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SAMPLE PURIFICATION USING A C_{18}-BONDED REVERSED-PHASE CARTRIDGE FOR THE QUANTITATIVE ANALYSIS OF CORTICOSTEROIDS IN ADRENAL CELL CULTURES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OR GAS CHROMATO-GRAPHY—MASS SPECTROMETRY*

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SUMMARY

Quantitative extraction and subsequent purification of small biological samples often involve cumbersome procedures. We have devised a short and efficient method for the quantitative extraction of the corticosteroid and the 20α reduced steroid series from culture medium containing 20% sera in a single, pure fraction with separation from cholesterol. Passage through a C_{18}-bonded reversed-phase Sep-Pak® cartridge of the acidified culture medium and subsequent extraction of the steroid fraction with methanol yields a single fraction containing all steroids in 90% recovery and reduced quantities of cholesterol down to 30%. The extract can then be used without further purification for quantitative analysis by high-performance liquid chromatography or derivatized and analyzed by gas chromatography and gas chromatography—mass spectrometry.

INTRODUCTION

The quantitative analysis of small biological samples is often difficult due to the minute quantities of the compounds to be assayed mixed together with large quantities of interfering materials. The isolation of the compounds to be identified and quantified is usually done by methods of low resolution but which can handle large amounts of materials, separating compounds into groups. The individual separation which allows identification and quantitation is done by methods of low capacity but of high efficiency. While many advances have been achieved in the methods of separation and detection, the

methods of extraction remain either laborious with minimal recovery or yielding samples of insufficient purity.

The most common extraction methods used for steroids from biological fluids are solvent partitioning and chromatography, using adsorbents such as Amberlite XAD-2 or Sephadex derivatives [1, 2]. Solvent partitioning gives fairly good steroid yield, but usually necessitates some other type of purification such as paper chromatography, thin-layer chromatography (TLC) or some form of column chromatography. Adsorption chromatography is usually done as a multi-column step to yield well-separated, pure samples.

Recently, Sep-Pak® cartridges have been available from Waters Assoc. These are small, pre-packed columns of various supports which have a large variety of uses. The C_{18}-bonded support packed cartridges are especially useful for biological applications. Among other applications they have been used by Hartwick et al. [3] for extraction of serum nucleotides. Very recently, Shackleton and Whitney [4] have described a method using these cartridges for extraction of urinary steroids.

In our laboratory newborn rat adrenal cells grown in culture have been shown to secrete steroid hormones under adrenocorticotropic hormone (ACTH) stimulation, for several weeks [5, 6]. The studies leading to the biochemical definition of this system as well as the evaluation of the effect of hormonal stimulators or inhibiting drugs need a sensitive, accurate analysis of steroid hormones down to the nanogram level. The method used until now has been the classical solvent extraction method, after deproteinization and delipidation, followed by TLC which fractionates the sample into three main fractions [7]. The least polar fraction contains pregnenolone, progesterone, 20α-dihydroprogesterone plus other $C_{21}O_2$ steroids. An intermediate fraction contains deoxycorticosterone and other mono-oxygenated progesterones of the general formula $C_{21}O_3$. The most polar fraction contains 18-hydroxy-11-deoxycorticosterone, corticosterone and other $C_{21}O_4$ steroids. Although cholesterol migrates with the solvent front, it tends to overload the plate and is thus present in all the fractions. This thin-layer purification step yields pure samples, serves to concentrate a compound or a family of compounds into a single fraction, and allows fast analysis of metabolites of radioactive precursors by radioscanning. However, it presents the disadvantage in quantitative analysis of differential steroid extraction according to their polarity. Another inconvenience is the length of this extraction—purification method.

We have developed a one-step extraction procedure using a C_{18} Sep-Pak cartridge system, which allows the processing of numerous samples in a very short period of time, with accurate, high yields. The samples so extracted in a single fraction are pure enough to be analyzed directly by high-performance liquid chromatography (HPLC), gas chromatography (GC), or gas chromatography—mass spectrometry (GC—MS).

MATERIALS AND METHODS

Steroids and reagents

Most of the authentic steroids were purchased from Makor Chemicals (Jerusalem, Israel) and Steraloids (Wilton, NH, U.S.A.); 18-hydroxy-11-deoxycorti-

costerone* was from Searle (Naucalpan, Mexico) and aldosterone from Sigma (St. Louis, MO, U.S.A.).

Labeled [$7n$-^{3}H] pregnenolone (specific activity, 21 Ci/mmol), [$7n$-^{3}H] cholesterol (specific activity, 15 Ci/mmol), [4-^{14}C] progesterone (specific activity 51.6 mCi/mmol) and [4-^{14}C] pregnenolone (specific activity, 59 mCi/mmol) were obtained from the Commissariat à l'Énergie Atomique (Saclay, France).

[4-^{14}C] Deoxycorticosterone (specific activity, 55 mCi/mmol) and [4-^{14}C]-cholesterol (specific activity, 59 mCi/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). [4-^{14}C] Corticosterone (specific activity 50 mCi/mmol) and [4-^{14}C] cortisol (specific activity, 50 mCi/mmol) were obtained from The Radiochemical Centre (Amersham, Great Britain). All solvents were of the pure for analysis quality from Merck (Darmstadt, G.F.R.). The following reagents were used: O-methoxyamine hydrochloride (MO, Cl) from Pierce Chemical Co. (Rockford, IL, U.S.A.), bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) from Supelco (Bellefonte, PA, U.S.A.).

