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QUANTITATIVE ANALYSIS OF CORTICOSTEROIDS IN ADRENAL CELL CULTURES BY CAPILLARY COLUMN GAS CHROMATOGRAPHY COMBINED WITH MASS SPECTROMETRY

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SUMMARY

A quantitative method using glass capillary column gas–liquid chromatography (GLC) has been established for the analysis of corticosteroids in small biological samples such as a few milliliters of medium from steroidogenic cultured cells. The steroid extract is separated in three main thin-layer chromatographic fractions. The steroids assayed in these fractions are: less polar fraction ($C_{21}O_2$ steroids), 20α-dihydroprogesterone, pregnenolone and 20α-dihydro-pregnenolone; intermediate fraction ($C_{21}O_3$ steroids), deoxycorticosterone, 11-oxo- and several hydroxy-20α-dihydroprogesterones; corticosteroid fraction ($C_{21}O_4$ steroids), corticosterone and 18-hydroxydeoxycorticosterone.

The method has been assessed by characterizing the steroid structures and checking the purity of GLC effluents through direct coupling of the capillary column to the electron-impact or chemical-ionization source of a computerized mass spectrometer. Applications to the quantitative evaluation of the endocrinological status of newborn rat adrenocortical cell cultures under various conditions of development, hormonal stimulation or specific inhibition by drugs are described.

INTRODUCTION

Normal adrenocortical cells isolated from newborn rats and grown in tissue culture have been shown to secrete the same corticosteroid hormones as *in vivo* under the stimulation by the adrenocorticotropic hormone (ACTH)[1,2]. These cells in primary culture proliferate for 4–5 days after seeding and can be maintained secreting for 2 weeks or longer. They represent an appropriate model system for studies on the regulation of enzymes linked to the steroid biosynthetic pathway. Measurements of the quantitative changes in differentiated steroidogenesis or in vicarious pathway of steroid metabolism due to various developmental stages, hormonal stimulation or specific inhibition by drugs demand an accurate, sensitive and specific analytical method.

Usually the methods employed by endocrinologists for the assay of steroids in

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adrenal tissue culture are essentially non-separative methods such as fluorimetry[3–9], reverse isotope dilution[10], radioimmunology[11] or competitive protein binding[12]. Thin-layer chromatography (TLC) has also been used for the separation of some steroids and the estimation of their production from radioactive precursors.

In 1974, a gas–liquid chromatographic (GLC) technique using a packed column was used for the separation of rat adrenal corticosteroids, including 18-hydroxysteroids[13]. This method was applied to the analysis of the two main steroids excreted from adrenal cultured cells by using a mass spectrometer as a detector of few specific ions[1,2] or a common flame-ionization detector[14]. High-performance liquid chromatography was also used for the analysis of a few steroids from adult adrenal cultured cells[15].

Taking advantage of the high efficiency of capillary columns and the high sensitivity of detection of their effluents due to the sharpness of the elution peaks, we have developed a quantitative method that permits the separation of numerous steroids at the nanogram level. Some of them have been newly identified in this biological system. Quantitation of the main corticosteroids and pregnane steroids is carried out on down to one hundredth of the culture medium extract of a 20-cm² dish.

MATERIALS AND METHODS

Reference compounds and reagents

Most of the authentic steroid samples were purchased from Ikapharm (Ramat-Gan, Israel) and Steraloids (Wilton, N.H., U.S.A.), 18-hydroxydeoxycorticosterone and 18-hydroxyprogesterone from Searle (Naucalpan, Mexico) and aldosterone from Sigma (St. Louis, Mo., U.S.A.). The steroid nomenclature, trivial names and abbreviations are reported in the Table I and in the legend of Fig. 2.

[4-^{14}C]Progesterone (specific activity 51.6 mCi/mmole) and [4-^{14}C]pregnenolone (specific activity 59 mCi/mmole) were obtained from the Commissariat à l'Energie Atomique (Saclay, France).

The following reagents and solvents were used: O-methyloxyamine hydrochloride (MO,Cl) from Pierce (Rockford, Ill., U.S.A.), pyridine (Merck, Darmstadt, G.F.R.) on potassium hydroxide, bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Supelco (Bellefonte, Pa., U.S.A.).

Derivative formation

The MO-TMS derivatives of corticosteroids were prepared by a modification of a previously described method[13].

At the tenth of a milligram level for GLC on a packed column. A 100-µl sample of a solution of MO,Cl in dry pyridine (16 mg/ml) was added to 100 µg of steroid obtained by evaporation under nitrogen of 100 µl of an ethanolic solution of the steroid (1 mg/ml). The reaction was carried out in a 2-ml vial with a PTFE cap at 65° for 3 h. Pyridine was evaporated under a stream of nitrogen at 65° in a sand-bath. Then 100 µl of a mixture of BSTFA and TMCS (4:1) was added and the mixture was heated at 65° overnight.

At the microgram level for GLC on a capillary column. Samples were obtained by dilution of the preceding derivatives with BSTFA to obtain 10–80 ng/µl solutions. Cholesteryl butyrate was added as an internal standard (STD) at a final concentration

of 20 ng/μl (measurement of response coefficient). A direct procedure was also developed for corticosteroid mixtures or for adrenal cell culture extracts. The dry extract, to which was added 1 μg of cholesteryl butyrate (STD), was taken up in 50 μl of MO,Cl in pyridine solution, allowed to react for 3 h at 65°, evaporated to dryness under nitrogen and the residue taken up in 100 μl of BSTFA–TMCS (4:1) and allowed to react at 65° overnight. The final concentration of STD was 10 ng/μl.

Thin-layer chromatography

The method used was as described previously[16]. The reference steroids and the steroid extracts were taken up in 100 μl of ethyl acetate and fractionated by TLC on 0.25-mm silica gel 60 F-254 plates (Merck). The following solvent systems were used: first run (15 cm), diisopropyl ether; second run (12 cm), diisopropyl ether–acetone (60:40). Spots of corticosteroids were detected under UV light and by radioscanning of the TLC plate. Each localized zone was scraped off and steroids were extracted from the silica gel by sonication in several solvents consecutively (methanol, ethyl acetate and dichloromethane).

