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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF STEROIDS SECRETED BY HUMAN ADRENAL AND TESTIS CELLS IN MONOLAYER CULTURE

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SUMMARY

Reversed-phase high-pressure liquid chromatography with gradient elution on Zorbax-ODS columns has been used to separate, identify, and measure, spectrophotometrically, the steroids secreted by both human adrenal and testis cells in primary monolayer culture. Three related systems using exponential concave gradients have been developed with the specific objective of resolving the steroids produced by these two tissues. A methanol–water gradient has been used to separate most adrenal steroids, an acetonitrile–water gradient to separate testis steroids, and a dioxane–water gradient to separate polar steroids, including aldosterone. These three systems together permit the resolution of at least 43 naturally occurring steroids, plus four synthetic steroids with adrenocortical activity, with overall total elution times of 1 h or less for each system. Retention data for these steroids are given and the separation of steroids in the biological samples illustrated.

INTRODUCTION

In order to compare the functional activity of normal and neoplastic human adrenal and testis cells in monolayer culture, it is necessary to identify and measure a wide range of steroids. We describe here several high-pressure liquid chromatography (HPLC) systems with which this can be achieved, and illustrate their application to this biological system.

We have shown, using fluorimetry[1], radioimmunoassay[2], and radioactive steroid metabolism[3], that certain specialised functions are retained for long periods of time by steroidogenic cells in primary monolayer culture. Some changes in function have, however, been observed[2,4]. The fact that changes can occur in this system makes it necessary to identify and measure all steroids secreted by the cultured cells before valid comparisons can be drawn between normal and neoplastic cells. Radioimmunoassay is not suited to this task because, apart from the labour that it entails, it does not recognise unusual or unexpected products that may occur in pathological tissues.

HPLC has been successful in separating artificial steroid mixtures[5–7] and has also been applied on a limited scale to the measurement of individual plasma steroids[8–10]. Furthermore, the levels of most steroids secreted by the cultures were

within the capabilities of this technique. We have therefore used HPLC to identify and measure, spectrophotometrically, the physiologically important 4-en-3-one steroids synthesised by the cells. As an aid in identifying the products of the cultures, we have determined the retention times of a large number of naturally occurring steroids on the three reversed-phase partition systems that have been used.

MATERIALS AND METHODS

Materials

The methanol, acetonitrile, 1,4-dioxane, and dichloromethane used were Nanograde (Mallinckrodt, St. Louis, Mo., U.S.A.) or HPLC grade (Rathburn, Walkerburn, Great Britain). Solvents for HPLC were degassed under vacuum immediately before use. Water was deionised and glass-distilled.

Radioactive steroids were from the Radiochemical Centre (Amersham, Great Britain) and non-radioactive steroids from Sigma (Kingston-upon-Thames, Great Britain), Steraloids (Pawling, N.Y., U.S.A.), Ikapharm (Ramat-Gan, Israel), and the MRC Steroid Reference Collection. Their purity was checked by thin-layer chromatography (TLC) and HPLC, and non-radioactive steroids were recrystallised when necessary.

The trivial names and abbreviations used are listed in Table I.

Chromatography

We used a DuPont Model 830 high-pressure liquid chromatograph (DuPont, Wilmington, Dela., U.S.A.), equipped with a Model 837 variable-wavelength spectrophotometer and a Model 838 programmable gradient elution module. Samples were injected via a Rheodyne septumless valve. The spectrophotometer was operated in the double-beam mode with an air reference cell and chromatograms were recorded on a Phillips PM 8000 flat-bed recorder and integrated with a Time Electronics TS 100A digital integrator.

We selected reversed-phase chromatography with gradient elution because of the wide range of polarities of the steroids that needed to be separated in the samples. These systems have the advantages of high selectivity and rapid re-equilibration[11]. The columns used were 25 cm × 2.1 mm I.D. stainless-steel precision-bore tubing, commercially packed with Zorbax-ODS (DuPont).

