REVERSED- AND NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 18-HYDROXYLATED STEROIDS AND THEIR DERIVATIVES

COMPARISON OF SELECTIVITY, EFFICIENCY AND RECOVERY FROM BIOLOGICAL SAMPLES

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SUMMARY

The chromatographic behaviour of adrenal 18-hydroxysteroids, and a series of derivatives thereof, has been studied in reversed-phase and normal-phase high-performance liquid chromatography (HPLC). Both 18-hydroxycorticosterone and 18-hydroxy-11-deoxycorticosterone exhibited a marked loss of chromatographic efficiency when separated on incompletely-covered C_{18} reversed-phase packings with methanol–water gradient elution. Maximum-coverage reversed-phase supports (as determined by methyl red adsorption) showed no such effect, nor was it seen when aprotic solvents, such as dioxane, were used. This phenomenon is unique among the wide range of adrenal and testicular steroids that we have studied and affords a useful test, applicable under aqueous conditions, of coverage by the alkylsilane reversed phase, a factor of considerable importance in the successful resolution of complex mixtures of adrenal steroids by reversed-phase HPLC. The retention times of the 20-methoxy, 20-ethoxy, 21-acetoxy, etiolactone, 11β, 18-ether and dimeric derivatives of the naturally-occurring 18-hydroxysteroids have been determined in relation to major adrenal steroids. Procedures for extraction and reversed-phase HPLC of 18-hydroxysteroids from tissues without the formation of these less polar forms, which can complicate their separation by other chromatographic techniques, are illustrated with respect to a human malignant adrenocortical tumour which caused hypermineralocorticism.

INTRODUCTION

The C_{21} 18-hydroxylated steroids (*e.g.*, 18-hydroxycorticosterone, 18-hydroxy-11-deoxycorticosterone and 18-hydroxyprogesterone) are naturally-occurring compounds of adrenal origin, some of which possess weak mineralocorticoid activity[1], which they share with their more potent biosynthetic relative aldosterone. Such

steroids may be specifically elevated in certain disorders, but their biological and pathophysiological role remains, as yet, unclear[1]. Methods for their successful separation and measurement are, therefore, of interest. These compounds are relatively labile in comparison with other steroid hormones, and may form a variety of less polar derivatives, including dimers, etiolactones and 20-alkoxy (mixed ketal) compounds and, in some cases, $11\beta,18$-ethers[2-7] (Fig. 1). This potential instability of 18-hydroxy-steroids can complicate their chromatographic separation by conventional methods and their subsequent measurement by radioimmunoassay[7].
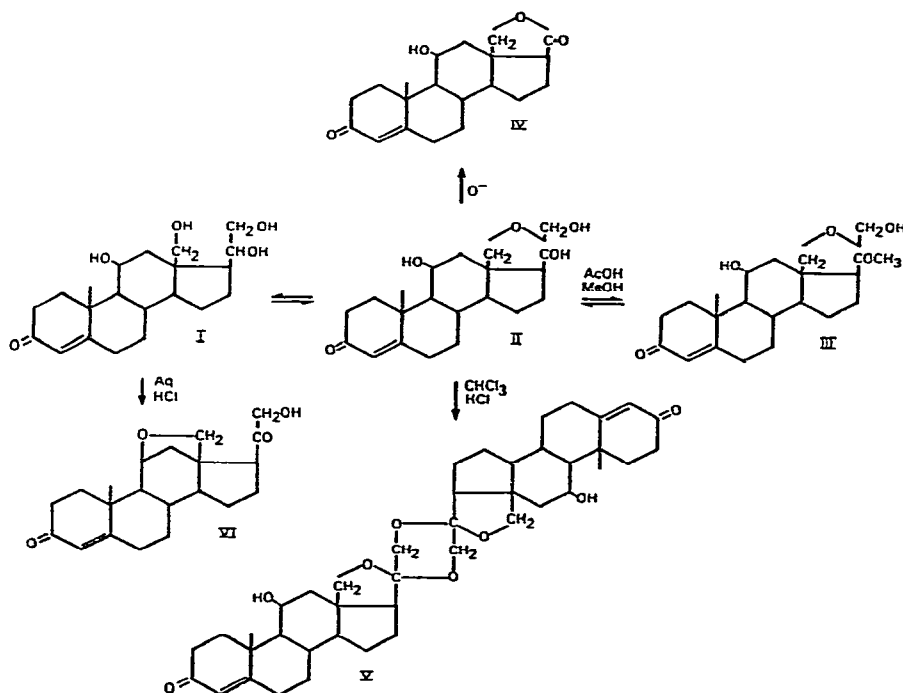

Fig. 1. 18-Hydroxysteroid interconversions. Solvent and acid-mediated reactions are illustrated in respect of 18-hydroxycorticosterone (I) and its tautomeric cyclic hemiketal (II), which can form 20-alkoxysteroids (III), an etiolactone (IV), a dimer (V) and an $11\beta,18$-ether (VI).

In order to separate, measure and identify the complete range of physiologically important UV-absorbing (4-en-3-one) adrenal and testicular steroid hormones secreted by *in vitro* preparations of human endocrine cells we developed, several years ago, gradient-elution high-performance liquid chromatographic (HPLC) systems based on a then novel spherical porous microparticulate (5 μm) alkylsilane-bonded silica[8]. We have successfully used these reversed-phase (RP) systems to monitor steroid secretion by cells in tissue culture, and have also applied them to the analysis of steroids in solid tissue samples[9]. These studies have revealed that to achieve the separation of complex natural mixtures of steroid hormones requires as precise a choice of RP packing from amongst the wide range now available[10] as of the specific solvent system used[8]. By exploiting both packing and solvent selective effects virtually all desired steroid separations can, in our experience, be obtained with such RP systems.

Nevertheless, two recent publications[11,12] have both opted for a normal-phase system based on the hydrophilic LiChrosorb DIOL (Merck, Darmstadt, G.F.R.) packing for separating some steroid hormones. In these cases, however, it was reported that 18-hydroxylated steroids could either not be chromatographed at all in microgram amounts[11], or were not eluted in their original form[12], clearly serious limitations if such systems are to be applied to biological samples of adrenal origin. In the light of these observations we have, therefore, examined the chromatographic behaviour of a wide range of 18-hydroxylated steroids (and their derivatives) on both these normal-phase HPLC systems and RP-systems of the type we have used extensively in other studies[8]. Previous studies of such steroids by HPLC have been limited to only a few compounds[8,9,13] and have not included their derivatives.