Gas–liquid chromatography and mass spectrometry (GLC–MS)

GLC on a packed column. A silanized glass column (4 m × 3 mm I.D.) packed with 1% OV-1 (Supelco) on Gas-Chrom Q with a nitrogen flow-rate of 30 ml/min, temperature programmed from 200° to 290° at 1°/min, was used. A Packard-Becker (Delft, The Netherlands) Model 420 gas chromatograph equipped with a flame-ionization detector was employed.

GLC on a capillary column. Glass columns from LKB (Bromma, Sweden) of dimensions 25 m × 0.21 mm I.D. (110,000 theoretical plates) or 25 m × 0.34 mm I.D. (70,000 theoretical plates) coated with SE-30 were employed. The nitrogen flow-rate was 1 ml/min. A Packard-Becker Model 427 chromatograph equipped with an all-glass solid injector[17] and a flame-ionization detector was used.

Methylene unit (MU) values were calculated by simultaneously running the steroids under study and C_{28}–C_{34} *n*-alkanes (Fluka, Buchs, Switzerland).

Response coefficients were calculated by comparison of the height (or the area) of the peak with that of 20 ng of cholesteryl butyrate. Several amounts of steroids were employed in the 10–160-ng range, with two or three measurements for each value.

GLC–MS instrument. A quadrupole mass spectrometer coupled with a glass capillary column and monitored by a computer was used (Finnigan 3300-6100). The capillary column was directly connected to the mass spectrometer source by a silanized glass line. The instrument was equipped with either an electron-impact (EI) source (electron energy 50 or 70 eV; filament current 0.5 mA) or a chemical-ionization (CI) source (reagent gas methane; ion source pressure 0.8 torr; electron energy 130 eV; filament current 0.25 mA).

Cell culture

The method was adapted from that described by Maume and Prost[1]. Adrenals were taken from newborn Wistar US/Commentry rats (INRA, Dijon, France) and were trypsinized with a solution of B-grade trypsin (3000 I.U./mg) (Calbiochem, San Diego, Calif., U.S.A.) in Hank's solution without calcium or magnesium at a concentration of 1.50 mg/ml in a cell-stir vial. The trypsin action was stopped by addition

of foetal calf serum and the trypsinized fractions were centrifuged. The cell pellets were isolated and suspended in cell-culture medium of the following composition: Ham F10 medium (Microbiological Assoc., Walkersville, Mass., U.S.A.), penicillin and streptomycin, 10% of foetal calf serum and 10% of human AB serum. Each fraction was poured into a Cooper tissue culture dish (Falcon, Oxnard, Calif., U.S.A.). The dishes were kept in a culture oven at 37° and under an air–carbon dioxide (95:5) atmosphere saturated with water.

Incubation

The radioactive steroid precursor [^{14}C]pregnenolone or [^{14}C]progesterone (68 nCi) was added to the dishes in 5 or 10 μl of absolute ethanol. Incubation times were usually 24 h. The media were gathered and kept at −20° until steroid extraction was performed.

Cell culture medium extraction, purification and derivatization

Before steroid extraction, the culture media were deproteinized and delipidated by precipitation with methanol (70%) at −20° and by centrifugation at −20°. The methanol was evaporated at 65° under a stream of nitrogen. The steroids were extracted from the remaining aqueous solution with ethyl acetate (three times) and dichloromethane (three times). The organic phases were pooled, dried over anhydrous magnesium sulphate and evaporated to 100 μl at 65° under a stream of nitrogen. TLC fractionation was carried out as described above. Each TLC fraction extracted by solvents was evaporated to dryness under nitrogen. The extraction yields were measured by radioactive counting before extraction and on each TLC fraction extract. The mean whole yield was 70%. The addition of an internal standard and the formation of MO-TMS derivatives for gas-phase analysis were carried out according to the procedure described above for derivatives at the microgram level.

RESULTS AND DISCUSSION

Characterization of corticosteroids by capillary gas–liquid chromatography

The steroids under study were characterized by their R_F values[2,16]. The MU values measured on both packed and capillary columns according to the method described by VandenHeuvel *et al.*[18] are given in Table I; the precision is 0.02 unit. On the capillary columns *syn* and *anti* isomers of some 3-oxo-4-ene corticosteroids are separated as MO-TMS derivatives, as can be seen in Fig. 1 for corticosterone. However, the separation of the 3-oxo-4-ene-MO *syn* and *anti* isomers is strongly dependent on the other functional groups present on the steroid skeleton. For instance, 18-hydroxydeoxycorticosterone (18-OH-DOC) as the MO-TMS derivative gives only one peak (see Fig. 1). On the basis of twenty cases studied here, 3-oxo-4-ene steroids can be divided into four groups with respect to the separation of the *syn* and *anti* isomers of their MO derivatives: (i) type 1, giving two well separated peaks, the first peak eluted being the higher; this group includes 11-deoxycorticosterone (DOC) and 11β-hydroxysteroids such as corticosterone, 11β-hydroxyprogesterone, and aldosterone; (ii) type 2, giving two well separated peaks, the first peak eluted being the lower; this group corresponds to the 17α-hydroxysteroids such as 11-deoxycortisol, 17α-hydroxyprogesterone and 17α-hydroxy-20α-dihydroprogesterone (persilylated deriv-

ative); (iii) type 3, giving a separation between two *syn* and *anti* isomer peaks of equal intensity; this group includes progesterone and its derivatives, 20α-dihydroprogesterone (20α-DHP) showing a beginning of separation (δMU = 0.04) and 6β-hydroxysteroids (6β-hydroprogesterone and 6β-hydroxy-DOC) with a large separation (δMU = 0.14); (iv) type 4, leading to a single peak and including steroids that bear 18-hydroxy or 11-oxo functions, such as 18-OH-DOC or 11-dehydrocorticosterone.