Empirical studies showed that the best solution to our "general elution problem"[12] was a concave exponential gradient of methanol–water to separate adrenal steroids, an acetonitrile–water gradient to separate testis steroids, and a dioxane–water gradient to separate polar steroids such as aldosterone. Details of these systems are given in Table II.

UV-absorbing steroids were detected at 240 nm (254 nm with dioxane gradients) and non-UV-absorbing radioactive steroids by liquid scintillation counting of eluate fractions. Careful selection of solvent batches, particularly of acetonitrile, eliminated or minimised the tendency of the gradient profile to contribute to background drift.

Quantification of steroids was routinely based on peak heights, which were shown to be directly proportional to the mass of steroid injected over at least a fifty-fold range (40–2000 ng). This covered the range of values encountered in the samples.

TABLE I

TRIVIAL NAMES AND ABBREVIATIONS

Trivial name	*Abbreviation*	*Chemical name*
Adrenosterone	(G)	androst-4-ene-3,11,17-trione
Aldosterone	(ALDO)	11β,21-dihydroxy-18-al-pregn-4-ene-3,20-dione
Androstenediol		androst-5-ene-3β,17β-diol
Androstenedione	(AD)	androst-4-ene-3,17-dione
Cholesterol		cholest-5-en-3β-ol
Corticosterone	(B)	11β,21-dihydroxypregn-4-ene-3,20-dione
Cortisol	(F)	11β,17α,21-trihydroxypregn-4-ene-3,20-dione
Cortisone	(E)	17α,21-dihydroxypregn-4-ene-3,11,20-trione
11-Dehydroaldosterone	(DHALDO)	21-hydroxy-18-al-pregn-4-ene-3,11,20-trione
11-Dehydrocorticosterone	(A)	21-hydroxypregn-4-ene-3,11,20-trione
Dehydroepiandrosterone	(DHA)	3β-hydroxyandrost-5-en-17-one
11-Deoxycorticosterone	(DOC)	21-hydroxypregn-4-ene-3,20-dione
11-Deoxycortisol	(S)	17α,21-dihydroxypregn-4-ene-3,20-dione
21-Deoxycortisol	(21-deoxyF)	11β,17α-dihydroxypregn-4-ene-3,20-dione
Dexamethasone		9-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione
20α-Dihydroprogesterone	(DHP)	20α-hydroxypregn-4-en-3-one
20β-Dihydroprogesterone		20β-hydroxypregn-4-en-3-one
5α-Dihydrotestosterone	(DHT)	17β-hydroxy-5α-androstan-3-one
17β-Estradiol		estra-1,3,5(10)-triene-3,17β-diol
Estriol		estra-1,3,5(10)-triene-3,16α,17β-triol
Estrone		3-hydroxyestra-1,3,5(10)-trien-17-one
11β-Hydroxyandrostenedione	(11βOH-AD)	11β-hydroxyandrost-4-ene-3,17-dione
19-Hydroxyandrostenedione		19-hydroxyandrost-4-ene-3,17-dione
18-Hydroxycorticosterone	(18OH-B)	11β,18,21-trihydroxypregn-4-ene-3,20-dione
6β-Hydroxycortisol	(6βOH-F)	6β,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione
18-Hydroxy-11-dehydrocorticosterone	(18OH-A)	18,21-dihydroxypregn-4-ene-3,11,20-trione
18-Hydroxy-11-deoxycorticosterone	(18OH-DOC)	18,21-dihydroxypregn-4-ene-3,20-dione
11β-Hydroxy-20α-dihydroprogesterone		11β,20α-dihydroxypregn-4-en-3-one
17α-Hydroxy-20α-dihydroprogesterone	(17αOH-DHP)	17α,20α-dihydroxypregn-4-en-3-one
17α-Hydroxy-20β-dihydroprogesterone		17α,20β-dihydroxypregn-4-en-3-one
17α-Hydroxypregnenolone		3β,17α-dihydroxypregn-5-en-20-one
6α-Hydroxyprogesterone		6α-hydroxypregn-4-en-3-one
6β-Hydroxyprogesterone		6β-hydroxypregn-4-en-3-one

