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COMPARISON OF DIFFERENT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS FOR THE PURIFICATION OF ADRENAL AND GONADAL STEROIDS PRIOR TO IMMUNOASSAY

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SUMMARY

The high-performance liquid chromatography of nineteen hormonal steroids with special respect to its suitability for routine purification of these steroids from crude, organic extracts of biological fluids prior to final quantitation by immunoassay has been studied. In all systems the gradient elution technique was applied. Separation of steroids has been investigated using different stationary phases chemically coated with non-polar, hydroxyl, NO_2 and CN groups. Reproducibility of retention times was studied on a stationary phase coated with hydroxyl groups (DIOL column) using different organic eluents. Coefficients of variation range from 0.76 to 8.16%. Reproducibility was shown to be unequivocally better in the gradient part than in the isocratic part of the chromatographic run. In contrast to the other steroids, 18-hydroxylated steroids were more or less unstable in certain systems studied. As to resolution and reproducibility, the DIOL column run with an *n*-hexane—dioxane gradient has been shown to be superior to the other systems studied.

INTRODUCTION

The direct and specific measurement of hormonal steroids in biological fluids by immunoassay only is feasible if the steroid occurs in relatively high amounts, such as cortisol in serum [1, 2], or if highly specific antisera are available [3]. However, for most steroid determinations — especially for those of urinary free steroids — these prerequisites are not valid and purification steps are necessary prior to the final immunological quantitation [4] if the estimation is to be specific. Similarly, if several steroids have to be determined simultaneously in one sample, a separation of these steroids is obligatory [5—7]. Numerous methods of purification or separation including nearly all known techniques of chromatography have been reported in the literature hitherto. All these procedures, however, are rather time-consuming and scarcely fit to be

TABLE I

THE SYSTEMS OF COLUMN PACKINGS AND SOLVENTS EXAMINED IN THE PRESENT STUDY

System	Trademark of column	Origin	Chemically bonded group	Mean diameter of particles (μm)	Internal diameter of column (mm)	Components of solvents	
						A	B
1	RP-18	Hewlett-Packard, Böblingen, G.F.R.	Octadecylsilyl	10	4	Water	Methanol
2	DIOL	Merck	OH	10	3	*n*-Hexane	*n*-Hexane–isopropanol (70:30)
3	DIOL	Merck	OH	10	3	*n*-Hexane	*n*-Hexane–dioxane (50:50)
4	DIOL	Merck	OH	10	3	*n*-Hexane	CH_2Cl_2
5	DIOL	Merck	OH	5	4	*n*-Hexane	*n*-Hexane–isopropanol (70:30)
6	Nucleosil NO_2	Chrompack, Middelburg, The Netherlands	NO_2	5	4.6	*n*-Hexane	*n*-Hexane–ethanol (50:50)
7	Nucleosil CN	Chrompack	CN	5	4.6	*n*-Hexane	*n*-Hexane–isopropanol (70:30)

automated for use in the routine laboratory. The rapid development in the field of high-performance liquid chromatography (HPLC) seems to establish this technique as a version of chromatography suitable for complete automation [8]. Separation of steroids by HPLC meanwhile has been documented by several authors using isocratic systems [9—12] or using gradient elution with a non-polar reversed-phase system [13].

The purpose of the present study was to examine the separation of nineteen physiologically important steroids by HPLC using different polar bonded phases and different eluents with special respect to the suitability of these systems for a practicable purification of the steroids prior to immunoassay.

MATERIALS AND METHODS

A Hewlett Packard high-performance liquid chromatograph (Model 1084A), equipped with a fixed UV detector at 254 nm, two solvent and two pump systems, a variable volume (10—250 μl) injector, and a plot/print terminal was used throughout the study. All operating parameters of the chromatograph, such as flow-rate, temperature of solvents and column room, composition of the solvents as well as print or plot specifications, were regulated and controlled by microprocessors. In addition, changes of these parameters during a run, for example changing the solvent composition during gradient elution, were time programmable.

The eluents were fractionated using a time-regulated sample collector (LKB, Ultrorac®, Type 7000). ^{3}H radioactivity was measured in a liquid scintillation spectrometer (Packard Instruments, Model 2480) using Biofluor® (New England Nuclear) as scintillation cocktail.

Packing material

Stationary phases chemically bonded to silica gel as supporting material were examined exclusively in this study. The length of all columns was 25 cm. Trade names, origins of the various columns, chemically bonded groups, mean particle diameters, internal diameter of columns and the various eluents are listed in Table I.

Chemicals

n-Hexane, propan-2-ol, dioxane and dichloromethane were of p.a. grade and purchased from Merck (Darmstadt, G.F.R.). Solvents were degassed under vacuum immediately before use. Water was deionized and glass-distilled. Extrelut® for extraction of steroids from serum or urine samples was purchased from Merck. Radioactive steroids were from New England Nuclear (Dreieichenhain, Frankfurt/Main, G.F.R.) and non-radioactive steroids from Steraloids (Pawling, N.J., U.S.A.) except 18-OH-deoxycorticosterone and 18-OH-corticosterone, which were from Makor Chemicals Ltd. (Jerusalem, Israel). The purity of steroids absorbing at 254 nm was checked by thin-layer chromatography (system: chloroform—methanol, 9:1, v/v). The trivial names and abbreviations used throughout are listed in Table II.