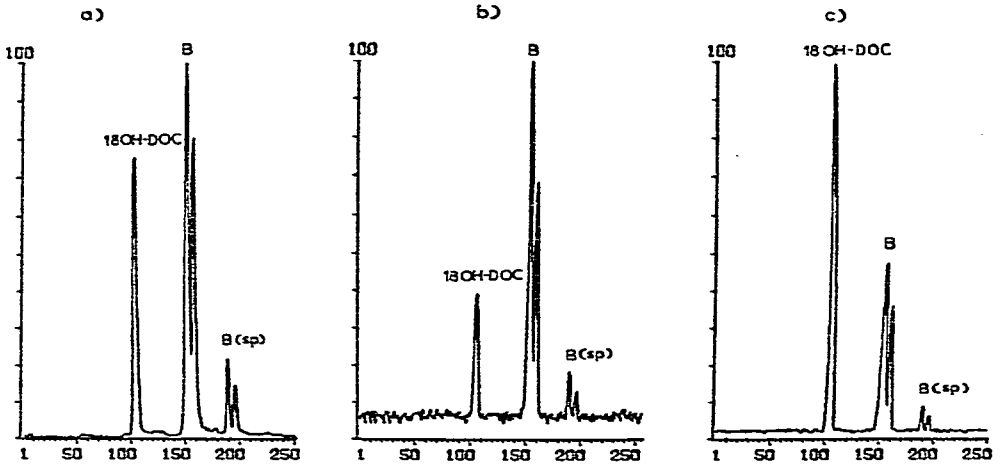


Fig. 1. Separation of 18-hydroxydeoxycorticosterone (18-OH-DOC) and corticosterone (B) isomers as O-methyloxime-trimethylsilyl (MO-TMS) derivatives by GLC with a glass capillary column (CAP-GLC) combined with a quadrupole mass spectrometer. (a) Detection of the total ionization current; (b) detection of the *m/e* 548 molecular ion intensity; (c) detection of the *m/e* 517 (M − OCH_3) ion intensity. The two major peaks of B correspond to *syn/anti* isomers of the 3-MO group; the two secondary peaks (sp) of B are *syn/anti* isomers of a corticosterone rearrangement form of unknown configuration. The *syn/anti* isomers of the 18-OH-DOC-3-MO group are not separated. B and 18-OH-DOC are also characterized by distinct response at the *m/e* 517 and 548 ions. GLC conditions: all-glass solid injection system; SE-30 capillary column (25 m × 0.21 mm I.D.); 110,000 theoretical plates; temperature programming from 200° at 1°/min. MS conditions: Finnigan 3300-6100 GLC–MS–computer instrument with electron-ionization source; electron energy, 50 eV; filament current, 0.5 mA.

Response coefficients of standard steroids

The chromatogram in Fig. 2 shows the response of the usual reference steroids compared with that of 10 ng of cholesteryl butyrate. Table II gives the response coefficients of thirteen steroids of interest for the quantitative study of the adrenal biosynthetic pathway. These coefficients were calculated from the areas (R_A) or heights (R_H) of the peaks; the R_A values range from 0.32 to 1.50 ($R_A = 1$ for cholesteryl butyrate) and the R_H values from 0.36 to 1.68 (sum of the isomeric derivatives) or 0.25 to 1.68 (major derivative) if the specially low response values of 17α-hydroxyprogesterone are excluded. The linearity of the steroid response towards the injected amount was ascertained from 10 to 80 ng, as shown in Fig. 3. The slopes of the response lines led to a mean value with a standard deviation of less than 3%.

These coefficients do not depend on the evaporation time on the glass needle of the solid injector between 1.5 and 17 min (variation 2%) and were similar to those

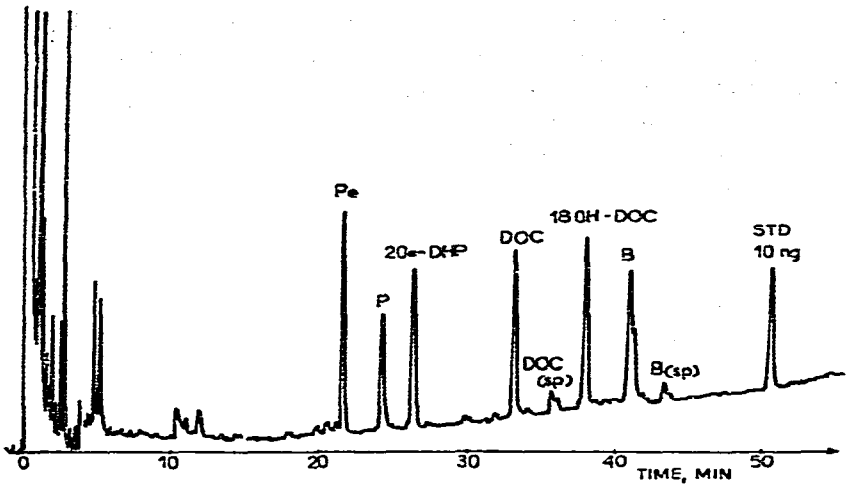


Fig. 2. Separation of a synthetic steroid mixture as MO-TMS derivatives by GLC with a glass capillary column. Responses of each steroid are compared with that of a 10 ng injection of cholesteryl butyrate as internal standard (STD). Pe = pregnenolone, 10 ng; P = progesterone, 10 ng; 20α-DHP = 20α-dihydroprogesterone, 10 ng; DOC = 11-deoxycorticosterone and its secondary peak (sp), 20 ng; 18-OH-DOC = 18-hydroxydeoxycorticosterone, 40 ng; B = corticosterone and its secondary peak (sp), 40 ng. GLC conditions: all-glass solid injection system; SE-30 capillary column (25 m × 0.34 mm I.D.); 70,000 theoretical plates; temperature programming from 240° at 1°/min; flame-ionization detection.

obtained on packed columns. The best reproducibility was obtained for R_H, and in practice R_H was used for the calculation of amounts of corticosteroids from adrenal cell cultures. From time to time the authentic steroid responses were checked by running the test mixture shown in Fig. 2.