(Continued on p. 360)

TABLE I (*continued*)

Trivial name	*Abbreviation*	*Chemical name*
11β-Hydroxyprogesterone		11β-hydroxypregn-4-en-3-one
16α-Hydroxyprogesterone	(16αOH-P)	16α-hydroxypregn-4-en-3-one
17α-Hydroxyprogesterone	(17αOH-P)	17α-hydroxypregn-4-en-3-one
7α-Hydroxytestosterone	(7αOH-T)	7α,17β-dihydroxyandrost-4-en-3-one
11β-Hydroxytestosterone		11β,17β-dihydroxyandrost-4-en-3-one
16α-Hydroxytestosterone		16α,17β-dihydroxyandrost-4-en-3-one
19-Hydroxytestosterone		17β,19-dihydroxyandrost-4-en-3-one
17-Isoaldosterone		11β,21-dihydroxy-18-al-(17β)-pregn-4-ene-3,20-dione
11-Ketoprogesterone		pregn-4-ene-3,11,20-trione
Prednisolone		11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione
Prednisone		17α,21-dihydroxypregna-1,4-diene-3,11,20-trione
Pregnenolone		3β-hydroxypregn-5-en-20-one
Progesterone	(P)	pregn-4-ene-3,20-dione
Testosterone	(T)	17β-hydroxyandrost-4-en-3-one

TABLE II

CHROMATOGRAPHIC CONDITIONS FOR THE SEPARATION OF ADRENAL, TESTIS AND POLAR STEROIDS BY HPLC

	Adrenal steroids	*Testis steroids*	*Polar steroids*
Column	Zorbax-ODS	Zorbax-ODS	Zorbax-ODS
Solvent (start)	40% (v/v) methanol–water	32% (v/v) acetonitrile–water	20% (v/v) dioxane–water
Solvent (finish)	100% methanol	100% acetonitrile	100% dioxane
Gradient	$y = x^3$	$y = x^3$	$y = x^3$
Time, min	50	50	50
Temperature, °C	45	45	45
Pressure, p.s.i.	2500	2000	2500
Flow (start), ml/min	0.38	0.38	0.38
Flow (finish), ml/min	0.67	0.80	0.34

The precision of individual values, determined by repeated injections, was ±3% (coefficient of variation, CV). In practice, integration of peak areas resulted in a reduced precision and accuracy, particularly at low attenuations.

Culture methods

These have been described previously[2,13] for adrenal and testis cells. The present results were obtained with primary monolayer cultures of human adrenocortical and testis cells set up from tissues obtained at surgery or from hysterotomy foetuses, and containing 0.2–1.0 × 10^6 cells per culture. The culture medium, which contained 15% serum, was changed every 24 h and retained for steroid measurement by HPLC.

Sample preparation

Culture medium samples (2–5 ml) were extracted with 10 ml dichloromethane. This was washed with 3 ml 0.1 *N* NaOH solution to remove saponifiable lipidic materials which otherwise contributed several small UV-absorbing (max. <219 nm) peaks to the chromatograms. Dichloromethane extracts were evaporated under nitrogen and redissolved in 40% ethanol or primary solvent, and aliquots were injected into the chromatograph. Experiments showed recoveries of 85 ± 3% S.D. for relevant steroids using this method with internal recovery standards (tritiated steroids). Because the extraction procedure was so reproducible, internal recovery standards were not included routinely in the culture medium samples.