Cell cultures

The method was adapted from the one described by Maume and Prost [5]. Adrenals were taken from newborn rats of Wistar US/Commentry (INRA, Dijon, France) strain. They were trypsinized by a solution of trypsin (B Grade, 3000 IU/mg) from Calbiochem (San Diego, CA, U.S.A.) in Ham's F10 medium without Ca^{2+} and Mg^{2+} salts, at a concentration of 1.75 mg/ml in a cell-stir vial. The trypsin action was stopped by the 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's F10 medium (Gibco, Paisley, Great Britain), penicillin (10 U/ml) and streptomycin (10 μg/ml), 10% of foetal calf serum and 10% of newborn calf serum. The total cholesterol content in this combination of sera is about 260 μg/ml of culture medium; of this, approximately 62 μg/ml is unesterified cholesterol. Each fraction was poured into a Cooper tissue-culture dish (Falcon, Oxward, CA, U.S.A.) of 5 ml capacity (bottom area 20 cm^2). At confluency the cell monolayer attached to the dish bottom contained between 2 and 3 million adrenocortical cells.

The dishes were kept in a culture oven at 37°C and under an air—CO_2 (95:5, v/v) atmosphere saturated with water. When the cultures were seven days old, ACTH from Choay (Paris, France) was added to the dishes in 0.1 ml of medium without serum.

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*The following code and trivial names are used: pregnenolone = 3β-hydroxy-5-pregnen-20-one; pregnanediol = 5α-pregnane-3β,20α-diol; progesterone = 4-pregnene-3,20-dione; pregnanolone = 20α-hydroxy-5α-pregnan-3-one; deoxycorticosterone = 21-hydroxy-4-pregnene-3,20-dione; deoxycorticosterone-21-acetate = 21-acetoxy-4-pregnene-3,20-dione; corticosterone = 11β,21-dihydroxy-4-pregnene-3,20-dione; corticosterone-21-acetate = 21-acetoxy-11β-hydroxy-4-pregnene-3,20-dione; deoxycortisol = 17α,21-dihydroxy-4-pregnene-3,20-dione; cortisol = 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; 18-hydroxy-11-deoxycorticosterone = 18,21-dihydroxy-4-pregnene-3,20-dione; 20α-dihydroprogesterone = 20α-hydroxy-4-pregnen-3-one; 2α-hydroxyprogesterone = 2α-hydroxy-4-pregnene-3,20-dione; 11β-hydroxy-20α-dihydroprogesterone = 11β,20α-dihydroxy-4-pregnen-3-one; trilostane (WIN 24540) = 4,5α-epoxy-17β-hydroxy-3-oxo-2α-androstane carbonitrile.

Incubations

Cell-culture medium was usually removed each 24 h. The steroid precursors were added to the dishes in 5 or 10 μl of absolute ethanol. The culture media were gathered and kept at $-30°C$ until steroid extraction was performed.

Reversed-phase chromatography

The steroid extraction was performed on Sep-Pak C_{18} columns from Waters Assoc. (Milford, MA, U.S.A.). These are cartridges, about 1 cm in diameter and 1.5 cm high, with octadecylsilane-bonded packing between two filters. The Sep-Pak columns were first rinsed with 4 ml of methanol, then with 4 ml of 0.01 M acetate buffer (pH 4.5). The cell-culture medium buffered to pH 4.5 was then passed through the column at a rate of one drop per sec (3 ml/min). The column was rinsed by 6 ml of 0.01 M acetate buffer (pH 4.5) and the steroids were eluted from the column by 3 ml of methanol at a rate of 1.5 ml/min. The sample was dried under a nitrogen stream and the humidity removed by adding a drop of benzene and evaporating again. Extraction yield was measured by adding a radioactive steroid to the culture medium measuring the radioactivity before extraction and again in the methanol extract.

Delipidation, deproteinization and TLC method

This method, previously described [8], was used as a comparison to the Sep-Pak method. The culture media were deproteinized and delipidated by precipitation with methanol—water (7:3, v/v) at $-30°C$, followed by centrifugation at $-20°C$. The methanol was evaporated and the steroids extracted from the remaining aqueous solution three times with ethyl acetate and three times with dichloromethane. The organic phases were pooled, dried over anhydrous magnesium sulfate and concentrated to approximately 100 μl at 65°C under a stream of nitrogen. The steroid extracts were purified and fractionated by TLC on silica gel (60 F 254) with fluorescent indicator (Merck). They were run first with diisopropyl ether and then with diisopropyl ether—acetone (6:4, v/v). The silica gel of the different fractions was scraped off and the steroids extracted by methanol and dichloromethane using an ultra-sonicator. The supernatants were pooled after centrifugation to separate the silica gel. The extraction yields were measured by radioactive counting before extraction and on each fraction extracted from TLC on a Packard Tri-Carb 460C liquid scintillation counter.

Derivative formation

The MO-TMS derivatives were prepared according to a previously described method [9]. The dry extract, to which 1 μg of cholesteryl butyrate was added as internal standard, was taken up in 100 μl of a solution of O-methoxyamine hydrochloride in dry pyridine at 16 mg/ml and allowed to react for 3 h at 65°C. The solution was evaporated to dryness under nitrogen and the residue taken up in 100 μl of BSTFA—TMCS (4:1, v/v) which was then allowed to react at 65°C for 16 h.