Corticosteroid assay from adrenal cell cultures and assessment by GLC–MS

The TLC method described above led to a satisfactory purification of the steroid extracts and to precise marking of each steroid zone by radioscanning of the labelled metabolites on the plate. A typical TLC analysis of extract coming from ACTH stimulated or unstimulated cultures is shown in Fig. 4. Five zones are shown on the plate: zones a and b, corresponding to progesterone and 20α-DHP, respectively, can contain other $C_{21}O_2$ steroids. Zone c corresponds to $C_{21}O_3$ steroids such as DOC and various hydroxy-20α-dihydroprogesterones and zone d to $C_{21}O_4$ corticosteroids such as corticosterone and 18-OH-DOC. The analysis of individual steroids contained in each zone, after extraction, derivatization and addition of internal standard, was performed by capillary column GLC; it allowed the quantitation of the main steroids biosynthesized by adrenal cell cultures. Typical separations are shown in Figs. 5–7.

TLC fractions a and b ($C_{21}O_2$ steroids)

Fig. 5 shows a gas chromatogram obtained from TLC fractions a and b. The adrenal cell culture was incubated for 24 h with ACTH and with $5 \cdot 10^{-7}$ *M* of a cyanosteroid, the 4,5α-epoxy-17β-hydroxy-3-oxo-2α-androstane carbonitrile (WIN

TABLE I

METHYLENE UNIT VALUES OF MO-TMS DERIVATIVES OF STEROIDS BY CAPILLARY AND PACKED COLUMN GLC

GLC conditions: temperature programming from 240° at 1°/min on SE-30 capillary column (25 m × 0.35 mm I.D.) and from 180° at 1°/min on 1% OV-1 packed column (4 m × 3 mm I.D.). All oxo groups are derivatized as MO except for 11-oxo group; all the hydroxyl functions are silylated except for the 17α-hydroxy group in the 17α-20α-dihydroxy compound.

Steroid	*Packed column (1% OV-1)*	*Capillary column (SE-30)*
Pregnenolone (3β-hydroxy-4-pregnen-20-one)	27.78	28.20
21-Hydroxypregnenolone (3β,21-dihydroxy-4-pregnen-20-one)	30.00	30.00*
5α-Pregnanolone (3β-hydroxy-5α-pregnan-20-one)	27.88	28.00*
5β-Pregnanolone (3β-hydroxy-5β-pregnan-20-one)	27.22	27.24*
Progesterone (4-pregnene-3,20-dione)	28.35	28.79§§
11-Oxoprogesterone (4-pregnene-3,11,20-trione)	29.42*	29.91
11β-Hydroxyprogesterone (11β-hydroxy-4-pregnene-3,20-dione)	30.44	30.80**
		30.85**
17α-Hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione)	29.26*	29.47**
		29.56**
6β-Hydroxyprogesterone (6β-hydroxy-4-pregnene-3,20-dione)	28.95*,**	29.18**
	29.07*,**	29.32**
2α-Hydroxyprogesterone (2α-hydroxy-4-pregnene-3,20-dione)	29.30*	29.60
20α-Dihydroprogesterone (20α-hydroxy-4-pregnen-3-one)	28.87	29.28§§
20β-Dihydroprogesterone (20β-hydroxy-4-pregnen-3-one)	28.62	28.71*
17α-Hydroxy-20α-dihydroprogesterone (17α,20α-dihydroxy-4-pregnen-3-one)	30.49*	30.81
Deoxycorticosterone = DOC (21-hydroxy-4-pregnene-3,20-dione)	30.50	30.75§§
		31.26**,***
		31.35**,***
18-Hydroxydeoxycorticosterone (18,21-dihydroxy-4-pregnene-3,20-dione)	31.66	31.74
	32.30***	32.30***
Corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione)	32.12	32.32**
	32.70***	32.38**
		32.76**,***
		32.86**,***
6β,21-Dihydroxyprogesterone = 6β-hydroxy-DOC (6β,21-dihydroxy-4-pregnene-3,20-dione)	30.97*,**	31.09**
	31.09*,**	31.24**
		31.41**,***
		31.55**,***
11-Dehydrocorticosterone (21-hydroxy-4-pregnene-3,11,20-trione)	31.66	31.57*
Aldosterone (11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al: 11,18-hemiacetal form)	31.73*,§	31.74**
	32.14*,§	31.82**
		32.18**
		32.25**
11-Deoxycortisol (17β,21-dihydroxy-4-pregnene-3,20-dione)	30.98	31.08*,**
		31.18*,**
Cholesterol (5-cholestene-3β-ol)	31.07	31.35
Cholesteryl butyrate (3β-butyryloxy-5-cholestene)	34.00*	34.19

* From 200°.

** *Syn/anti* isomers.

*** Minor isomeric peak.

§ 18α/β isomers[21].

§§ A broad peak top; beginning of separation on 110,000 theoretical plate column or at lower starting temperature[19].