MATERIALS AND METHODS

Steroid standards were obtained from the following sources: 18-hydroxy-11-dehydrocorticosterone (18OH-A), 18-hydroxycorticosterone (18OH-B), 18-hydroxy-11-deoxycorticosterone (18OH-DOC), 18-hydroxy-11-deoxycorticosterone-1,2-diene ($\Delta^{1,2}$-18OH-DOC), 18-hydroxytestosterone (18OH-T), 18-hydroxyprogesterone (18OH-P), 18-hydroxycorticosterone dimer (di 18OH-B) and 18-hydroxy-11-deoxycorticosterone dimer (di 18OH-DOC) from the Medical Research Council steroid reference collection by courtesy of Professor D. Kirk (systematic names are given in Table I). Nuclear magnetic resonance spectra[14] indicate that the dimeric compounds used in this study are probably the 20,21:20′,21′ symmetrical "anhydro" dimers (see Fig. 1). Aldosterone and other non-18-hydroxylated compounds were from Steraloids (Pawling, NY, U.S.A.) and Sigma (Poole, Great Britain).

20-Methoxy and 20-ethoxy derivatives of these steroids were prepared, where appropriate, by treatment in methanol or ethanol containing 0.02 M acetic acid at 37°C for 18 h[7]. The 21-acetoxy compounds were synthesised by treatment with acetic anhydride–pyridine (4:1) at 60°C for 30 min, to monitor the possible formation of acetylated compounds during the preparation of 20-alkoxy derivatives; 18-hydroxysteroid diacetates are not formed under these conditions.

11β,18-Ethers were prepared by incubation of steroids in 10^{-2} M HCl (ref. 6). Etiolactones were formed by periodic acid oxidation according to Tait et al.[15] and the 20β-reduced derivatives of the 18-hydroxysteroids by incubation with 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (Sigma) and NADH.

18-Hydroxy-[1,2-^{3}H]corticosterone (sp.act. 44 Ci/mmol) and 18-hydroxy-11-deoxy-[1,2-^{3}H]corticosterone (sp.act. 51 Ci/mmol) were obtained from The Radiochemical Centre (Amersham, Great Britain) and their purity checked by HPLC before use. Radiolabelled 16α,18-dihydroxy-11-deoxycorticosterone (16α,18-diOH-DOC) was prepared by incubation of [^{3}H]18OH-DOC with human adrenal tissue minces, according to the procedure described by Dale and Melby[16], except that foetal rather than adult tissue was used.

RP-HPLC of the steroids was carried out on 150 × 4.6 mm I.D. stainless-steel columns, using a Spectra-Physics SP-8000 chromatograph programmed for an exponential concave binary gradient (NL2), or an Altex Model 322 chromatograph programmed for an equivalent gradient. The solvent systems used for RP-HPLC were 40–100% methanol in water, 32–100% acetonitrile in water and 20–100% dioxane in

TABLE I

18-HYDROXYSTEROID NOMENCLATURE

Number	Abbreviation	Systematic name
1	16α,18diOH-DOC	16α,18,21-Trihydroxypregn-4-ene-3,20-dione
2	20β-dihydro 18OH-A	18,20β,21-Trihydroxypregn-4-ene-3,11-dione
3	18OH-A	18,21-Dihydroxypregn-4-ene-3,11,20-trione
4	18OH-B (II)*	11β,18,21-Trihydroxypregn-4-ene-3,20-dione-20,18-hemiacetal
5	Δ¹·²-18OH-DOC	18,21-Dihydroxypregna-1,4-diene-3,20-dione-20,18-hemiacetal
6	18OH-B 11β,18 ether (VI)	21-Hydroxy-11β,18-epoxy-4-pregnene-3,20-dione
7	20β-dihydro 18OH-B	11β,18,20β,21-Tetrahydroxypregn-4-en-3-one
8	18OH-B Me (III)	20-Methoxy-11β-hydroxy-18,20-epoxy-4-pregnen-3-one
9	18OH-B lactone (IV)	11β-Hydroxyandrost-4-en-3-one-17β,18-carbolactone
10	18OH-DOC	18,21-Dihydroxypregn-4-ene-3,20-dione-20,18-hemiacetal
11	18OH-B Et	20-Ethoxy-11β-hydroxy-18,20-epoxy-4-pregnen-3-one
12	18OH-A Me**	20-Methoxy-18,20-epoxy-4-pregnen-3,11-dione
13	18OH-T	17β,18-Dihydroxyandrost-4-en-3-one
14	20β-dihydro 18OH-DOC	18,20β,21-Trihydroxypregn-4-en-3-one
15	18OH-DOC lactone	3-Oxo-4-androstene-17β,18-carbolactone
16	18OH-B Ac	21-Acetoxy-11β,18-dihydroxypregn-4-ene-3,20-dione
17	18OH-DOC Me	20-Methoxy-18,20-epoxy-4-pregnen-3-one
18	18OH-DOC Ac	21-Acetoxy-18-hydroxypregn-4-ene-3,20-dione
19	18OH-B Me Ac	21-Acetoxy-20-methoxy-11β-hydroxy-18,20-epoxy-4-pregnen-3-one
20	18OH-DOC Et	20-Ethoxy-18,20-epoxy-4-pregnen-3-one
21	18OH-DOC Me Ac	21-Acetoxy-20-methoxy-18,20-epoxy-4-pregnen-3-one
22	18OH-P Me	20-Methoxy-18,20-epoxy-4-pregnen-3-one
23	18OH-P	18-Hydroxypregn-4-ene-3,20-dione
24	di 18OH-B (V)***	20,21:20',21'-Anhydrodi(11β,18,21-trihydroxypregn-4-ene-3,20-dione)
25	di 18OH-DOC	20,21:20',21'-Anhydrodi(11β,18-dihydroxypregn-4-ene-3,20-dione)

* See Fig. 1 for structures.
** The 20-ethylate of 18OH-A was not formed under the conditions used here for preparation of 20-alkoxy-18-hydroxysteroids (see Materials and methods), and nor were 20-propylates of 18OH-B or 18OH-DOC.
*** Probable structure.

water gradients. These were developed previously for the separation of, respectively, adrenal, testicular and polar steroid hormones of natural origin[8]. The Altex system was used for the dioxane gradients. Solvents were HPLC-grade from Rathburn Chemicals (Walkerburn, Great Britain), except chloroform (AnalaR; BDH, Poole, Great Britain), which was washed with water to remove ethanol stabiliser immediately prior to use. Water used for chromatography was doubly glass-distilled. RP-HPLC was carried out under conditions optimised for steroid hormones, i.e. at 45°C and a constant flow-rate of 1 ml/min[8,9]. Eluted compounds were detected with a Schoeffel 770 or Spectromonitor III (Laboratory Data Control) variable-wavelength spectrophotometer at 240 or 254 nm (dioxane gradients).