Lipidex purification of derivatized samples

The purification on the Sep-Pak cartridge does not remove a number of volatile, low-molecular-weight impurities, which in themselves do not interfere

with the sample analysis. In time, however these tend to cause deterioration of the capillary columns. Excess reagents and these polar residues were separated from the steroid MO-TMS derivatives by chromatography on Lipidex 5000 (Packard Instrument Co. Downers Grove, IL U.S.A.) following the method of Axelson and Sjöval [10]. The reaction mixture is passed through a 3-cm Lipidex column using hexane—pyridine—HMDS (98:1:1, v/v) concentrated under nitrogen and taken up in 100 μl of the same solvent mixture or a mixture of BSTFA—pyridine (8:2, v/v). Standard mixtures tested in this system give close to 100% recovery for all steroids tested, measured against a cholesteryl butyrate internal standard.

Gas chromatography and gas chromatography—mass spectrometry

Gas chromatography was carried out on 25-m long SE-30 wall-coated open tubular columns (0.21 mm I.D.) made by Spiral (Dijon, France). A Packard Model 427 chromatograph equipped with an all-glass solid injector and a flame ionization detector was used. Injector temperature was 260°C column temperature was programmed from 240°C to 295°C at 1°C/min, the detector temperature was 300°C. The flow-rate of nitrogen was 0.8 ml/min.

Determination of methylene unit (MU) values was achieved by simultaneously running the steroids under study and C_{28} to C_{34} n-alkanes (Fluka, Buchs, Switzerland). Quantitative measurements by GC have been done by using response coefficients calculated by comparing the height of the peak of each adrenal steroid with that of cholesteryl butyrate [7]. This response coefficient was then used to quantify the steroids in the samples using cholesteryl butyrate as internal standard.

A Finnigan 3300-6100 quadrupole mass spectrometer (Finnigan Corp., Sunnyvale. CA, U.S.A.) coupled with a glass capillary column was used with helium as carrier gas. The capillary column was directly connected to the mass spectrometer source by a silanized glass line. The instrument was equipped with a chemical-ionization source. The reagent gas was methane, ion source pressure was 0.8 Torr electron energy 130 eV, and filament current 0.25 mA.

Characterization and quantitation of steroids by GC and GC—MS

The characterization of steroids is accomplished by comparing their MU values with those of reference compounds already published [7] and by verification of the similitude of their mass spectra in the chemical-ionization mode to those of authentic samples. For the quantitation a test mixture containing the reference steroids and the internal standard is processed with each sample series and run with it. In this manner there is constant control of the possible changes in the response coefficients to cholesteryl butyrate. The changes which we have found to affect this coefficient are incomplete derivatization, humidity in the sample and selective adsorption on the needle due to the presence of impurities, or on the column if reactivated.

High-performance liquid chromatography

A Varian 5000 Liquid Chromatograph System (Varian Assoc., Palo Alto CA, U.S.A.), equipped with a UV detector (at 254 nm), a column heater and a solvent programmer was used. A stainless-steel column (30 cm × 4 mm I.D.)

prepacked with octadecylsilane (C_{18}) bonded to 10-μm silica particles, Micropak from Varian was used. The column temperature was 40°C and the solvent gradient was programmed linearly from 30% to 70% (v/v) acetonitrile in water in 30 min at a flow-rate of 0.8 ml/min.

Characterization and quantitation of steroids by HPLC

Steroids are characterized by their elution times relative to corticosterone-21-acetate and deoxycorticosterone-21-acetate, which are used as internal standards. Response coefficients towards these two steroid derivatives have been calculated by comparing their peak heights at different concentrations. A steroid test mixture is run before each series and analyzed to ensure proper functioning of the system.

RESULTS AND DISCUSSION

Comparative yield of extracted steroids

Steroids of varying polarity were extracted from the cell-culture incubation

TABLE I

RECOVERY OF RADIOLABELED STEROIDS FROM CULTURE MEDIUM IN DIFFERENT FRACTIONS OF SEP-PAK® EXTRACTION

Each experiment was performed on 6 ml of culture medium with 10% foetal calf serum and 10% newborn calf serum. Quantities added corresponded to both ^{14}C-labeled (specific activity, 50 mCi/mmol) and unlabeled steroids. The quantity of [^{3}H] cholesterol (4 Ci/mmol) added was less than 10 ng. Free cholesterol in the culture medium came from the sera and was measured by capillary GC. Values are expressed as the percentage of initial radioactivity added.