RESULTS

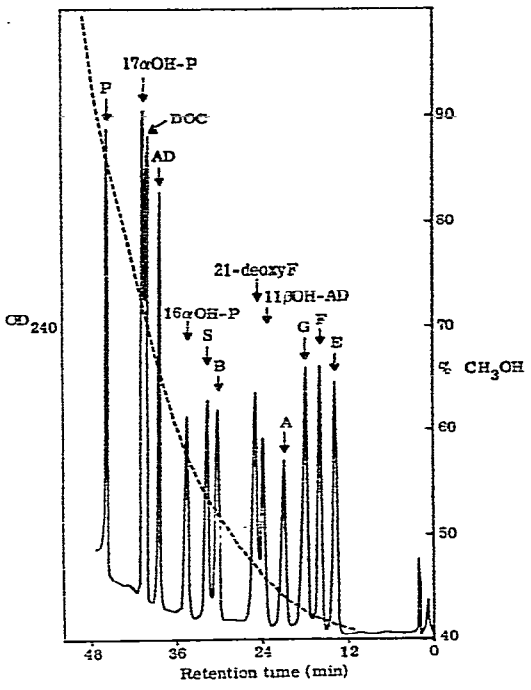
Adrenal steroids

Experiments with [^{3}H]pregnenolone as an exogenous precursor indicated that cultured adult human adrenocortical cells had the capacity to form six major unconjugated steroids: cortisol, deoxycortisol, corticosterone, deoxycorticosterone, androstenedione, and 11β-hydroxyandrostenedione. These metabolites were separated by TLC and identified by established procedures of chromatography, derivatisation, and recrystallisation to constant specific activity. However, this type of experiment cannot determine whether all these steroids are synthesised from endogenous precursors. Nor does it necessarily reflect the proportions in which they are secreted, because of potential differences in the metabolism of exogenous and endogenous precursors. Nevertheless, it does indicate a probable pattern of steroidogenesis and therefore a HPLC system was devised to separate primarily these steroids.

Fig. 1 shows a chromatogram of adrenal steroid standards separated by the methanol–water system described in Table II. Fig. 2 shows the separation of endogenous steroids secreted by an adult human adrenal culture. Peaks corresponding to the six steroids definitively identified as radiometabolites were obtained. A further peak was found with the retention time of authentic 16α-hydroxyprogesterone. This steroid had not been identified as a radiometabolite because of the limited resolution of the TLC system used. However, when sought, it was found and conclusively identified.

Forty-three naturally occurring and four synthetic steroids with adrenal activity were chromatographed with methanol–water and their retention times recorded (Table III). All steroids were eluted with band widths of 1–2 min. These data made it possible to rule out a large number of other steroids as significant products of the cultured normal cells. The reproducibility of the retention times varied between 1 and 4% (CV) provided that (i) primary solvent composition was closely controlled, and (ii) columns were re-equilibrated with primary solvent for 25 min before starting the gradient. By holding the gradient at 100% methanol for 10 min after each run, non-polar serum constituents present in the culture medium extracts (*e.g.*, cholesterol) were eluted from the columns.

The minimum detectable quantity of steroid in the least sensitive region of the gradient was 2 ng, measured as twice baseline noise. In practice, medium samples containing >5 ng, well below the levels encountered in most experiments, could be easily distinguished from the background.



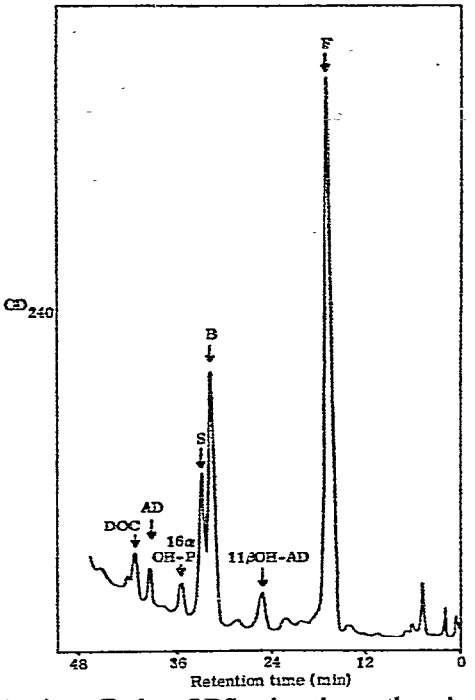


Fig. 1. Separation of adrenal steroid standards (210–440 ng) on Zorbax-ODS using the methanol–water gradient (— — —) described in Table II. Attenuation, 0.16 a.u.f.s. For abbreviations, see Table I.