The following 5–7 μm C_{18}-alkylsilane–bonded silica packings were tested: Ultrasphere-ODS (5 μm; Altex Scientific, Berkeley, CA, U.S.A.), ODS-Hypersil

(5 μm; Shandon, Runcorn, Great Britain), Zorbax ODS (7 μm; DuPont, Hitchin, Great Britain) and Spherisorb S5 ODS (5 μm; Phase Separations, Queensferry, Great Britain) (Table II). Residual accessible silanol groups were compared by spectrophotometric measurement of methyl red adsorbed from carefully dried toluene[8]. This test, like several others, is not without its drawbacks[17], but it does afford a rapid means of screening bulk RP packings and ranking them objectively.

TABLE II

DETAILS OF CHEMICALLY-BONDED MONOLAYER PACKINGS AND COLUMNS USED

PP = prepacked by manufacturer; LP = laboratory-packed; S = spherical; I = irregular; A = dimethyloctadecylchlorosilane; B = octadecyltrichlorosilane; C = not divulged by manufacturer but probably glycidoxypropyltrimethoxysilane (hydrolysed) (ref. 17) or related compound; D = trimethylchlorosilane; E = trimethylsilylimidazole; ND = not divulged by manufacturer; NA = not applicable.

Packing	Length (cm)	I.D. (mm)	Type	Efficiency (plates/m × 10^{-3})*	Particle size (μm)	Pore diameter (nm)	Pore volume (ml/g)	Specific surface area (m²/g)	Shape	Derivatisation reagent	Capping reagent	% C loading	Methyl red adsorption***
Ultrasphere-ODS	15	4.6	PP	90	4–6	8	0.57	180	S	A	D	12	<2
ODS-Hypersil	15	4.6	LP	60	5–7	10	0.7	200	S	B	E	10	20
Zorbax ODS	15	4.6	LP	56	7–9	9	ND	370	S	A	—	16	252
Spherisorb S5 ODS	15	4.6	LP	50	3–7	8	0.57	220	S	ND	D**	8	512
LiChrosorb DIOL	25	4.6	LP	30	4–7	10	ND	250	I	C	—	4.5	NA

* Efficiencies of RP packings determined using progesterone ($k' \approx 25$) eluted isocratically with aqueous methanol. Efficiency of DIOL column determined using cortisone ($k' \approx 4$) with n-hexane–isopropanol (4:1).
** Partially capped to constant carbon loading.
*** μg methyl red adsorbed from dry toluene (0.7 mg/ml)/100 mg packing.

Normal-phase chromatography of the 18-hydroxylated steroids was carried out isocratically at ambient temperature, using a 250 × 4.6 mm I.D. column of LiChrosorb DIOL (5 μm; Merck) with the n-hexane–isopropanol solvent system of Schöneshöfer and Dulce[11] and the water-saturated chloroform–isopropanol system reported by Cavina et al.[12]. Standards were dissolved in the primary solvent for injection with the chloroform-based system; in all other systems, including RP-HPLC, they were injected in 1–10 μl of ethanol. All 18-hydroxysteroids were freshly dissolved.

All columns tested were, with the exception of Ultrasphere-ODS, packed in the laboratory at 5000 p.s.i. from a methanol slurry using a Magnus P5000 pump.

RESULTS

Experiments in other laboratories[2-7], in which "conventional" methods of steroid chromatography (e.g., silica gel thin layer, paper, Sephadex LH-20) were

used, have demonstrated several potential transformations of C_{21} 18-hydroxylated steroids such as 18OH-B (I, Fig. 1) to less polar forms. Thus 20-alkoxyl (mixed ketal) derivatives (III) and dimeric compounds (V) can be formed, probably from the thermodynamically more stable cyclic hemiketal form (II) in which these steroids primarily exist in solution[1,3]. These transformations may take place inadvertently in the course of the isolation and separation of the steroids and on long-term storage in solution. The solvents used for this purpose (e.g. ethanol, methanol, acetone and chloroform) appear to play a key role in the process, particularly if they contain traces of organic acidic impurities[4,5,7]. Mineral acids, on the other hand, promote hydrolysis of the 20-alkoxyl compounds[4], although they may result in the formation of other products, including 11β,18-ether-linked forms (VI) of the 11β-hydroxylated steroids, such as 18OH-B[5,6]. The 18-hydroxysteroids are also susceptible to oxidation. We have, for example, noted the facile decomposition of 18OH-B into a product with the mobility of its γ-lactone (IV) when it was applied in trace amounts to glycol-impregnated glass-fibre (ITLC) sheets prior to chromatography[18].

In the present HPLC study we have prepared a variety of these potential 18-hydroxysteroid transformation products. Their retention times and chromatographic behaviour have been established, using both the methanol–water and dioxane–water gradient-elution systems that we have used extensively for RP-HPLC of adrenal steroids in biological samples[8,9]. A range of different RP-packings of the C_{18} alkylsilane-bonded type have also been evaluated in respect of their suitability for high-efficiency 18-hydroxysteroid HPLC and compared with normal-phase systems described by other groups[11,12]. Finally, the behaviour of endogenous 18-hydroxysteroids in biological samples has been studied with the aid of appropriate radiolabelled steroids.

RP-HPLC of 18-hydroxylated steroids and their derivatives

Effects of different C_{18} alkylsilane-bonded RP packings. Our attention was first drawn to the anomalous behaviour of certain 18-hydroxysteroids in RP-HPLC during the course of a study of the different patterns of selectivity of a wide range of alkylsilane-bonded RP packings towards a large number of UV-absorbing adrenal and testicular steroids[9]. In contrast to all the other steroids that we tested, including aldosterone, 18OH-B and 18OH-DOC exhibited a substantially lower chromatographic efficiency when they were separated on RP packings which contained a significant number of residual accessible silanol groups, as determined by methyl red adsorption (Table II). This effect manifested itself, however, only when a methanol–water elution system was used (Fig. 2).