Steroid	Quantity (μg)	Percentage in buffered medium	Percentage in buffered wash			Percentage in methanol eluate		
			2 ml	+ 2 ml	+ 2 ml	2 ml	+ 2 ml	+ 2 ml
Pregnenolone*	0.001	2	0	0	0	92	0.4	0
Cholesterol	720	51	3.9	1.3	1	27	11	3
Pregnenolone	0.5	4.4	1.1	0.3	0	92	1	0
Cholesterol	720	31	11	3	1	29	9	2.3
Pregnenolone	50	3.4	0.4	0	0	91	0.5	0.3
Cholesterol	720	31	12	1.2	0.4	30	11	3
Progesterone	0.02	0	0	0	0	94	0	0
Cholesterol	720	46	4.1	1.1	0.5	24	13	0
Progesterone	1	3	0.1	0	0	94	1.7	0.1
Cholesterol	720	32	10	0.5	0.1	30	10	1
Progesterone	50	2.5	0.3	0	0	93	1	0
Cholesterol	720	30	14	1	0.2	30	3.5	0.6
Corticosterone	0.02	7	0.3	0	0	86	0.3	0
Cholesterol	720	39	8	4	1	20	13	0
Corticosterone	0.2	3.8	0.1	0	0	89	0.2	0
Cholesterol	720	27	2.4	0.1	0	36	19	0.6
Corticosterone	50	6	0.4	0	0	88	2	0.2
Cholesterol	720	47	19	6	2	17	4	0.4
Cortisol	0.02	6	0.8	0.2	0.4	88	0.6	0
Cholesterol	720	47	2.2	0.8	0.5	18	4	0.5
Cortisol	0.3	4.4	0.1	0	0	93	1	0
Cholesterol	720	22	1.3	0	0	38	9.4	1.5
Cortisol	50	3.3	0.1	0	0	91	0.3	0
Cholesterol	720	45	3.8	0.1	0.1	24	11	0.3

*In this experiment labeled steroids were [^{3}H] pregnenolone (21 Ci/mmol) and [^{14}C] cholesterol (50 mCi/mmol).

medium using the Sep-Pak method. [³H] Cholesterol plus the ¹⁴C-labeled steroid to be tested were added to the culture medium and separated on a Sep-Pak cartridge. The results (Table I) show that the yield of pregnenolone, progesterone, corticosterone and cortisol in the first 2 ml of methanol eluate are all approximately equal to about 90% and that this yield is independent of the quantity of steroid present; that is 1 ng to 50 μg of pregnenolone and 20 ng to 50 μg of the others. There seems to be slightly less retention of the more polar steroids such as cortisol and corticosterone in the passage of the medium, but not enough to change the final percentage recovery in the methanol fraction. Although 2 ml of methanol suffice to recover the bulk of the steroid, we usually collected 3 ml to ensure maximum recovery.

The yields obtained when the same steroids are extracted from the culture medium with the deproteinization, delipidation, TLC method are not as good (Table II). Although the total steroid extraction by ethyl acetate and methylene chloride is 86%, the individual steroid extractions from TLC are very different depending on their polarity. While there is 61% of the original pregnenolone extracted, only 50% corticosterone and 33% cortisol is extracted from the silica plate. The steroid recoveries from TLC only, also seen in Table II, show that the differential extraction occurs at this step.

Removal of interfering cholesterol

The sera which make up 20% of the culture medium (10% foetal calf serum and 10% newborn calf serum) contain an average 1.3 mg/ml total cholesterol of which approximately 0.3 mg/ml is unesterified cholesterol. The TLC step is performed in order to purify the steroid fractions and separate them as much

TABLE II

RECOVERY OF RADIOLABELED STEROIDS FROM CULTURE MEDIUM IN SUCCESSIVE STEPS OF DELIPIDATION, DEPROTEINIZATION, SOLVENT EXTRACTION AND TLC

Each experiment was performed on 6 ml of culture medium with sera as indicated in Table I. Steroids added were 4-¹⁴C-labeled (specific activity, approximately 50 mCi/mmol) and unlabeled compounds: cholesterol was ³H-labeled (4 Ci/mmol). Cholesterol content of the culture medium supplemented with sera was approximately 720 μg. In the TLC extraction only recoveries were calculated in a separate experiment after chromatography of the pure steroids. Recoveries by solvent extraction were 85—87% for all ¹⁴C-labeled steroids and 82—85% for [³H]cholesterol. Values are expressed as percentage of initial radioactivity added.

Steroids	Initial quantity (μg)	Solvent extraction and TLC extraction		TLC extraction only	
		Steroid (%)	Cholesterol (%)	Steroid (%)	Cholesterol (%)
Pregnenolone	0.5	61	15	62	11
Corticosterone	0.2	50	15	55	4
Cortisol	0.3	33	15	37	5
Pregnenolone	50	75	35	—	—
Corticosterone	50	52	35	—	—
Cortisol	50	34	35	—	—

as possible from this cholesterol. Attempts to analyse directly on GC the steroid fraction extracted by ethyl acetate and methylene chloride show a large amount of cholesterol which overloads the capillary columns and makes quantitation of steroids impossible. As can be seen in Table II, there always remains some cholesterol in all the fractions from TLC since it overloads the plate and tails. With the Sep-Pak method all the esterified cholesterol is removed as well as 64—83% of the unesterified cholesterol (Table I), so that quantitation is always possible.

Extraction of steroid mixture from the culture medium

A mixture of the standard steroids used as a test mixture for quantitation was added to 6 ml of culture medium and passed through a Sep-Pak cartridge as described in the Methods section. Recovery was calculated both by using [^{14}C] - corticosterone to monitor extraction and by adding the appropriate internal standards after extraction.