Fig. 2. Chromatogram of endogenous steroids secreted by adult human adrenocortical cells in monolayer culture, separated using the methanol–water gradient. Attenuation, 0.16 a.u.f.s.

Testis steroids

Experiments with [^{3}H]pregnenolone showed that the major radioactive metabolites obtained from human testis cultures were testosterone, 17α-hydroxyprogesterone, progesterone, 20α-dihydroprogesterone, and 17α-hydroxy-20α-dihydroprogesterone, with smaller amounts of polar metabolites.

To resolve these compounds, it was necessary to use an acetonitrile–water gradient (Table II) because no variation of the methanol–water system used to separate adrenal steroids was capable of resolving all these steroids within a reasonable period of time (<1 h). Fig. 3 shows the separation of testis steroid standards with acetonitrile and Fig. 4 of the endogenous steroids secreted by a foetal human testis in monolayer culture, including a peak corresponding to 16α-hydroxyprogesterone in addition to the above-mentioned steroids.

The retention times of steroid standards using the acetonitrile–water gradient are given in Table III. In addition to separating testis culture steroids, this system gave enhanced separations between several steroids incompletely resolved using methanol–water (*e.g.*, testosterone/17α-hydroxy-20α-dihydroprogesterone and 17α-hydroxyprogesterone/DOC). Conversely, separations not effected by acetonitrile–water were obtained with methanol–water (*e.g.*, testosterone/androstenedione and deoxycortisol/11β-hydroxyandrostenedione). These two systems were therefore com-