TABLE II

TRIVIAL NAMES AND ABBREVIATIONS

Trivial name	Abbreviation	Peak number in figures	Chemical name
Progesterone	P	(1)	Pregn-4-ene-3,20-dione
Androstenedione	AD	(2)	Androst-4-ene-3,17-dione
Pregnenolone	PL	(3)	3β-Hydroxypregn-5-en-20-one
5α-Dihydrotestosterone	α-DHT	(4)	17β-Hydroxy-5α-androstan-3-one
Dehydroepiandrosterone	DHEA	(5)	3β-Hydroxyandrost-5-en-17-one
Deoxycorticosterone	DOC	(6)	21-Hydroxypregn-4-ene-3,20-dione
Testosterone	T	(7)	17β-Hydroxyandrost-4-en-3-one
Estrone	E_1	(8)	3-Hydroxyestra-1,3,5(10)-trien-17-one
17-OH-Progesterone	17-OH P	(9)	17α-Hydroxypregn-4-ene-3,20-dione
17-OH-Pregnenolone	17-OHPL	(10)	17α, 3β-Dihydroxypregn-5-en-20-one
Estradiol	E_2	(11)	Estra-1,3,5(10)-triene-3,17β-diol
11-Deoxycortisol	S	(12)	21,17α-Dihydroxypregn-4-ene-3,20-dione
18-OH-Deoxycorticosterone	18-OH-DOC	(13)	18,21-Dihydroxypregn-4-ene-3,20-dione
Corticosterone	B	(14)	11β,21-Dihydroxypregn-4-ene-3,20-dione
Cortisone	E	(15)	17α,21-Dihydroxypregn-4-ene-3,11,20-trione
Aldosterone	Aldo	(16)	11β,21-Dihydroxy-18-al-pregn-4-ene-3,20-dione
Estriol	E_3	(17)	Estra-1,3,5(10)-triene-3,16α,17β-triol
Cortisol	F	(18)	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione
18-OH-Corticosterone	18-OH-B	(19)	11β,18,21-Trihydroxypregn-4-ene-3,20-dione

Chromatographic procedure

Appropriate amounts of ethanolic solutions of steroids were evaporated to dryness in a stream of nitrogen. The residues were redissolved in the eluents exhibiting the initial composition of the gradient elution patterns, thus yielding concentrations of 500 ng of each steroid per 100 μl of eluent. In all systems studied, 100 μl of the steroid mixture were transferred to glass microvials and injected with the variable-volume injector. If not otherwise stated the temperature of the column was 30°. UV-absorbing steroids were detected at 254 nm. In all chromatograms described, attenuation was 64×10^{-4} a.u./cm. Radioactive steroids were located by liquid scintillation counting of 0.5-ml eluate fractions. Gradients were run linearly with intermittent isocratic periods. In each system studied, gradient profiles were varied until the best separation of the steroid compounds in the corresponding system was achieved. The final gradient was held at 100% of solvent B in order to eliminate polar components in the case of serum or urine extracts.

Extraction procedure

Samples of urine or serum were extracted using a solid phase technique [14].

Procedure 1. Two millilitres of urine diluted 1 to 20 with water were pipetted onto 1.2 g of Extrelut and pre-extracted with 20 ml of carbon tetrachloride followed by 20 ml of dichloromethane.

Procedure 2. Two millilitres of urine or serum diluted with one part of water were extracted correspondingly with 20 ml of ether.

The organic extracts were evaporated to dryness and redissolved in the eluent for chromatography.

RESULTS

Separation on different stationary phases

In Fig. 1 the chromatogram of eleven steroids absorbing at 254 nm on a reversed-phase system (see system 1 in Table I) is shown. The band widths of all steroids range from 0.6 ml for P to 1.3 ml for F. Peaks are broader at the end than at the beginning of an isocratic part of the gradient profile. While a distinct resolution between progesterone derivatives differing in one hydroxyl group is obvious, adequate separation of isomeric steroids is scarcely achieved; for example, separation of the double-hydroxylated progesterone derivatives S, 18-OH-DOC and B. The retention time is longer for steroids containing an hydroxyl group than for the analogous steroid with a C=O group (see 18-OH-B and Aldo or AD and T).

The chromatogram of adrenal and gonadal steroids on a stationary phase chemically coated with hydroxyl groups (DIOL column) is shown in Fig. 2 (system 2 in Table I). Steroids without absorbance at 254 nm were localized by radiofractionation. The elution pattern of steroids is nearly inverse to that in system 1. Band widths of absorbing steroids are comparable to those measured in system 1.

Similarly, separation of isomeric steroids is moderate (S and 18-OH-DOC or F and 18-OH-B). However, this system enables the distinct resolution of at least nine individual steroids, while the other steroids may be separated in terms of steroid groups.