Figs. 1 and 2 show representative analyses of the test mixture before and after Sep-Pak extraction. Fig. 1a shows the separation of the mixture by HPLC with the internal standards deoxycorticosterone-21-acetate and corticosterone-
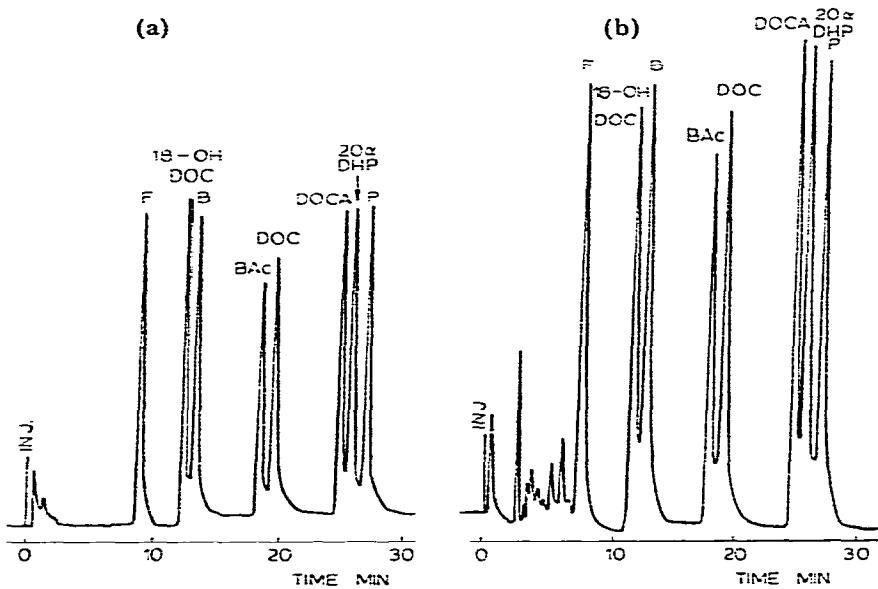

Fig. 1. Separation by HPLC of reference steroid test mixture extracted by Sep-Pak® cartridge. (a) Test mixture containing reference steroids run on a C$_{18}$-bonded reversed-phase column in the HPLC system. (b) Same test mixture after extraction from the cell-culture medium in identical conditions to incubation mixtures. The internal standards corticosterone-21-acetate and deoxycorticosterone-21-acetate were added after extraction. Chromatographic conditions: prepacked C$_{18}$-bonded, stainless-steel column (30 cm × 4 mm I.D.); 7200 theoretical plates; column temperature 40°C; solvent gradient programmed linearly from 30% to 70% (v/v) acetonitrile—water in 30 min at a flow-rate of 0.8 ml/min; UV detection at 254 nm. Abbreviations: F = cortisol (1 μg); 18-OH DOC = 18-hydroxy-11-deoxycorticosterone (1 μg); B = corticosterone (1 μg); BAc = corticosterone-21-acetate (1 μg); 20α-DHP = 20α-dihydroprogesterone (1 μg); P = progesterone (1 μg); DOC = deoxycorticosterone (1 μg).
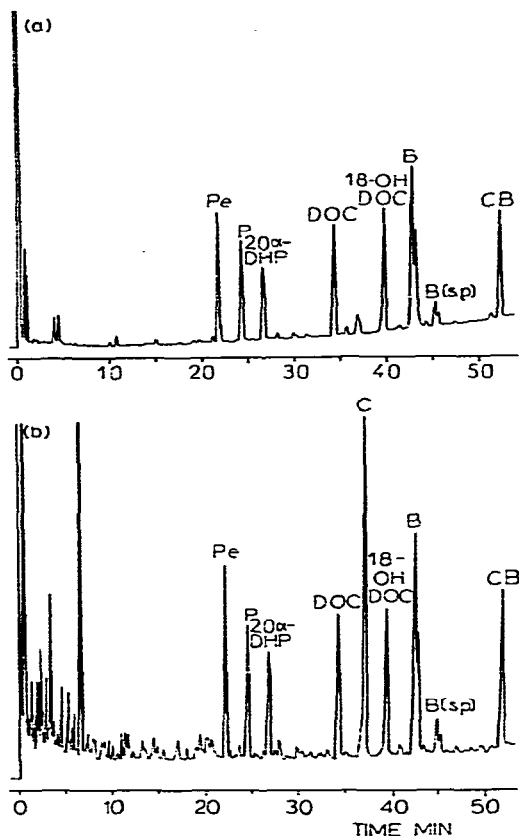
Fig. 2. Separation by GC of reference steroid test mixture extracted by Sep-Pak® cartridge. (a) MO-TMS derivatives of a test mixture containing reference steroids run on an SE-30 capillary column in the GC system. (b) Same test mixture after extraction from the cell-culture medium in identical conditions to the incubation mixtures. The internal standard cholesteryl butyrate was added after extraction and before MO-TMS derivatization. Chromatographic conditions: all-glass solid injection system; SE-30 coated glass capillary column (25 m × 0.21 mm I.D.); 75,000 theoretical plates; temperature programming from 240°C at 1°C/min. Abbreviations: Pe = pregnenolone (10 ng); P = progesterone (10 ng); 20α-DHP = 20α-dihydroprogesterone (10 ng); DOC = deoxycorticosterone (20 ng); C = cholesterol; 18-OH DOC = 18-hydroxy-11-deoxycorticosterone (40 ng); B = corticosterone (40 ng); B(sp) = secondary peak of corticosterone; CB = cholesteryl butyrate (10 ng).

21-acetate added. Using the response coefficients measured in this chromatogram the recoveries of each steroid from the culture medium are calculated from the chromatogram shown in Fig. 1b. The recoveries are between 88% and 95%. This is in agreement with the respective recovery of the radioactive standard. Previous experiments show that no interfering impurities are extracted from the culture medium.