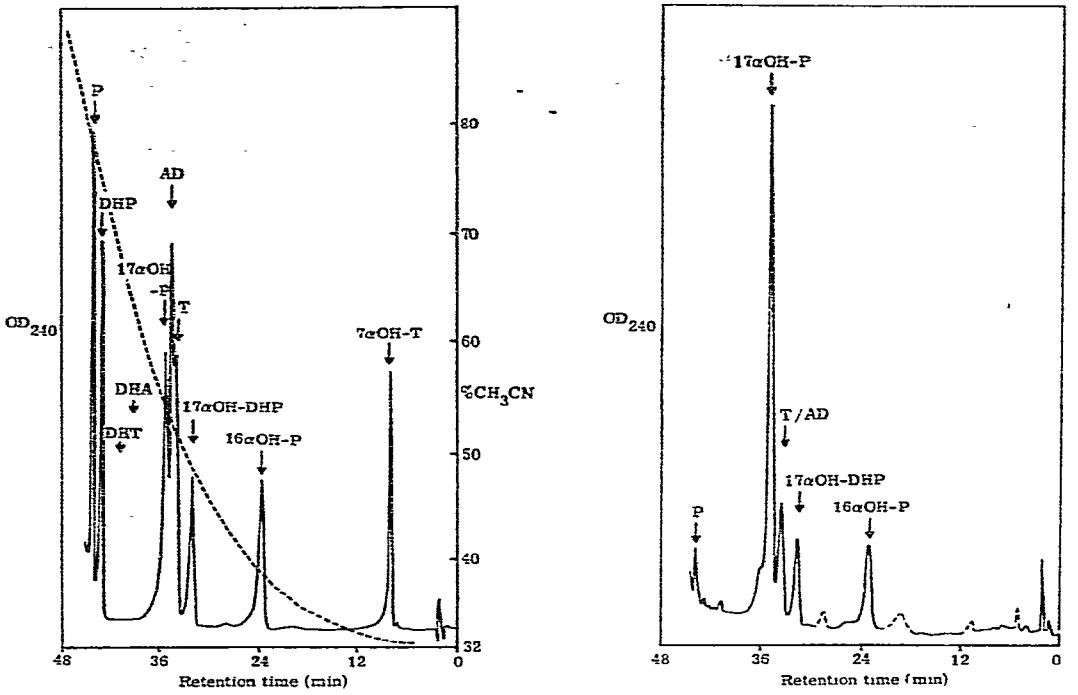


Fig. 3. Separation of testis steroid standards (330–750 ng) on Zorbax-ODS using the acetonitrile–water gradient (— — —) described in Table II. Attenuation, 0.32 a.u.f.s. For abbreviations, see Table I.

Fig. 4. Chromatogram of endogenous steroids secreted by foetal human testis cells in monolayer culture, separated using the acetonitrile–water gradient. Non-steroidal medium components are shown with broken lines. Attenuation, 0.16 a.u.f.s.

plementary. Most of the compounds tested could, with the exception of a group of polar steroids, be identified by the use of one or both systems.

Polar steroids

Using the systems described above, polar steroids such as aldosterone were not retained for sufficient time to obtain useful separations. These compounds are, however, an important group of physiologically active substances and a further system was devised to separate them.

A partial separation of this group was obtained by reducing the initial concentration of acetonitrile in the acetonitrile–water system to 19% (v/v), but aldosterone was inadequately resolved from cortisol. Methanol–water systems of increased polarity were less satisfactory, failing to resolve aldosterone and cortisone. The relative retention times of these steroids on Zorbax-ODS were, however, altered by using a dioxane–water mixture of appropriate strength (Table II) so that aldosterone was well separated from other UV-absorbing adrenal steroids. Fig. 5 shows the separation of polar adrenal steroid standards with dioxane–water and Fig. 6 of the endogenous steroids, including aldosterone, secreted by a cultured aldosteronoma (Conn's syndrome). In addition to separating these steroids, this system served equally well to resolve polar testis steroids (Table III).

TABLE III

RETENTION TIMES (MIN), MEASURED FROM INJECTION, FOR STEROID STANDARDS ON ZORBAX-ODS USING THE GRADIENT SYSTEMS DESCRIBED IN TABLE II

Steroid	*Methanol 40–100%*	*Acetonitrile 32–100%*	*Dioxane 20–100%*
6β-Hydroxycortisol	3	3.5	4
11-Dehydroaldosterone	8	5	15
17-Isoaldosterone	11	6	15
18-Hydroxy-11-dehydrocorticosterone	11.5	6.5	17.5
Prednisone	12	9	23
Aldosterone	13.5	8	19
Estriol	14	8	25
Cortisone	14	9	25.5
19-Hydroxyandrostenedione	15	11.5	20
7α-Hydroxytestosterone	15.5	9	19.5
18-Hydroxycorticosterone	15.5	7	21
Prednisolone	15.5	9	26.5
Cortisol	16	9	27
Adrenosterone	18	17	28
19-Hydroxytestosterone	19	10	24
16α-Hydroxytestosterone	21	11	27
11-Dehydrocorticosterone	21.5	15	29.5
Dexamethasone	24	13	35
11β-Hydroxyandrostenedione	24	17	32
21-Deoxycortisol	26.5	16	34.5
Corticosterone	31	18	36
11β-Hydroxytestosterone	31	15	33.5
18-Hydroxydeoxycorticosterone	31.5	16	34.5
11-Deoxycortisol	33	20	37.5
6α-Hydroxyprogesterone	34	24.5	38.5
16α-Hydroxyprogesterone	35	24	38
11-Ketoprogesterone	37	30	40
Estrone	37	30.5	42.5
11β-Hydroxy-20α-dihydroprogesterone	38	26	39
Androstenedione	39	36	42
6β-Hydroxyprogesterone	39	30	41
Estradiol	40	29	43
11β-Hydroxyprogesterone	41	33	43
11-Deoxycorticosterone	41	36	43.5
17α-Hydroxyprogesterone	41.5	38	45
Testosterone	42	35	43.5
17α-Hydroxy-20α-dihydroprogesterone	42.5	32	42
17α-Hydroxy-20β-dihydroprogesterone	43	32.5	43.5
Dehydroepiandrosterone	44	41	44
Androstenediol	45	38	—
17α-Hydroxypregnenolone	45.5	39	—
5α-Dihydrotestosterone	46.5	42	—
Progesterone	47	45	49
20α-Dihydroprogesterone	47.5	43.5	47.5
20β-Dihydroprogesterone	48.5	46	49.5
Pregnenolone	48.5	47	50
Cholesterol	54	>60	—

