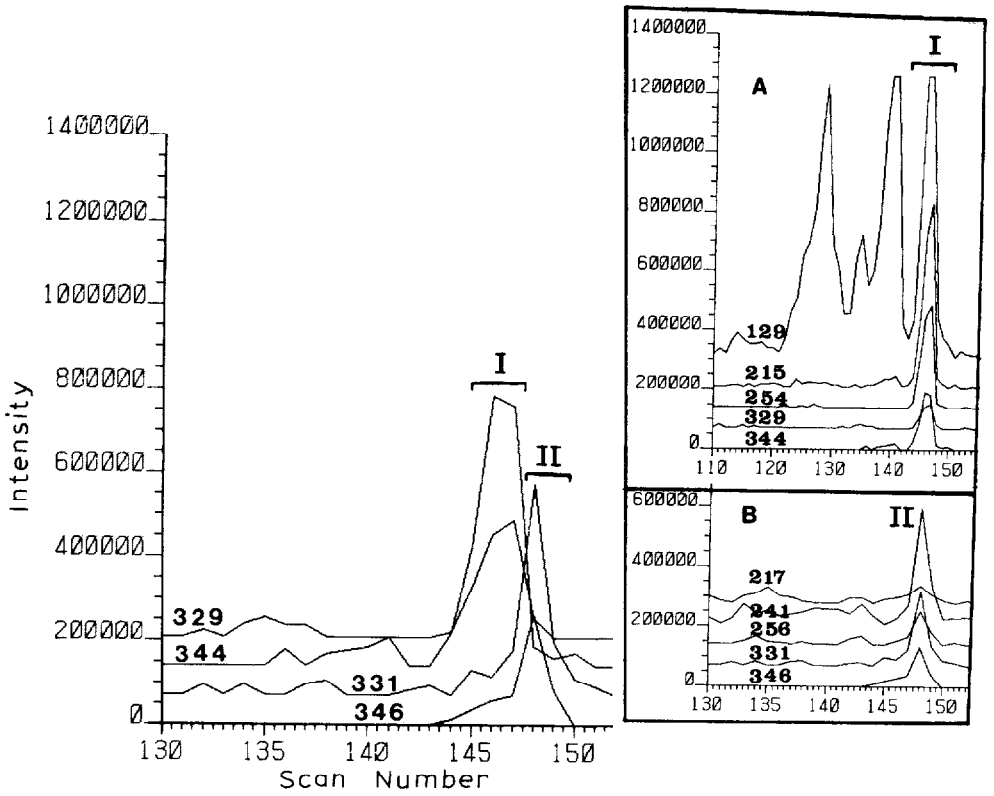

Fig. 3. Extracted ion current profile (EICP) of 5,16-androstadien-3β-ol and 5α-androst-16-en-3β-ol from microsomal incubation of pregnenolone. A group of ions (I) characteristic of 5,16-androstadien-3β-ol is partially separated from another group (II) of ions belonging to 5α-androst-16-en-3β-ol. Insets A and B show the separate EICP plot for 5,16-androstadien-3β-ol and 5α-androst-16-en-3β-ol, respectively. The presence of these two compounds is indicated by the coincidence of their individual characteristic ion peaks.

was achieved; an even better separation was obtained with argentation TLC using pre-coated silica gel 80A plates (see Table II).

DISCUSSION

By using capillary GC and GC–MS, our studies of pregnenolone metabolism in neonatal porcine testis have revealed the formation of numerous metabolites, including the very non-polar 16-androstenes that have become well-documented in this species[16,17].

Three pathways for biosynthesis of 16-androstenes are currently suggested; one involving 16-dehydropregnenolone as intermediate[7,8], one involving 5-pregnene-3β,20β-diol[9] and one involving 16α-hydroxypregnenolone[18]. The results of short-term kinetic studies, which may help to elucidate their relative importance will be presented elsewhere. A pre-requisite for such studies is the identification and quantification of a large number of intermediates. Some of these have a narrow polarity range and present particular difficulties with respect to separation of compounds of

analogous structure. For example, although the separation of pregnenolone and 16-dehydropregnenolone has been reported[7] using TLC with multiple runs in chloroform–ethanol (98:2, v/v), we have been unable to achieve resolution using this system or several other TLC and column chromatographic procedures.

Capillary GC has permitted use of a single analytical method to separate all the compounds of interest with the exception of 5,16-androstadien-3β-ol and 5α-androst-16-en-3β-ol, which could be only partially resolved by capillary GC on OV-1. However, using the extracted ion current profiles, the presence of both was indicated by coincidence of their individual characteristic ion peaks (Fig. 3). Earlier studies exploited the formation of π-complexes on $AgNO_3$-impregnated thin layers in the resolution of 5,16-androstadien-3β-ol and 5α-androst-16-en-3β-ol[1,3]. In our hands, this method resulted in serious streaking of spots but we have found that pre-coated TLC plates, which have been dipped in a solution of $AgNO_3$, give excellent resolutions without streaking. This separation is important because in all of the postulated pathways, 5,16-androstadien-3β-ol appears to be the first 16-androstene formed from pregnenolone while 5α-androst-16-en-3β-ol may be formed later in the pathway.

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