The GC traces of these test mixtures are shown in Fig. 2a (pure standards) and Fig. 2b (standards extracted from culture medium). The recoveries, as determined by comparing these two chromatograms by their response coefficients to cholesteryl butyrate, were also between 88% and 95%. Note the fairly clean solvent front and the relatively small amount of cholesterol in comparison with its high ratio towards others steroids in the culture medium itself.

Stability of 18-hydroxylated steroids

The formation of methyl and ethyl ketals of 18-hydroxy-11-deoxycorticosterone catalyzed by an acid medium has been previously described [11]. Since C_{21} 18-hydroxylated steroids are among the steroids to be measured, the method was tested to see if the methyl ketal of 18-hydroxy-11-deoxycorticosterone could be detected. This method uses a final concentration of 0.01 M acetate buffer in the aqueous phase and then pure methanol (final pH of the residue in water approximately 4.5). After evaporation the sample is taken up in absolute ethanol (pH after evaporation, 5.5). Thus the acetic acid concentration in methanol or ethanol is much lower than that (0.1 M) used by Roy et al. [11] to produce the ketal in a short period of time. Therefore, methyl or ethyl ketal formation in the methanolic solution of 18-hydroxy-11-deoxycorticosterone during Sep-Pak elution, or during storage of its ethanolic solution at −30°C is unlikely. Nevertheless, a supplementary precaution is to add 0.1% triethylamine [4, 11] or pyridine to the alcohlol extracts.

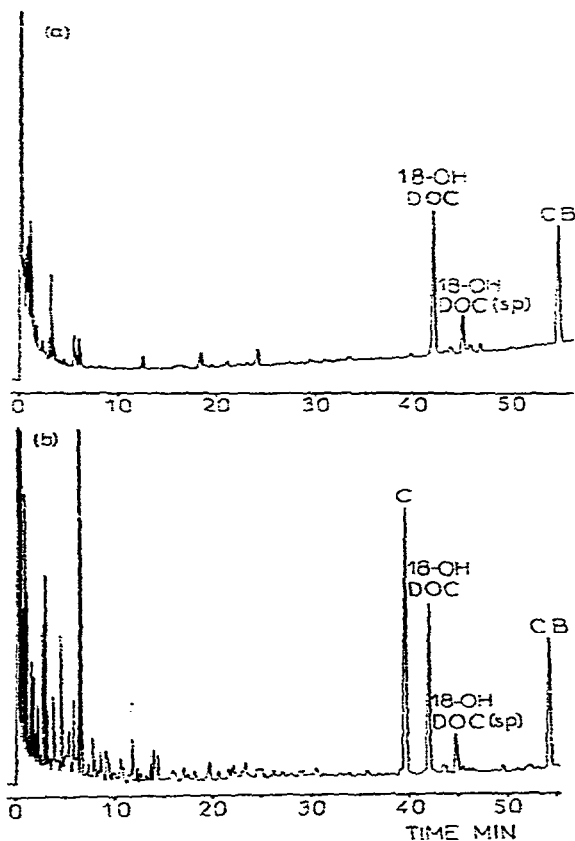Fig. 3. Analysis by GC of 18-hydroxy-11-deoxycorticosterone extracted by Sep-Pak® cartridge. (a) MO-TMS derivative of 18-hydroxy-11-deoxycorticosterone run on an SE-30 capillary column in the GC system. (b) Same steroid after addition to and extraction by Sep-Pak cartridge from the cell-culture incubation medium. The internal standard, cholesteryl butyrate (1 μg), was added after extraction and before MO-TMS derivatization (10 ng injected). Chromatographic conditions, abbreviations and steroid quantities: same as in Fig. 2, except 18-OH DOC(sp) = secondary peak of 18-hydroxy-11-deoxycorticosterone.

A sample of this steroid added to the culture medium and extracted with the described Sep-Pak method is shown in Fig. 3b. The recovery here is 96% and there was no methyl ketal detected by mass chromatography of the protonated molecular ion m/z 462 of the MO-TMS derivative. Furthermore, there is no degradation of 18-hydroxy-11-deoxycorticosterone by the acid-catalyzed Beckman degradation as shown in Fig. 3b.

Analysis of endogenous steroid production using two methods of extraction

A comparison of the GC separations obtained through the deproteinization, delipidation, extraction and TLC method and the method using a single Sep-Pak column is shown in Fig. 4. As can be seen from the chromatograms, both methods are well adapted for the quantitation of steroids from one cell-culture dish, where we can easily detect 100—200 ng of a single steroid in a complex mixture with a limit down to 10—20 ng per culture dish.

In both cases we analyzed the endogenous steroid production by newborn rat adrenal cultured cells after treatment with 100 mU of ACTH. Fig. 4a and b show the two fractions eluted from the TLC plate. The samples are fairly pure and quantitation of the steroids through their response coefficients to cholesteryl butyrate is possible. Note there are large amounts of squalene in both fractions as well as cholesterol.