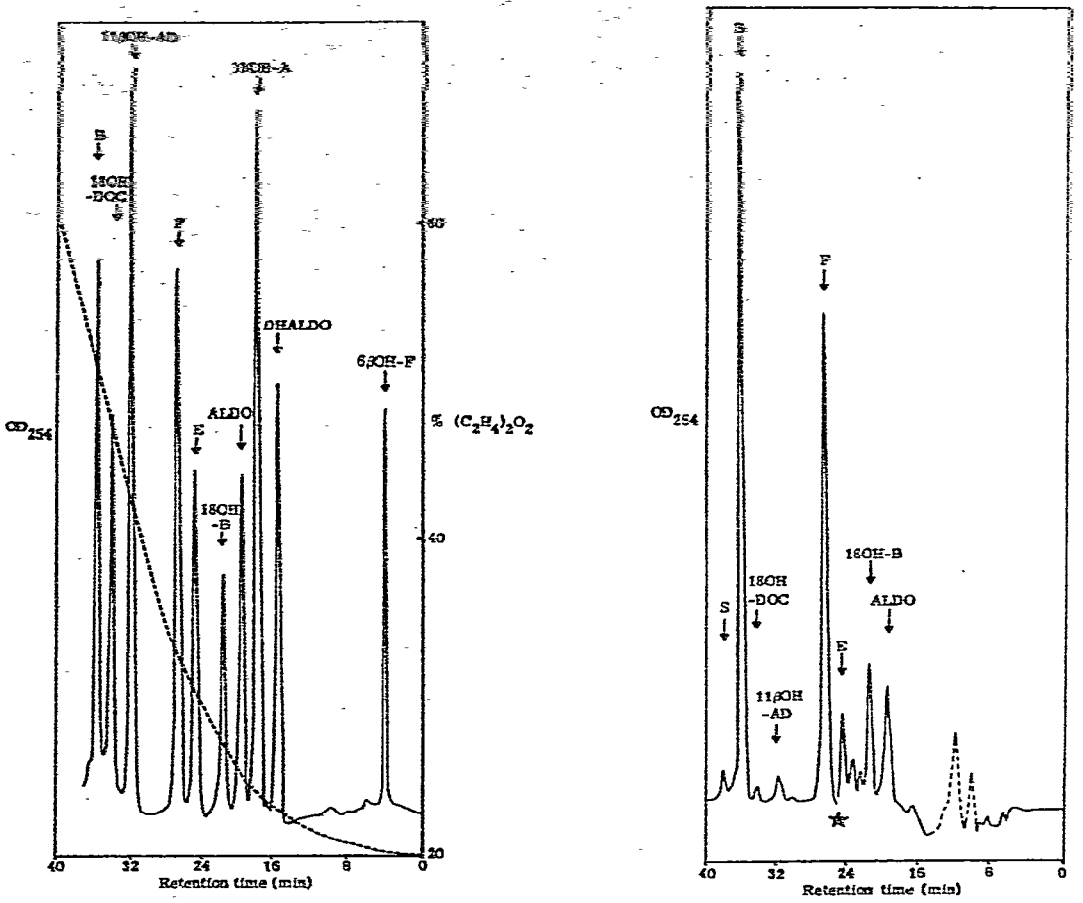


Fig. 5. Separation of polar adrenal steroid standards (250–750 ng) on Zorbax-ODS using the dioxane–water gradient (— — —) described in Table II. Attenuation, 0.16 a.u.f.s. For abbreviations, see Table I.