One possible explanation was the on-column formation of less polar derivatives of the 18-hydroxysteroids. Both the leading and trailing edges of the broad peaks of 18OH-B and 18OH-DOC on the uncapped RP-supports were, therefore, separately trapped. They were rechromatographed, without evaporation to dryness, using the same solvent system but with a maximum-coverage RP packing, on which broadening of the 18-hydroxysteroid peaks does not occur (Fig. 2). Both zones of the broad peaks of both 18OH-B and 18OH-DOC from the low-coverage packings showed two distinct peaks on rechromatography, an early eluted compound, corresponding to the original steroid, and a late-eluted component ($< 50\%$), which had the retention time of the synthesised 20-methoxy derivative (see Figs. 3 and 4). Rechromatography of 18-
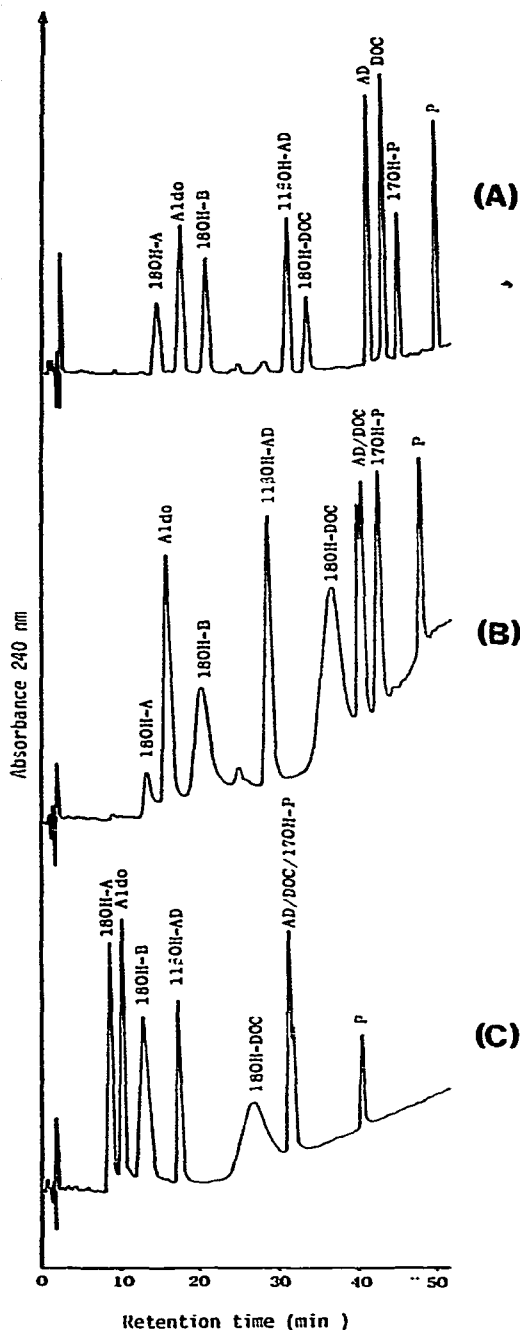
Fig. 2. RP-HPLC of adrenal standards using (A) Ultrasphere-ODS, (B) Zorbax ODS and (C) Spherisorb S5 ODS. Details of columns and packings are given in Table I. All separations were carried out with the same exponential concave gradient of 40–100% methanol in water (illustrated in Fig. 3), at a constant flow-rate of 1 ml/min and 45°C. Note the marked loss of efficiency with some 18-hydroxysteroids on the incompletely covered C_{18} packings. Compounds tested were 18-hydroxy-11-deoxycorticosterone (18OH-A), aldosterone (Aldo), 18-hydroxycorticosterone (18OH-B), 11β-hydroxyandrostenedione (11βOH-AD), 18-hydroxy-11-deoxycorticosterone (18OH-DOC), androstene-3,17-dione (AD), 11-deoxycorticosterone (DOC), 17α-hydroxyprogesterone (17OH-P) and progesterone (P). Note also differing steroid selectivities of these RP packings.

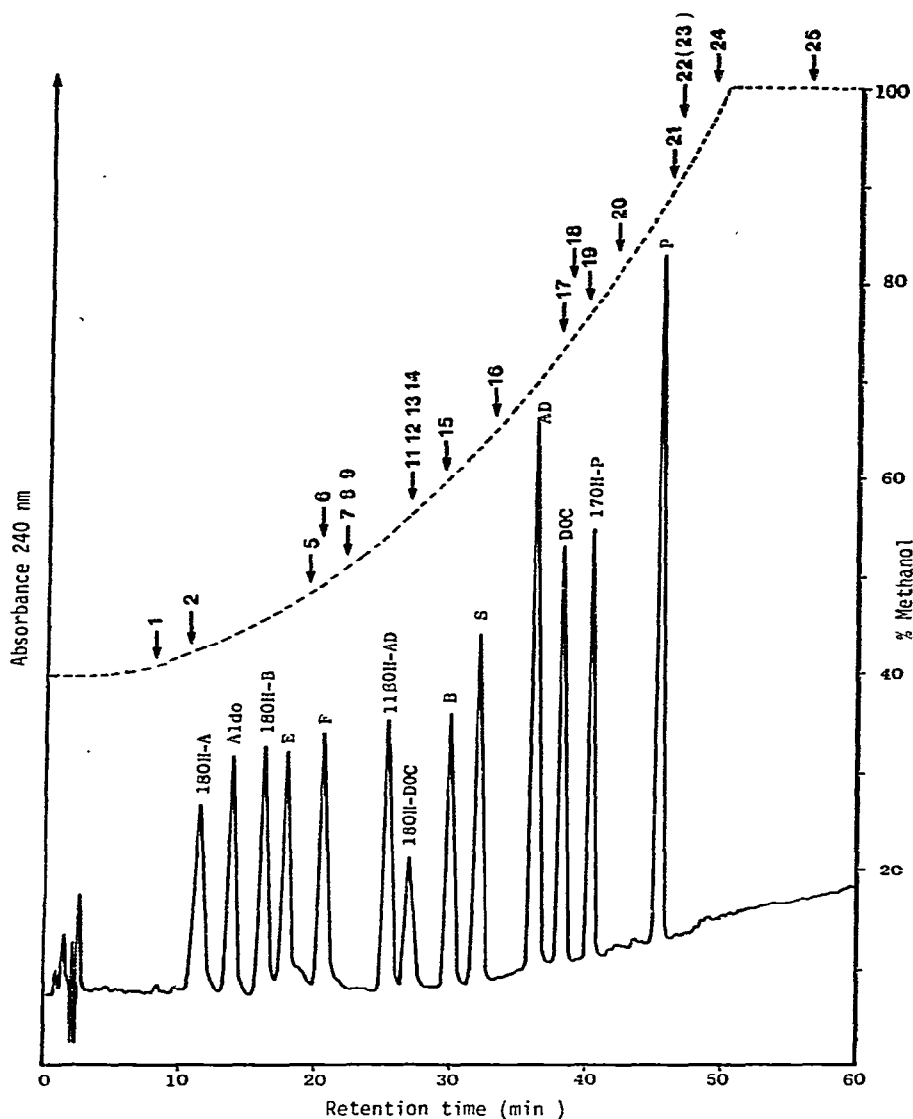Fig. 3. Retention times of 18-hydroxysteroid derivatives (arrows) on a 150 × 4.6 mm I.D. ODS-Hypersil column, with methanol–water gradient elution (broken line). Other chromatographic conditions as in Fig. 2. Positions of the numbered derivatives (see Table I for key) are shown in relation to the separation of a mixture of major adrenal steroid standards. In addition to the compounds named in Fig. 1, the latter include cortisone (E), cortisol (F), corticosterone (B) and 11-deoxycortisol (S).