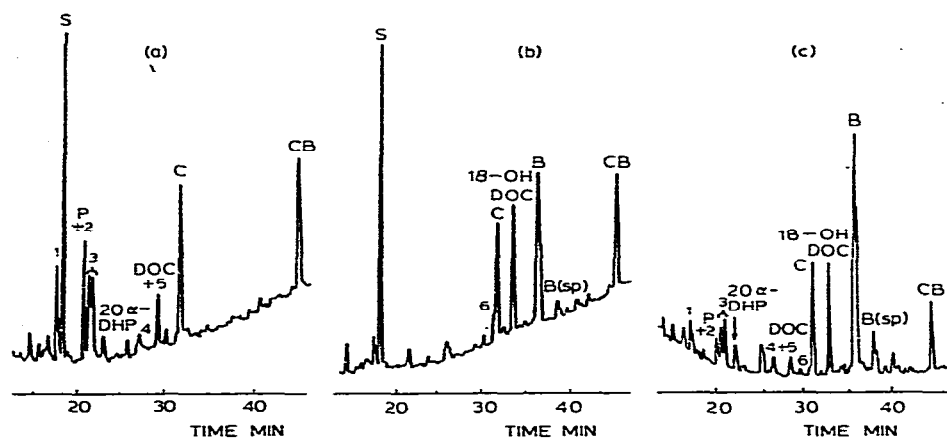The analysis performed by Sep-Pak extraction is shown in Fig. 4c. It is a

Fig. 4. Separation by GC of endogenous steroids produced in two cell cultures using two different methods of extraction. Culture 1, (a) and (b), was extracted by the deproteinization, delipidation, solvent extraction method and chromatographed on TLC. It was divided into two fractions, one containing the least polar steroids and some of intermediate polarity such as deoxycorticosterone and 18-hydroxy-20α-dihydroprogesterone (a), and the other containing the most polar steroids, together with others of intermediate polarity (b). Both fractions contain squalene and cholesterol. Culture 2 was extracted by the Sep-Pak® method and was chromatographed directly after MO-TMS derivatization. Cholesteryl butyrate (1 μg) was added to all three samples before MO-TMS derivatization, as internal standard. Chromatographic conditions: same as in Fig. 2. Abbreviations: 1 = 5α-pregnane-3α,20α-diol; S = squalene; 2 = 5α-pregnane-3β,20α-diol; 3 = 20α-hydroxy-5α-pregnan-3-one; 4 = 18-hydroxyprogesterone and 11-oxo-20α-dihydroprogesterone; 5 = 18-hydroxy-20α-dihydroprogesterone; 6 = 11β-hydroxy-20α-dihydroprogesterone; CB = cholesterylbutyrate (10 ng injected); other abbreviations are the same as in Fig. 2.

single fraction, as free of impurities as the TLC fractions, with no squalene and little cholesterol. Quantitation of all steroids produced is also possible, though mass spectrometry was necessary in both cases to identify mixed peaks.

The interference of cholesterol with the quantitation of 2α-hydroxyprogesterone, 11β-hydroxy-20α-dihydroprogesterone and deoxycortisol still remains, since they have similar MU values and are eluted together [7]. But the reduced amounts of cholesterol make an approximate quantitation possible. When the analysis necessitates mass fragmentography the reduced amounts of cholesterol are especially helpful.

The range of excretion of the individual steroids is between 0.2 and 30 μg per dish per day (up to 70 μg in maximum stimulation conditions). Each analysis is done on one hundredth of the sample. We can increase the sensitivity of the detection to 1 ng or less. By increasing the sample concentration and the volume injected on the column, both possible due to the purity of the sample, one tenth of the sample can be analyzed. A total production of less than 10 ng per dish per day can be measured. Of course with GC—MS and especially using mass fragmentography one can quantify much smaller amounts.

Analysis of exogenous steroid production by both HPLC and GC

An incubation of an exogenous steroid using an inhibitor was analyzed by the Sep-Pak method. A single culture dish was incubated with 10^{-4} M trilostane (as an inhibitor of endogenous steroid production), 50 μg of deoxycorticosterone and 500 mU of ACTH, for 24 h. The recovered medium was processed through a Sep-Pak cartridge and analyzed both by HPLC and GC. The resulting chromatograms are shown in Fig. 5.

The HPLC trace (Fig. 5a) shows a very clean scan with only corticosterone and 18-hydroxy-11-deoxycorticosterone as products. There is no remaining deoxycorticosterone. The most polar peaks, those that elute near the solvent front, have been identified as arising from the medium itself. The peak which elutes before 18-hydroxy-11-deoxycorticosterone is always present when a culture incubation with trilostane is analyzed and has not been identified. Trilostane has not been detected since it does not possess any function with intense UV absorption at 254 nm. This UV detection method does not allow the steroid products that do not possess a 3-oxo-4-ene ring A structure to be seen. Thus, pregnenolone, the pregnanediols and any of the ring A reduced metabolites will not be detected. Therefore, it is often of interest to analyze the same sample by GC.