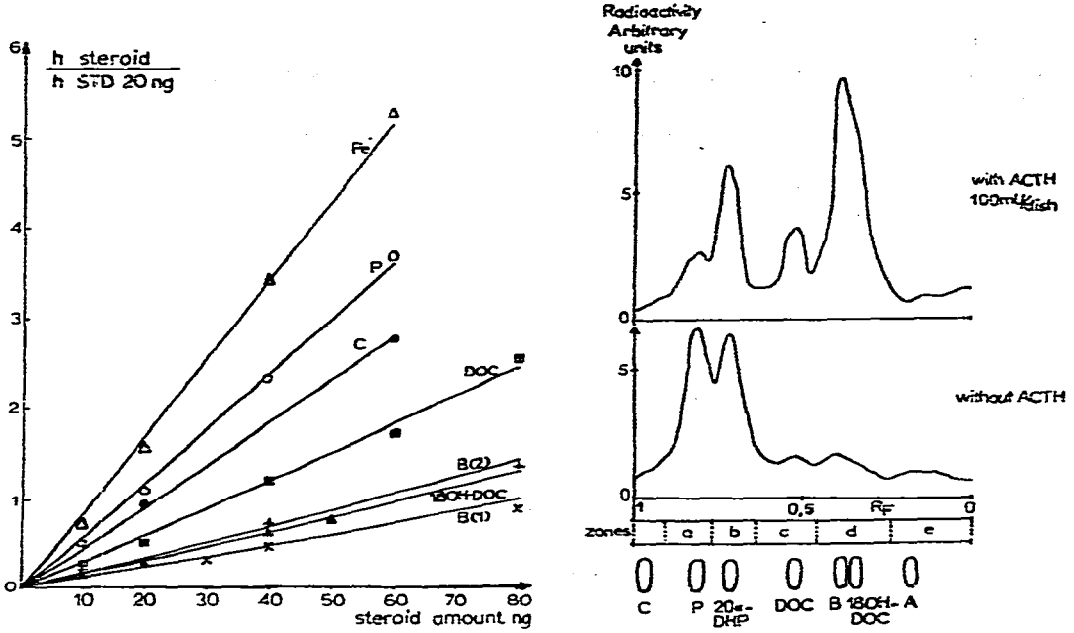


Fig. 3. Response curves of six steroids detected by flame ionization after GLC on a glass capillary column. Ratios of the height (h) of each steroid peak and of the height of 20-ng internal standard peak are plotted *versus* the steroid amount (nanograms). Linear graphs are obtained for amounts varying from 10 to 80 ng. Abbreviations: pregnenolone (Pe), △; progesterone (P), ○; cholesterol (C), ●; deoxycorticosterone (DOC), ■; 18-hydroxydeoxycorticosterone (18-OH-DOC), ▲; corticosterone (B). B (1) curve (+) is obtained by measurement of the height of the highest peak of B; B (2) (×) corresponds to the sum of the heights of the two major peaks. Each point corresponds to the mean of two or three measurements. Chromatographic conditions as in Fig. 2.

Fig. 4. Separation by TLC of labelled steroids biosynthesized from [4-^{14}C]progesterone by adrenal cells in monolayer culture. Above, cultured cells stimulated by 100 mU per dish of adrenocorticotropic hormone (ACTH); below, corresponding unstimulated cultured cells. For quantitation of individual steroid amounts, each zone is scraped off the plate, derivatized and assayed by CAP-GLC with internal standard; an aliquot is analysed by GLC–MS for the assessment of the GLC peak purity. Cell culture conditions: age of the donor animals, 6 days; age of cell culture, 8 days; Ham's F10 medium with 10% human serum and 10% of foetal calf serum; incubation time, 24 h. Steroid abbreviations as in Fig. 2; C = cholesterol; A = aldosterone. TLC conditions: first migration (15 cm), diisopropyl ether as eluent; second migration (12 cm), diisopropyl ether–acetone (60:40) as eluent.

24540), an inhibitor of 3β-hydroxysteroid dehydrogenase-5(4)-ene isomerase. In this culture, the production of progesterone and corticosteroids was strongly inhibited; pregnenolone (Pe) accumulated in large amounts (15.9 μg per 20-cm^2 culture dish per day) and a metabolite (peak 1) present only in trace amounts in non-inhibited cultures appeared. Compound 1 was identified as 20-dihydropregnenolone by its MU value and characteristic ions in CI–MS: m/e 461 (MH), 447 (M—15), 371 (MH—90) and 281 (MH—2·90). A small amount of 20α-DHP from the reduction of progesterone was present at the rate of 1.8 μg per culture dish per day. In the same culture non-inhibited by the cyanosteroid, Pe was present only in trace amounts and 20α-DHP at the rate of 11.3 μg per culture dish per day.

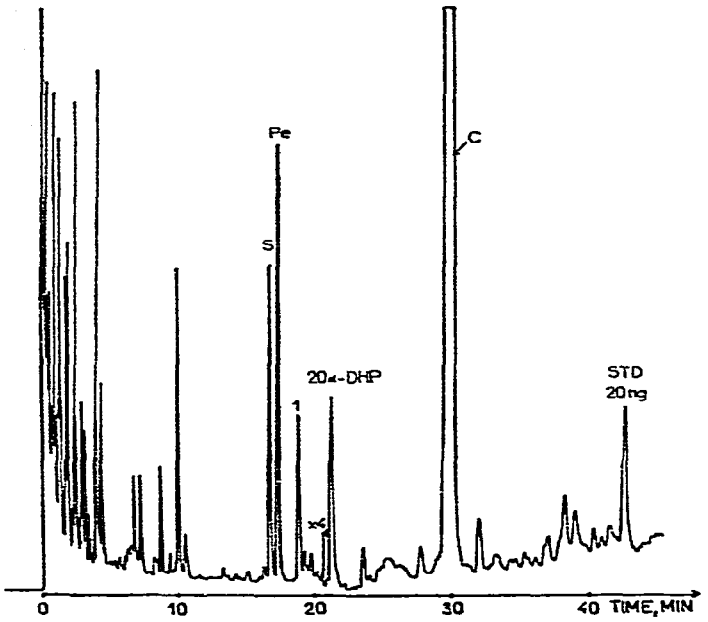


Fig. 5. CAP-GLC separation of steroids synthesized by adrenal cell culture: TLC fractions a and b as indicated in Fig. 4, corresponding to the less polar pregnane steroids ($C_{21}O_2$). Adrenal cell culture stimulated by ACTH and treated with $5 \cdot 10^{-7}$ *M* of 4,5α-epoxy-17β-hydroxy-3-oxo-2α-androstane carbonitrile, an inhibitor of 3β-hydroxysteroid dehydrogenase; age of donor rats, 3 days; age of the culture, 6 days; incubation time, 24 h. Pregnenolone (Pe), present in only small amounts in non-inhibited culture is here the major steroid (15.9 μg per culture dish). The amount of 20α-dihydroprogesterone (20α-DHP) (1.8 μg per culture dish) is much smaller than in non-inhibited cultured cells. Peak 1 is a steroid identified in this adrenal cell culture as 20α-dihydro-pregnenolone. Cholesterol (C) and squalene (S) are derived mainly from serum-supplemented medium. Chromatographic conditions as in Fig. 2, except for the starting temperature (250°). The internal standard (STD) is cholesteryl butyrate (20 ng). The analysis corresponds to a 1/50th aliquot of a 20-cm² culture dish.