Fig. 6. Chromatogram of endogenous steroids secreted by an aldosteronoma (Conn's syndrome) in monolayer culture, separated using the dioxane–water gradient. Non-steroidal medium components are shown with broken lines. Attenuations, 0.04 [redacted] *0.64 a.u.f.s. (24–40 min).

DISCUSSION

In this paper we have described three HPLC systems using reversed-phase chromatography in conjunction with gradient elution to separate a wide variety of naturally occurring steroids. Using 25 cm Zorbax-ODS columns, it was not possible to separate all such steroids within a reasonable period of time (<1 h) using a single system. Separate systems were therefore developed to solve particular analytical problems associated with different types of biological sample, and they have been used to separate, identify, and measure the steroids secreted by cultured human adrenal and testis cells.

The use of different eluting solvents afforded a useful degree of selectivity. Thus, 20-hydroxy- and 20-ketosteroids were not completely resolved using methanol,

but were separated with acetonitrile (*e.g.*, progesterone/20α-dihydroprogesterone and 17α-hydroxyprogesterone/17α-hydroxy-20α-dihydroprogesterone). Conversely, the separation of 11-hydroxy- and 11-ketosteroids was enhanced by methanol as compared with acetonitrile (*e.g.*, F/E, B/A, 11β-hydroxyandrostenedione/adrenosterone, and prednisolone/prednisone). Other effects of these types and numerous examples of reversals in the order of retention were encountered (see Table III). These facilitated the identification of unknown compounds in samples. Differential selective effects of this type presumably stem from the large differences in the Hildebrand proton-accepting (δa) and proton-donating (δh) solubility parameters (methanol: $\delta a/\delta h$ = 7.5/7.5; acetonitrile: $\delta a/\delta h$ = 2.5/0)[12]. Dioxane ($\delta a/\delta h$ = 3/0) in general resembled acetonitrile but nevertheless afforded an improved separation of aldosterone and 18-hydroxylated steroids from other polar adrenal steroids.

The fact that a wide range of compounds can be separated with these HPLC systems makes them particularly suited to identifying steroids secreted by pathological tissues in which significant changes of function may have occurred. We have successfully examined the patterns of steroidogenesis by cultures of adrenal carcinomas and adenomas, congenitally hyperplastic adrenals, male pseudohermaphrodite testes (testicular feminisation syndrome), and a testicular interstitial cell tumour, and compared them with those of normal adult and foetal adrenals and testes. Results of these studies will be presented elsewhere.

Levels of steroid measured from these cultures were, in most instances, well above the limits of sensitivity (>2 ng for 4-en-3-one steroids). The plasma steroids in many of the conditions referred to above could, therefore, be examined using these systems, since the pattern rather than the overall amounts of individual steroids secreted can often be the most significant differential diagnostic feature. Although plasma steroids have been measured with HPLC[8–10], it is clear that the major advantage of the technique, simultaneous estimation of several compounds, has not yet been fully exploited.

In experiments with cultured adrenal and testis cells, steroids without the 4-en-3-one configuration (*e.g.*, ring A-reduced metabolites) were not formed in significant amounts from tritiated precursors. HPLC can, however, be used to separate non-UV-absorbing steroids as 2,4-dinitrophenylhydrazine derivatives[14,15] and measure them with high sensitivity (>1 ng). While 5-en-3-one steroids can be measured at 200 nm (>150 ng) and oestrogens at 280 nm (>50 ng), the reduced sensitivity of direct spectrophotometric detection renders it, in general, unsuitable for the measurement of these steroids in biological samples. In the absence of a universal steroid detector of high sensitivity, there remains the use of radioimmunoassay in conjunction with the resolving power of HPLC to measure, with enhanced specificity, those steroids that fall below the present detection limits.

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