hydroxysteroid peaks trapped from high-coverage packings in methanol–water showed little (< 5%) or no 20-methylate, demonstrating that the nature of the packing plays a key role in generating these derivatives. However, as a similar peak broadening was noted when 18OH-B and 18OH-DOC were chromatographed on a bonded hydrophilic normal-phase packing with a completely different solvent system in which no
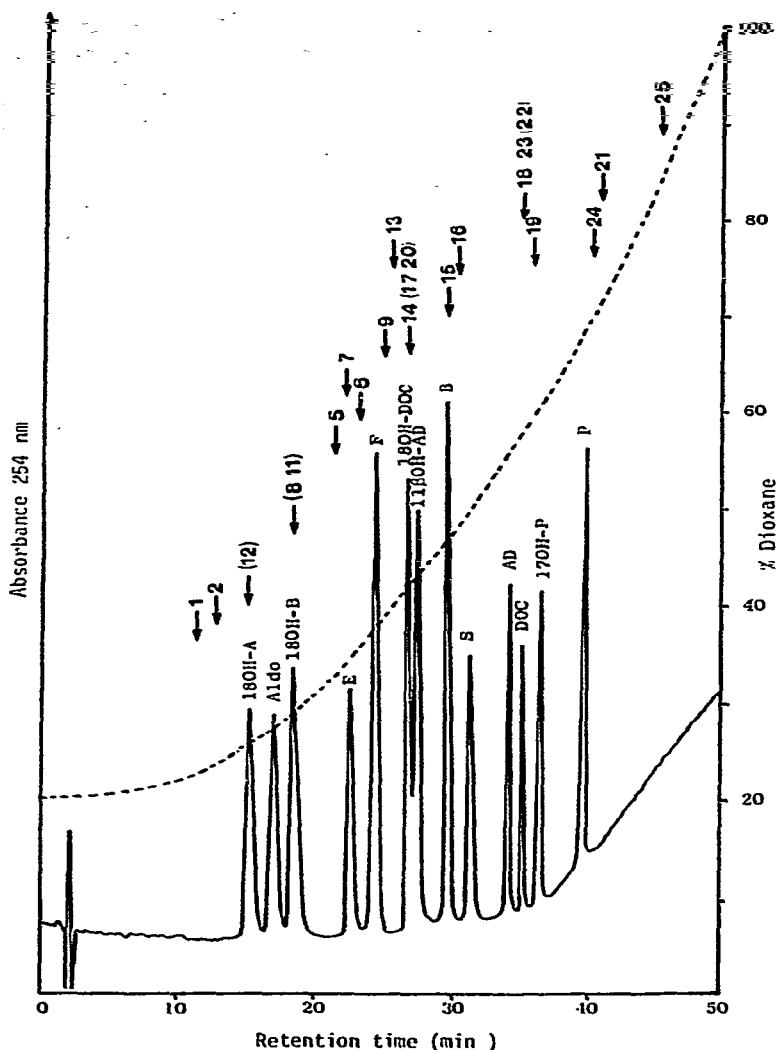
Fig. 4. Retention times of 18-hydroxysteroid derivatives (arrows) on a 150 × 4.6 mm ODS-Hypersil column, with dioxane–water gradient elution (broken line). Other chromatographic conditions as in Fig. 2. Enhanced separation of polar steroids is achieved with this system, but 20-methylated and ethylated 18-hydroxysteroids do not separate from their parent compounds (see text).

derivatives were formed (see below), on-column derivatisation to less polar forms does not seem to be the sole explanation of this phenomenon.

A specific interaction of some of the native 18-hydroxysteroids with active residual adsorptive sites on HPLC packings, therefore, also seems to contribute significantly to the observed loss of efficiency. This interpretation was confirmed by the observation that the addition of neutral salt (NaCl, 0.9%, w/v), acid (H_3PO_4, 0.01%, v/v) or base (triethylamine, 0.1 M) to the eluent substantially improved 18-hydroxy-steroid efficiencies on the low-coverage RP packings. The triethylamine also substan-

tially improved the selectivity of the low-coverage columns in respect of the steroids illustrated in Fig. 2. Although this organic base does not attack the silica matrix of RP packings as fast as inorganic bases, it does result in significant dissolution over a period of 100–200 h[19] and it does not, therefore, afford a permanent solution to the problem of different steroid selectivities with different RP supports.

Among the other 18-hydroxysteroids tested, this loss of efficiency when methanol–water gradients without ionic additives were used was noted only with $\Delta^{1,2}$-18OH-DOC. It was not observed with 18OH-DOC, 18OH-T, or any of the 20-methylated, 21-acetylated, lactonised or dimeric forms of 18OH-B or 18OH-DOC. These all chromatographed with high efficiency in methanol–water on both low- and high-coverage RP packings.

18OH-P, however, did not conform to either pattern. It could not be chromatographed efficiently on any RP support using methanol–water, even on maximum-coverage packings, such as Ultrasphere-ODS and ODS-Hypersil. In all cases it gave a very broad peak (5–10 min), eluted after progesterone. This may be related to an extremely facile solvent-mediated derivatisation that has been noted on the part of this steroid giving its 20-methoxy form (Fig. 3).

Effects of different solvent systems. "Maximum-coverage"-type RP packings, such as ODS-Hypersil and Ultrasphere-ODS, are evidently necessary both for high-efficiency 18-hydroxysteroid chromatography and for the best separation of other major adrenal products, when using methanol–water gradient elution (Fig. 2). For polar adrenal steroids, however, the dioxane–water system offers a pattern of selectivity which facilitates certain separations[8,9]. In order to be able to monitor the potential formation of 18-hydroxysteroid derivatives in biological samples we have, therefore, established the retention times with both systems of all 18-hydroxysteroids and of their derivatives likely to be formed in the course of extraction and chromatography. ODS-Hypersil columns were used for this study, and the results are illustrated in Figs. 3 and 4, from which it is apparent that many of these steroids can be separated effectively both from each other and from major non-18-hydroxylated adrenal steroids using these RP systems. Of particular note are the very late retention times of the dimers of 18OH-B and 18OH-DOC. Attempts to prepare the $11\beta,18$-ether of 18OH-B (VI) according to the procedure of Aragones *et al.*[5] with aqueous HCl yielded only a small quantity (10–15%) of a less polar derivative, together with an even smaller amount of a compound with the retention time of the etiolactone, from which it was clearly separated. The major derivative has, therefore, been named as the $11\beta,18$-ether in Figs. 3 and 4, although its identity must be regarded as provisional. All other derivatives were successfully prepared in high yield by the procedures described in Materials and methods.