TLC fraction c ($C_{21}O_3$ steroids)

Fig. 6 shows the separation of steroids contained in TLC fraction c of an extract of adrenal cell culture stimulated by ACTH and treated with $5 \cdot 10^{-6}$ *M* of 5-bromodeoxyuridine, a synthetic analogue of thymidine. This compound is incorporated in fast-growing cells such as fibroblasts, which are eliminated by photonic irradiation. The steroids in this fraction are essentially the same as in the non-treated culture. The main steroid peak, which has the retention time of DOC, was found to be heterogeneous by MS even if the capillary column GLC separation was carried out on an 110,000 theoretical plate column. EI–MS gave rise to a mass spectrum that showed two obvious series of ions; the first series at *m/e* 460 (M), 429 (M—31), 326, 285 and 103 is characteristic of DOC as the MO-TMS derivative as shown in Table II, giving the main ions in EI–MS of authentic samples of steroid MO-TMS derivatives. The second series of ions at *m/e* 505(M^+), 490 (M—15), 415 (M—90), 400 (M—90—15), 384 (M—90—31), 285 (M—SC—CH—90) and 117 (100%) is characteristic of a pregnendiolone with a side-chain (SC) corresponding to –CH(OTMS)–CH_3 (*m/e* 117). The CI mass spectrum of this peak confirmed the identification of DOC (ions at *m/e* 461 = M+H, 429 = MH—32, 327, 300 = MH—SC) by comparison with CI–MS of

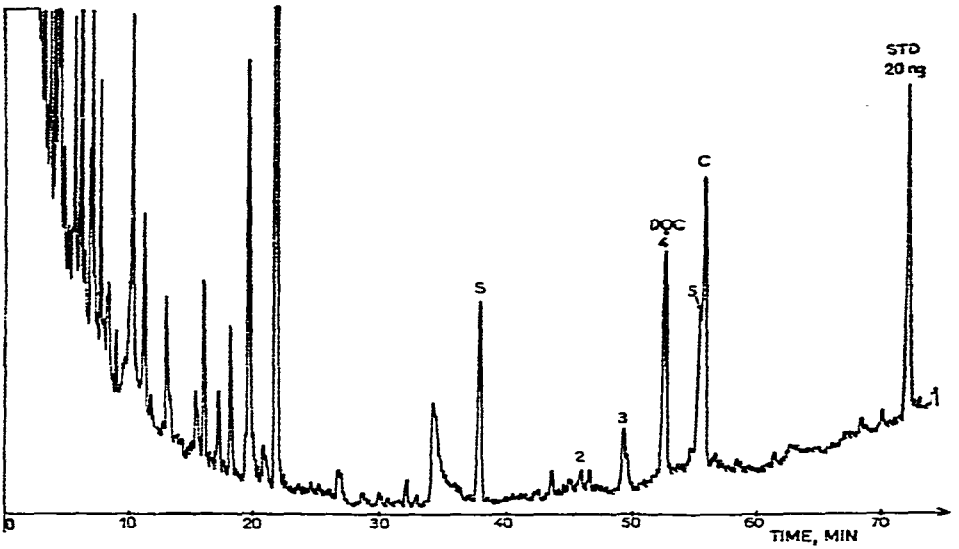


Fig. 6. CAP-GLC separation of steroids synthesized by adrenal cell culture: TLC fraction c as indicated in Fig. 4, corresponding to an intermediate fraction ($C_{21}O_3$ steroids). Adrenal cell culture stimulated by ACTH, 50 mU per 20-cm² culture dish, and treated with $5 \cdot 10^{-6}$ *M* of 5-bromodeoxyuridine, an analogue of thymidine, used to eliminate the fast-growing fibroblastic cells. Age of donnor rats, 6 days; age of the culture, 5 days; incubation time, 48 h. Deoxycorticosterone (DOC), several isomers of X-hydroxy-20α-DHP (peaks 2, 4 and 5) and 11-oxo-20α-DHP (peak 3) are present here and also in untreated cells. Other abbreviations as in Fig. 5. Chromatographic conditions: as in Fig. 2, except the starting temperature (200°).

an authentic sample[20] and that of a hydroxy-20α-DHP (peak 4) (ions at *m/e* 534 = M+29, 506 = MH, 490 = M−15, 474 = MH−32, 416 = MH−90, 326 = MH−2·90 and 117 = SC).

Other peaks were characterized by GLC–MS as hydroxy-20α-DHP (peaks 5 and 2). Peak 3 has the spectrum of an 11-oxo-20α-DHP. Peak 5 is partially mixed with the peak of cholesterol.

Individual assay of DOC and hydroxy-20α-DHP isomers was performed by mass fragmentography (MF) in the CI mode; the ions monitored by MS were *m/e* 506.4, 474.4 (hydroxy-20α-DHP), 461.4, 429.4 (DOC) and 369.3, which corresponds to the MH–butyric acid ion of the cholesteryl butyrate chosen as the internal standard. In the same analysis another group of ions containing *m/e* 517.4 and 549.4 can be monitored, allowing the analysis of 18-OH-DOC and corticosterone.

TLC fraction d, containing $C_{21}O_4$ corticosteroids

The two major peaks were identified as 18-OH-DOC and corticosterone by EI–MS and CI–MS. The EI mass spectra taken by repetitive scanning of the quadrupole mass spectrum showed that these two peaks were homogeneous and displayed the same ions as authentic samples reported in Table III. The quantitation of 18-OH-DOC and corticosterone indicated rates of 9.3 and 17 μg per culture dish and per

TABLE II

RESPONSE COEFFICIENTS OF STEROID MO-TMS DERIVATIVES ON SE-30 CAPILLARY COLUMN

Response coefficients are calculated as ratio of peak areas (R_A) or peak heights (R_H) of steroids and that of the same amount in nanograms of cholesteryl butyrate as internal standard (STD). R_A and R_H are the means of values obtained from steroid amounts varying from 10 to 80 ng (two or three measurements for each value); amount of STD = 20 ng. Chromatographic conditions: 25 m × 0.35 mm I.D. capillary column; temperature programming from 240° at 1°/min.