The corresponding GC trace (Fig. 5b) shows the efficiency of the sample cleaning. There are some impurities at the solvent front and several peaks in the area of pregnenolone and pregnanediol, which are present after all culture incubations. The large amount of pregnenolone (14.9 μg per dish per 24 h) is due to the inhibition of 3β-hydroxysteroid dehydrogenase by trilostane. As seen on the chromatogram, there is no remaining deoxycorticosterone detected and the only products are 18-hydroxy-11-deoxycorticosterone and corticosterone. GC—MS analysis confirmed the identify of the steroids. Only the peak eluting before cholesterol with a protonated molecular ion at m/z 429 has not been identified. It has been seen in all culture incubations with trilostane and might be a metabolite, though its structure is not known. Indeed, it does not corre-
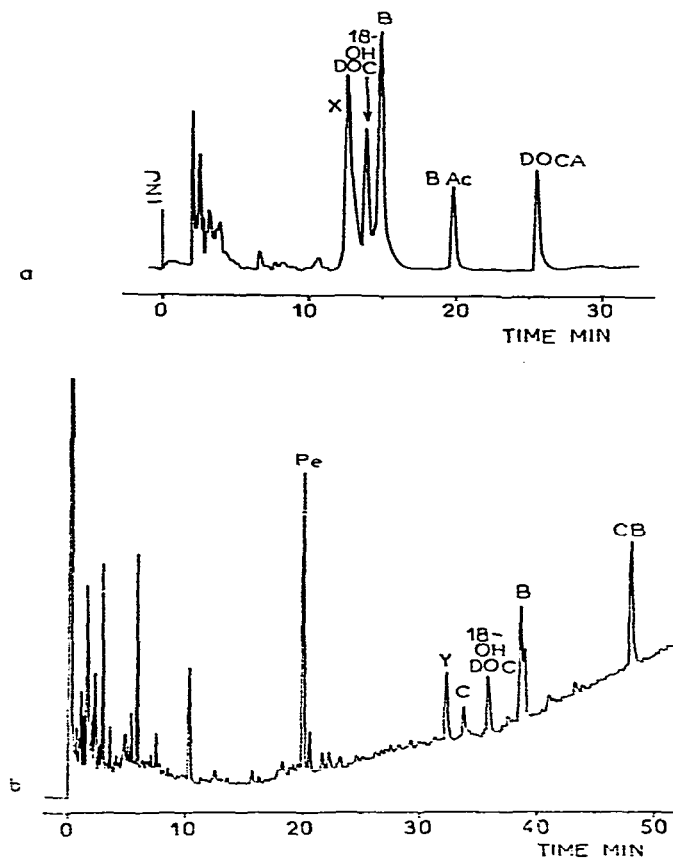
Fig. 5. Separations by HPLC and GC of exogenous steroid products by a single cell culture. An incubation using deoxycorticosterone as substrate and trilostane as inhibitor of endogenous steroid production was extracted by the Sep-Pak® method and analyzed by both HPLC and GC. (a) HPLC analysis with BAc (corticosterone-21-acetate, 1 μg) and DOCA (deoxycorticosterone-21-acetate, 1 μg) as internal standards. (b) GC analysis with cholesteryl butyrate (CB) as internal standard (10 ng injected). Both methods show no interfering impurities and are well adapted for quantitation. Pregnenolone is not detected by the UV detector at 254 nm in the HPLC system. Chromatographic conditions: same as in Figs. 1 and 2. Abbreviations: X or Y = unknowns; other abbreviations are the same as in Figs. 1 and 2.

spond to trilostane itself, which has a very low response coefficient to cholesteryl butyrate having four peaks as the MO-TMS derivative: the major one at 29.86 MU (0.034 response coefficient), the second largest at 29.28 MU (0.012 response coefficient) and two other very minor peaks at 29.58 and 30.99 MU. None of these peaks corresponds to the unknown.

Corticosterone and 18-hydroxy-11-deoxycorticosterone were quantitated using both methods (Table III). Deoxycorticosterone-21-acetate and corticosterone-21-acetate were added to the sample before HPLC analysis as internal standards. For the GC quantitation cholesteryl butyrate was added prior to MO-TMS derivatization. Both methods show similar results with 27.4 and 30.8 μg corticosterone per dish measured by HPLC using deoxycorticosterone-21-acetate and corticosterone-21-acetate as internal standards, respectively, and 26.4 μg per dish with the GC analysis. The results for 18-hydroxy-11-deoxy-

TABLE III

QUANTITATION OF STEROIDS PRODUCED BY AN ADRENAL CELL CULTURE: COMPARISON OF HPLC AND GC METHODS

Internal standards: HPLC A method = corticosterone-21-acetate, HPLC B method = deoxycorticosterone-21-acetate; GC method = cholesteryl butyrate. Incubation conditions: deoxycorticosterone (50 μg) as exogenous substrate, trilostane (10^{-4} M) as inhibitor of 3β-hydroxysteroid dehydrogenase, ACTH (500 mU per culture dish), incubation time = 24 h. Results are expressed as μg per dish per 24 h.

	HPLC methods		GC method
	A	B	
Pregnenolone	—	—	14.9
18-Hydroxy-11-deoxycorticosterone	15.8	16.7	16.1
Corticosterone	27.4	30.8	26.4

corticosterone are even closer, with 15.8 and 16.7 μg per dish with HPLC and 16.1 μg per dish with the GC methods. The agreement between the two different internal standards in the HPLC method indicates that there are no interfering substances in either measurement. There is also very good agreement between the two different quantitation methods, showing that there is a fairly general removal of interfering impurities by the Sep-Pak extraction.

CONCLUSION

This Sep-Pak extraction method is short and simple, saving not only analysis time but also materials, since there is a very limited amount of solvents and glassware necessary. In addition, this method provides a single, pure fraction, with excellent recoveries of all steroids in biological samples. The purification requires a single step and the samples are ready for analysis by HPLC or derivatization and analysis by GC, with no further work-up. Quantitation is reproducible and accurate down to 50—100 ng of steroid by HPLC, to 10 ng by GC and GC—MS methods and even more sensitive by mass fragmentographic methods.

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