The most significant observation, in the context of the present study, was the fact that none of the 20-methylated or 20-ethylated derivatives of the 18-hydroxy-steroids could be separated from their parent compounds on dioxane–water gradients, although they all gave distinct, later, retention times with methanol–water. After trapping from dioxane–water and rechromatography with methanol–water, it was evident that these derivatives had, in fact, been completely converted to products with the retention times of their non-alkylated forms during HPLC with the former solvent system. The only exception was the 18OH-B methylate which still showed traces of the 20-methoxy form after trapping from dioxane and rechromatography with methanol–

water. Even in this case, however, the greater part was recovered as the parent steroid. A similar reversion to non-derivatised forms was noted when the methylated and ethylated 18-hydroxysteroids were chromatographed with acetonitrile–water. In marked contrast, all etiolactones, 20β-reduced steroids, dimers and 21-acetates, including acetates of the 20-methoxy-18-hydroxysteroids, were stable to rechromatography with dioxane or acetonitrile. 18OH-P gave a high-efficiency peak on the dioxane–water system, eluted much earlier than progesterone, totally at variance with its behaviour with methanol–water.

Normal-phase chromatography of 18-hydroxylated steroids

We were able to chromatograph successfully both 18OH-B and 18OH-DOC, in amounts ranging from 30 μg–30 ng, and to recover them in an undegraded, underivatised form from a 5 μm LiChrosorb DIOL column, using the n-hexane–isopropanol (4:1) solvent systems of Schöneshöfer and Dulce[11], provided that the column had been previously exposed to salt-containing eluents and biopolymers (*e.g.* próteins). With n-hexane–isopropanol this type of "used" column gave good chromatography of all 18-hydroxysteroids, including 18OH-B and 18OH-DOC (Fig. 5). When a freshly-packed column was substituted, however, both the latter steroids gave very broad peaks (> 10 min) that virtually precluded their detection by UV-absorbance, even in microgram amounts, although radiolabelled steroids revealed their greatly extended profile. Addition of triethylamine (0.1 M) to the eluting solvent significantly improved the 18-hydroxysteroid peak shapes, indicating that adsorptive sites were probably responsible for the loss of efficiency.

A study of other 18-hydroxysteroids using the normal-phase n-hexane–isopropanol system revealed that those which gave broad peaks on the uncapped RP supports (*e.g.*, 18OH-B, 18OH-DOC and $\Delta^{1,2}$-18OH-DOC) also failed to chromatograph efficiently on fresh LiChrosorb DIOL columns in the absence of ionic additives. Conversely, those which chromatographed efficiently on the low-coverage RP packing (*e.g.*, 18OH-A, 18OH-T, and the methylated, acetylated and dimeric forms of 18OH-B and 18OH-DOC) all gave high-efficiency peaks on the untreated hydrophilic support (Fig. 5).

We did not experience the same difficulty as Cavina *et al.*[12] in recovering undegraded 18OH-DOC from DIOL columns when using a water-saturated chloroform–isopropanol (99:1) eluent[12]. This steroid could be chromatographed, trapped and rechromatographed without the formation of the less polar derivative reported by these workers, without special precautions other than the use of freshly prepared solvents. The solvents rather than the packing are, in fact, probably responsible for the results of Cavina *et al.*[12]. Ethanol-free chloroform rapidly becomes acidic, and such acid chloroform is known to promote the formation of dimeric 18-hydroxysteroids[14]. Both new and "used" DIOL columns can, however, be employed with water-saturated chloroform systems without loss of efficiency; the water therein presumably deactivates the residual adsorptive sites on this type of packing.

Chromatography of 18-hydroxysteroids in biological samples

The preparation of biological samples for HPLC also involves the use of various solvents which might react with 18-hydroxysteroids. To check the compatibility of the procedures we have used routinely for steroid extraction[8,9], we have
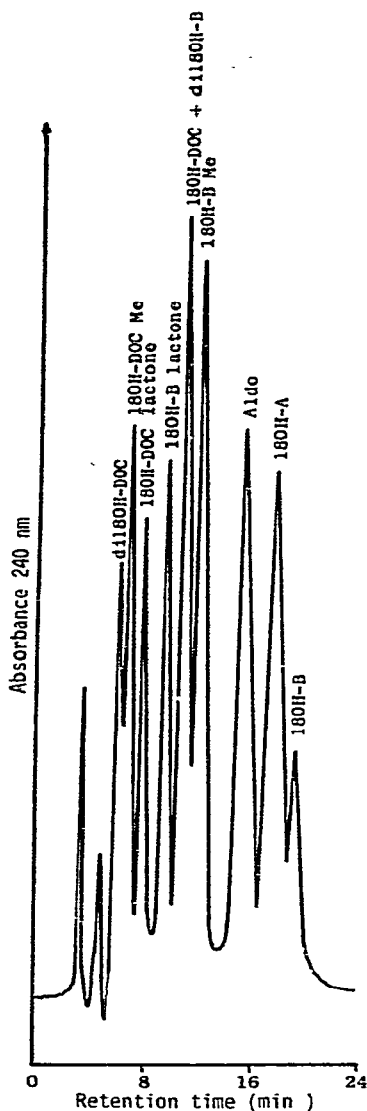
Fig. 5. Normal-phase chromatography of 18-hydroxysteroids and their derivatives (Table I) on a 250 × 4.6 mm I.D. LiChrosorb DIOL column with *n*-hexane–isopropanol (4:1) at 1 ml/min and ambient temperature. The column used had been exposed previously to proteins.

monitored the behaviour of trace amounts of both [³H]18OH-B and [³H]18OH-DOC added to tissue from a malignant human adrenocortical tumour which caused hyper-mineralocorticism and which contained such steroids.