Steroid MO-TMS	R_A	R_H
Pregnenolone	1.28	1.68
Progesterone	1.05	1.20
17α-Hydroxyprogesterone*	0.13	0.26 (with 2 peaks) 0.095 (with the highest peak)
6β-Hydroxyprogesterone	1.60	1.90 (with 2 peaks) 1.00 (with the highest peak)
2α-Hydroxyprogesterone	1.08	1.40
20α-Dihydroprogesterone	1.50	1.60
17α-Hydroxy-20α-dihydroprogesterone	0.75	0.70
Deoxycorticosterone	0.60	0.60
18-Hydroxydeoxycorticosterone (18-OH-DOC)	0.40	0.32
Corticosterone	0.32	0.36 (with 2 peaks) 0.25 (with the highest peak)
6β,21-Dihydroxyprogesterone (6β-hydroxy-DOC)	0.36	0.40 (with 2 peaks) 0.22 (with the highest peak)
Aldosterone	0.40	0.35 (with 4 peaks) 0.24 (with 2 highest peaks)
Cholesterol	0.84	0.92

day, respectively. Amounts of corticosterone and 18-OH-DOC ranged from 3 to 17 μg and from 1.7 to 9.5 μg, respectively, per culture dish containing $0.2 \cdot 10^6$–$1.0 \cdot 10^6$ cells stimulated by 23 mU/ml of ACTH and grown at different periods of the year. The ratio between the amounts of the two steroid hormones was found to be constant from one culture to another and equal to 1.7–1.8. This corresponds to values observed in the rat *in vivo*. The separation is shown in Fig. 7.

The proposed method is especially well adapted to the analysis of small amounts of corticosteroids in adrenal cell cultures. The limit of the detection is a few nanograms for corticosteroids and 1 ng for 21-deoxypregnane steroids. These amounts are much below the level usually encountered in the cultures, except for minor metabolites or for corticosteroids in inhibitory conditions. MF must be used in these instances to obtain accurate values. The specificity of the method is satisfactory, as was shown by GLC–MS; this is due to the association of liquid chromatography (TLC) and high-efficiency capillary column GLC. With deoxycorticosterone, MF analysis is suitable for discriminating between this steroid and another $C_{21}O_3$ steroid present in the cultures.

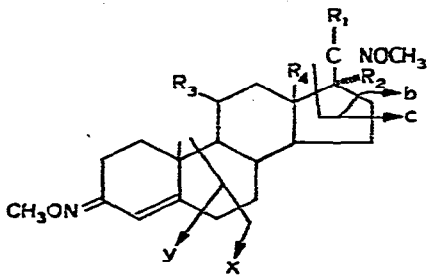
This method can be also adapted to the analysis of corticosteroids and related steroids in other small biological samples such as tissue biopsies and minute samples of blood.

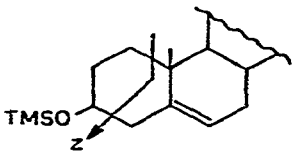
* A complete silylation for this compound was obtained with methyltrimethylsilyltrifluoroacetamide–trimethylbromosilane (80:20) giving rise to $R_H = 1.12$ (with 2 peaks) and $R_H = 0.72$ (with the highest peak).

TABLE III

VALUES OF MAIN ION *m/e* AND RELATIVE INTENSITIES (RI) OF MO-TMS DERIVATIVES OF CORTICOSTEROIDS WITH RESPECT TO THE BASE PEAK IN ELECTRON-IMPACT QUADRUPOLE MASS SPECTROMETRY

Electron energy, 50 eV; filament current, 0.5 mA. Sample introduction through a GLC SE-30 capillary column, temperature programmed from 240° at 1°/min. All oxo groups are converted to MO derivatives except in 11 position; all hydroxyl groups are as TMS ethers.





Compound	*Molecular ion m/e (RI)*	*Base peak*		*Masses and RI of the other fragments*
		m/e[a]	Σ_{40}%	
Pregnenolone	417 (1.1)	100^{23}	5	402^{2} (6.3), 386^{3} (12), 331^{5} (0.6), 327^{6} (1.5), 312^{9} (12.6), 296^{10} (9.6), 288^{11} (9.3), 129^{21} (61.7)
Progesterone	372 (100)	372^{1}	20	357^{2} (8.7), 341^{3} (71), 286^{5} (27.2), 273^{7} (37.9), 153^{17} (44.1), 151^{19} (24.6), 137^{20} (32.1), 100^{23} (65.8)
3α-Hydroxy-5β-pregnan-20-one	419 (0.4)	100^{23}	10	404^{2} (0.7), 388^{3} (8.6), 298^{10} (6.5), 243 (2.9)
3β-Hydroxy-5α-pregnan-20-one	419 (0.5)	100^{23}	10	404^{2} (1.7), 388^{3} (17.4), 298^{10} (2.3)
5α-Pregnane-3βs20α-diol	464 (0)	117^{29}	10	449^{2} (1.4), 374^{6} (1.3), 346 (1.6), 284^{25} (0.7), 269 (55.5), 359^{9} (0.5))
20α-Dihydroprogesterone	417 (3.1)	117^{29}	20	402^{2} (0.2), 386^{3} (0.4), 312^{9} (0.7), 301^{28} (2.4), 296^{10} (1.8), 153^{17} (21
20β-Dihydroprogesterone	417 (1.4)	117^{29}	20	402^{2} (0.1), 386^{3} (0.3), 327^{6} (0.2), 312^{9} (0.3), 301^{28} (1.4), 153^{17} (19.3), 296^{10} (2.0)
11-Deoxycortisol	548 (2.1)	273^{7}	20	517^{3} (72.1), 458^{6} (1.2), 445^{8} (17.4), 427^{10} (64.8), 396^{12} (4.9), 368^{25} (4.1), 337^{14} (12.4), 153^{17} (31.5), 151^{19} (36.8), 137^{20} (39.9)
11β-Hydroxyprogesterone	460 (31.5)	339^{10}	5	445^{2} (22), 429^{3} (32.6), 374^{5} (7.8), 370^{6} (36.1), 361^{7} (24.8), 355^{9} (8.5), 152^{18} (29.7), 137^{20} (2.8)
11-Deoxycorticosterone	460 (25.5)	286^{5}	15	429^{3} (5.9), 398^{4} (11), 357^{8} (15.3), 326^{24} (5.1), 308^{12} (5.1), 295^{13} (25.6), 273^{7} (79.5), 188^{15} (48.7), 175^{16} (41), 153^{17} (72), 151^{19} (43.5), 137^{20} (51), 103^{22} (71.9)
18-Hydroxy-11-deoxycorticosterone	548 (5.8)	396^{12}	5	517^{3} (51), 458^{6} (13.6), 445^{8} (1.8), 427^{10} (39.4), 414^{24} (2.9), 383^{13} (7.4), 368^{25} (33.7), 337^{14} (21), 297^{26} (38.9), 266^{27} (27.4), 153^{17} (24.7), 103^{22} (83)

TABLE III *(continued)*

Compound	*Molecular ion, m/e (RI)*	*Base peak*		*Masses and RI of the other fragments*
		*m/e**	$\Sigma_{40}\%$	
Aldosterone**	562 (0)	459[8]	10	547[2] (0.8), 531[3] (0.6), 413 (2.8), 103[22] (12.4)
21-Hydroxypregnenolone	505 (4.6)	129	5	490[2] (1.3), 474[3] (15.8), 402[8] (7.9), 384[10] (5.1), 188[15] (44.8), 175 (72.5), 103[22] (58)
11-Dehydrocorticosterone	474 (16.4)	152[18]	5	443[3] (16.4), 412[4] (1.4), 371[8] (3.7), 340[24] (2.6), 309[13] (21), 300[5] (37), 287[7] (25.1), 188[15] (32.8), 175[16] (11), 103[22] (65)
Corticosterone	548 (14.7)	103[22]	5	517[3] (25), 458[6] (14.7), 445[8] (4.4), 427[10] (54), 414[24] (2.9), 396[12] (32), 374[5] (27), 361[7] (82.1), 188[15] (87.2)

* [1], M^+; [2], M − 15; [3], M − 31; [4], M − 2 (31); [5], M − (b + H); [6], M − 90; [7], M − c; [8], M − 103; [9], M − 90 − 15; [10], M − 90 − 31; [11], M − z; [12], M − 152; [13], M − 165; [14], M − 31 − 2 (90); [15], c + H; [16], b + 2H; [17], x + 2H; [18], x + H; [19], x; [20], y; [21], z; [22], a, $(CH_2OTMS)^+$; [23], c + H, $R_1 = CH_3$, $R_2 = H$; [24], M − 103 − 31, [25], M − 2 (90); [26], side chain (SC)−H−90; [27], M − (SC + H) − 90 − 31; [28], M − SC, $R_1 = CH_3$; [29], SC, $R_1 = CH_3$.

** 3,20-Di-MO-18(hemiacetal), 21-di-TMS derivatives

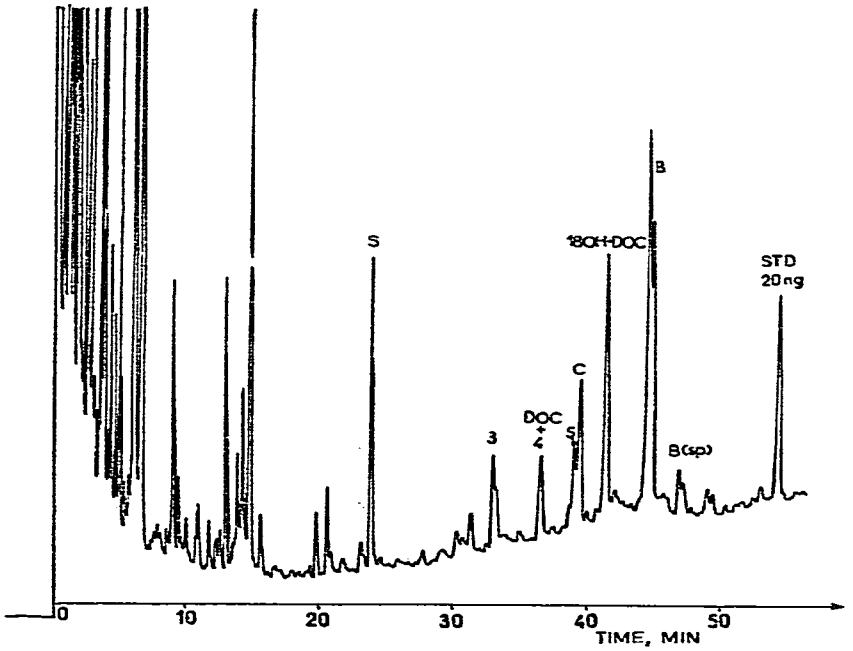


Fig. 7. CAP-GLC separation of steroids synthesized by adrenal cell culture: TLC fractions c and d as indicated in Fig. 4; fraction d corresponds to $C_{21}O_4$ corticosteroids. Adrenal cell culture stimulated by ACTH (100 mU per dish); age of donnor rats, 3 days; age of the culture, 5 days; incubation time, 24 h. The main steroids are 18-OH-DOC and corticosterone (B) at the rates of 9.3 and 17 μg per culture dish, respectively; other peaks (3, 4, 5 and DOC) are the same as in Fig. 6. Other abbreviations as in Fig. 5. Chromatographic conditions: as in Fig. 2.

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