Solid tumour tissue (< 0.2 g) was extracted with 8 ml ethanol, after homogenization and addition of the radiolabelled steroids (1 μCi). The supernatant after centrifugation was delipidated by diluting it with water to 80% (v/v) ethanol and pumping it through a Partisil-10 ODS column. Details of this procedure have been
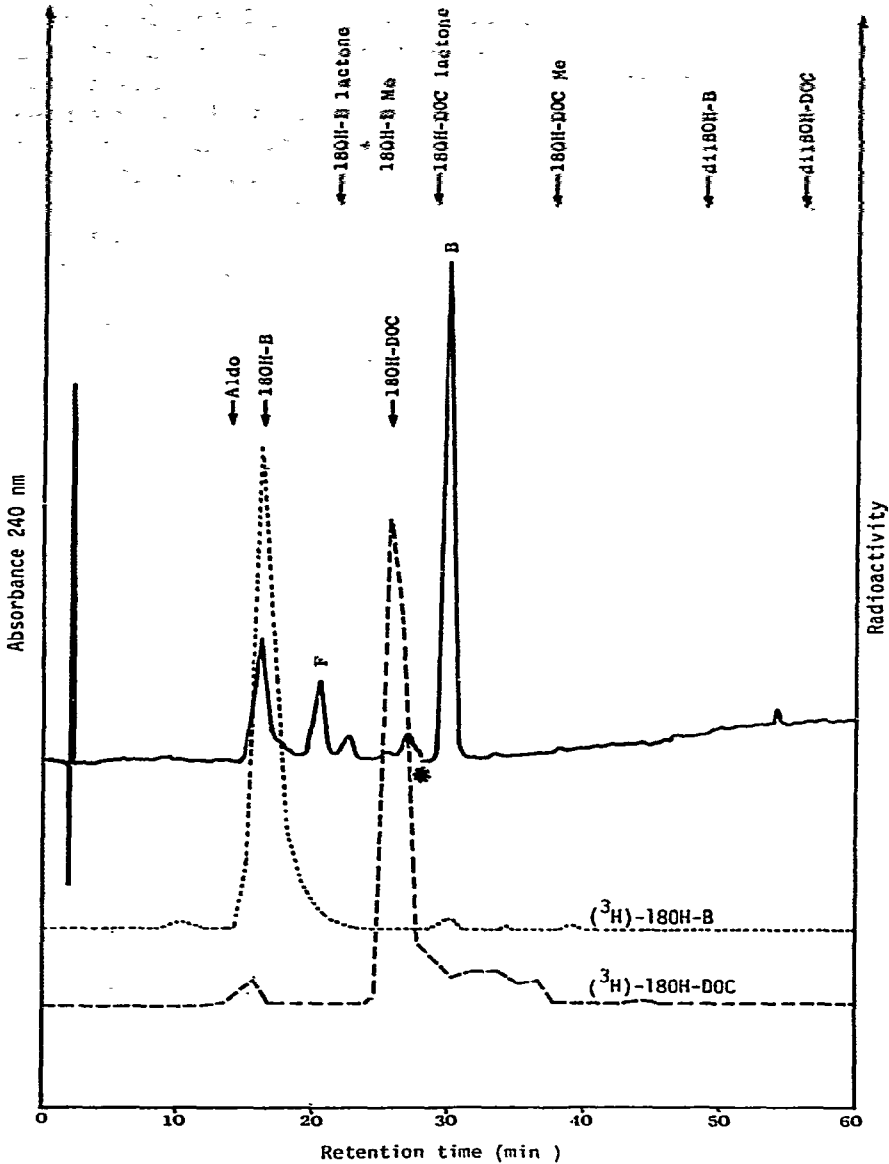
Fig. 6. HPLC of UV-absorbing steroids (solid trace), secreted *in vitro* by a malignant human adrenal tumour causing hypermineralocorticism, separated on a 150 × 4.6 mm I.D. ODS-Hypersil column with methanol–water gradient elution, as illustrated in Fig. 2. Major compounds include 18-hydroxy-corticosterone (18OH-B) and corticosterone (B), with lesser amounts of cortisol (F) and negligible levels of aldosterone (Aldo). At the point marked by the asterisk the attenuation was increased from 0.05 to 0.2 a.u.f.s. Trace amounts of both [³H]18OH-B and [³H]18OH-DOC (<10 ng) were added to tumour samples prior to their extraction with dichloromethane; the radioactivity in eluent aliquots (broken lines) indicated no significant formation of 18-hydroxysteroid derivatives (arrows). Identical scans were obtained from samples extracted with ethyl acetate.

given elsewhere[9]. The ethanol in the eluate was evaporated and the steroids were extracted from the aqueous residue with dichloromethane, which was in turn evaporated under nitrogen and the dry residue was taken up in microvolumes (25–50 μl) of ethanol for injection. HPLC scans of steroids extracted by this method from benign tumours causing hypermineralocorticism have been illustrated in another publication[9]. The samples from the malignant tumour studied here were chromatographed on ODS-Hypersil using both methanol–water and dioxane–water gradients. In neither case was formation of potential 18-hydroxysteroid derivatives (e.g. ethylates or lactones) detected, in spite of their exposure to low-coverage packings (Partisil-10 ODS) during delipidation.

Freshly disaggregated cell suspensions, prepared from the same tumour, were incubated in tissue culture medium with 10% serum and extracted, separately, with dichloromethane and ethyl acetate (5 volumes). The latter solvent was chosen for efficient extraction of expected polar steroids. Solvent extracts were evaporated to dryness in glass vessels under nitrogen and redissolved in ethanol for injection. The resultant scans of both endogenous UV-absorbing steroids and the radiolabelled steroids in eluent aliquots are illustrated in Fig. 6. No formation of lactones, dimers, ethylates or acetates was noted (see Figs. 3 and 4 for retention times). The endogenous steroids, including 18OH-B, which was formed in large amounts by this tumour, gave single high-efficiency peaks with both methanol–water and dioxane–water gradients, when maximum-coverage RP packings were used.

DISCUSSION

The chromatographic separation of naturally-occurring 18-hydroxysteroids poses special problems. These stem, in part, from the tendency of these compounds to form certain derivatives, some stable, some labile, in the presence of various types of solvent. This now well documented phenomenon[1–7] was first noted for 18OH-B by Raman et al.[20] and for 18OH-DOC by Dominguez[21], although the conversion of 18-hydroxyprogesterone to less polar forms during chromatography had been observed even earlier by Wettstein[22]. The molecular transformations involved have been partially clarified recently[1–7] (Fig. 1). This has permitted the preparation of some of these compounds and the examination of their behaviour in several HPLC systems, a technique which has thus far seen only limited application to the 18-hydroxysteroids[8,9,13].

By careful choice of chromatographic conditions, in which a maximum-coverage-type RP packing and/or the dioxane–water solvent system was used, it was possible to chromatograph all 18-hydroxysteroids as single peaks with efficiencies comparable to those of other less labile steroids, in spite of their tendency to undergo solvent-mediated derivatisation. The relative peak broadening of the 18OH-B and 18OH-DOC with methanol–water elution appeared to provide a very sensitive index of the stationary phase coverage, and we have, in fact, used this as a test to evaluate different alkylsilane-bonded RP packings. 18-Hydroxysteroids in the biological samples studies were, furthermore, stable during both extraction and chromatography. Using these techniques, we conclude, therefore, that RP-HPLC may be better suited to the analysis of these steroids than other chromatographic techniques. Although the proton-donating nature of methanol, as compared with dioxane or acetonitrile[8],
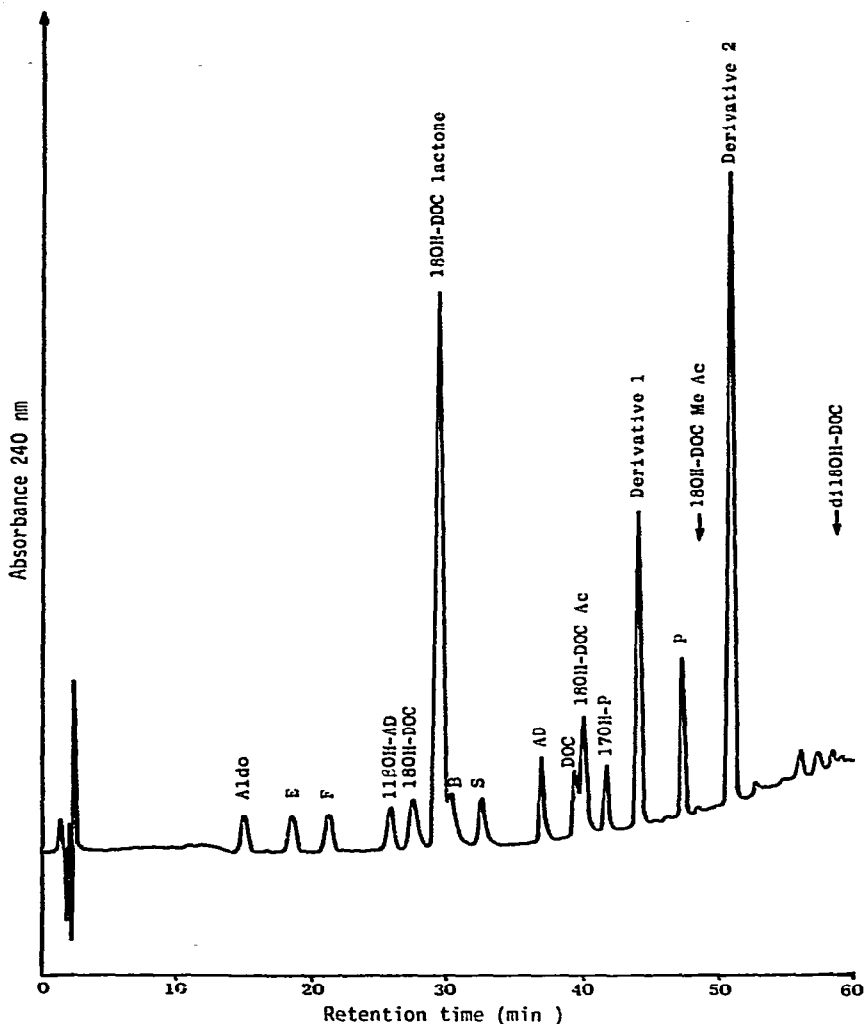
Fig. 7. RP-HPLC of UV-absorbing derivatives formed from 18-hydroxy-11-deoxycorticosterone (18OH-DOC) treated with chloroform–acetic acid (7:3) at ambient temperature for 18 h. This solvent mixture is reported as causing cyclisation and/or dimerisation of 18-hydroxysteroids when used for TLC (System 417; The Radiochemical Center, Amersham, Great Britain). Although peaks corresponding to the γ-lactone (18OH-DOC lactone) and 21-acetate (18OH-DOC Ac) are evident, the retention times of two major derivatives (1 and 2) did not correspond with either the 21-acetoxy-20-methoxy derivative (18OH-DOC Me Ac) or the 20; 21:20′, 21′ dimer of 18OH-DOC (di 18OH-DOC), and they were not formed when the γ-lactone or dimer were treated with chloroform–acetic acid. Steroids were separated on 150 × 4.6 mm I.D. ODS-Hypersil columns with methanol–water gradient elution as in Fig. 2. The non-18-hydroxysteroids in this chromatogram are components of a steroid standard mixture added to the sample for internal standardization (abbreviations used are given in legends to Figs. 2 and 3).

renders it generally unsuited to 18-hydroxysteroid HPLC, except under carefully controlled conditions, it must be used when isolation of the labile 20-alkoxysteroids is desired.

These efforts to develop HPLC methods suited to the 18-hydroxysteroids epitomise the search for the most effective means of separating all steroid hormones in tissue samples. On the basis of considerable experimentation[9] we remain convinced that RP-HPLC, in which both packing[10] and solvent selective effects[8] are exploited, offers the best method, at least in so far as UV-absorbing adrenal (and testicular) hormones are concerned. The major practical disadvantage of this method —that of selecting an RP packing with an appropriate pattern of selectivity from the wide range now available— can be obviated by the simple methyl red adsorption test and by testing the chromatographic behaviour of the 18-hydroxysteroids. The presence of residual accessible silanol groups does not, however, seem to be the only factor in-influencing the detailed steroid selectivity of these supports. The extent of coverage by the C_{18} phase, compared with the short alkyl chain capping compound, may also play a part, as the latter has its own intrinsic steroid selectivity[9].

LiChrosorb DIOL, a hydrophilic bonded normal-phase packing, is an alternative that has also been recommended for steroid hormone HPLC[11], and it may serve as a useful adjunct in achieving specific separations[12]. This packing has, however, several potential disadvantages; thus it requires "conditioning" with biological samples or masking of active residual adsorptive sites with ionic additives before high-efficiency 18-hydroxysteroid chromatography can be achieved. There is, furthermore, no simple method of determining the extent of bonded hydrophilic phase coverage prior to use, and the long-term stability of this type of support must be to some extent suspect, lacking, as it does, a hydrophobic stationary phase to protect the silica matrix from the eluents.

The systems for 18-hydroxysteroid separation that we have described should also offer a powerful method for the further exploration of solvent-induced transformations of these compounds. Thus, it would appear that the pathways illustrated in Fig. 1 do not necessarily represent the entire spectrum of reaction products associated with this class of hormone. We have noted the production of several additional derivatives in solutions of 18-hydroxysteroids exposed to acid conditions, in addition to products with the retention times of the symmetrical anhydro-dimers, 20-alkoxy-steroids, acetate and etiolactone standards, described in the present report (Fig. 7). RP-HPLC should, therefore, prove of value in separating and isolating such as yet unidentified transformation products prior to their characterisation by other methods.

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