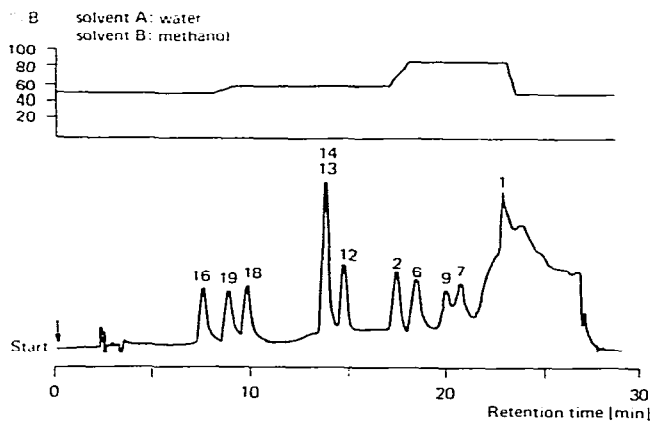

Fig. 1. Separation of adrenal steroids on a reversed-phase system using a water—methanol gradient (system 1 in Table I) and UV detection. For numbers of peaks, see Table II.

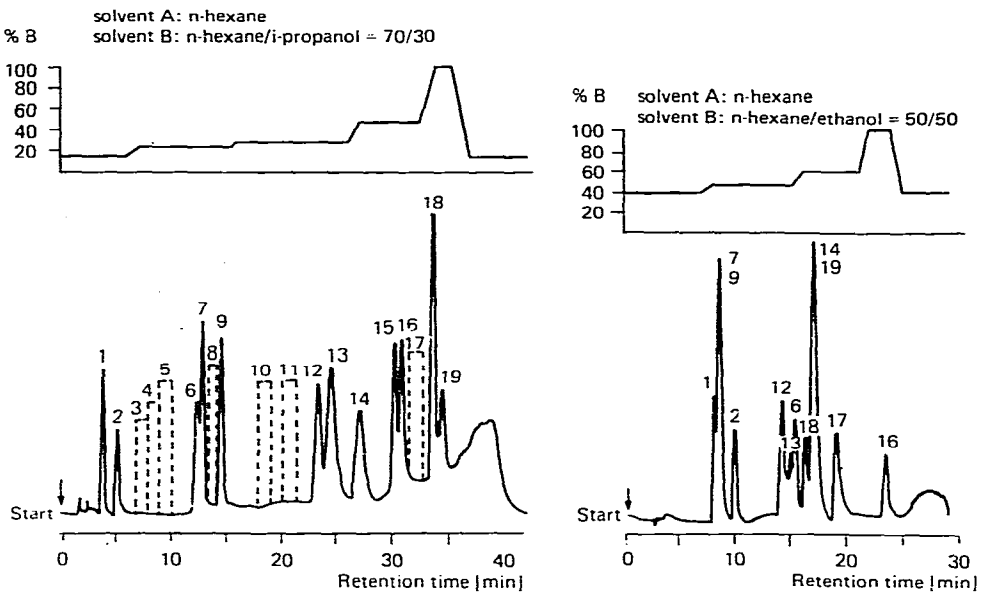

Fig. 2. Separation of adrenal and gonadal steroids on a DIOL column using an *n*-hexane—isopropanol gradient (system 2 in Table I). Steroids were detected by UV absorption or radioactivity.

Fig. 3. Separation of adrenal steroids on an NO_2 column using an *n*-hexane—methanol gradient (system 6 in Table I) and UV detection.

The elution pattern of steroids chromatographed on a stationary phase chemically coated with NO_2 groups (system 6 in Table I) is shown in Fig. 3. In this system, ethanol was used as polar component of the gradient mixture. In contrast to systems 1 and 2, resolution of the steroids differing in hydroxyl groups was rather impaired (P and 17-OH-P, DOC and 18-OH-DOC, B and 18-OH-B). However, a stronger affinity and selectivity of this polar phase to steroids differing in CO groups is apparent if the retention times of the steroids T and AD, as well as of E and Aldo, are considered.

In Fig. 4 the chromatogram of the steroids on a polar stationary phase coated with CN groups (system 7 in Table I) is demonstrated. Adequate resolution of steroids is nearly absent in this system. If comparing the retention times of 18-OH-DOC and DOC, or those of 18-OH-B and B, this system seems to operate — at least partially — like the reversed-phase system (system 1).

Influence of temperature and diameter of particles on separation

The influence of column temperature on the resolution of steroids is shown by the chromatogram in Fig. 5. The system is identical to system 2 except that the temperature of the column room was raised to 40°. By comparing the elution profiles of Figs. 2 and 5, it becomes evident that resolution of isomeric steroids is significantly impaired by increasing the temperature.

Fig. 6 shows a chromatogram of the DIOL column using particles of 5 μm diameter instead of 10 μm (system 5 in Table I). Band widths range from 0.6 to 1 ml. Resolution of the individual steroids is significantly superior to that

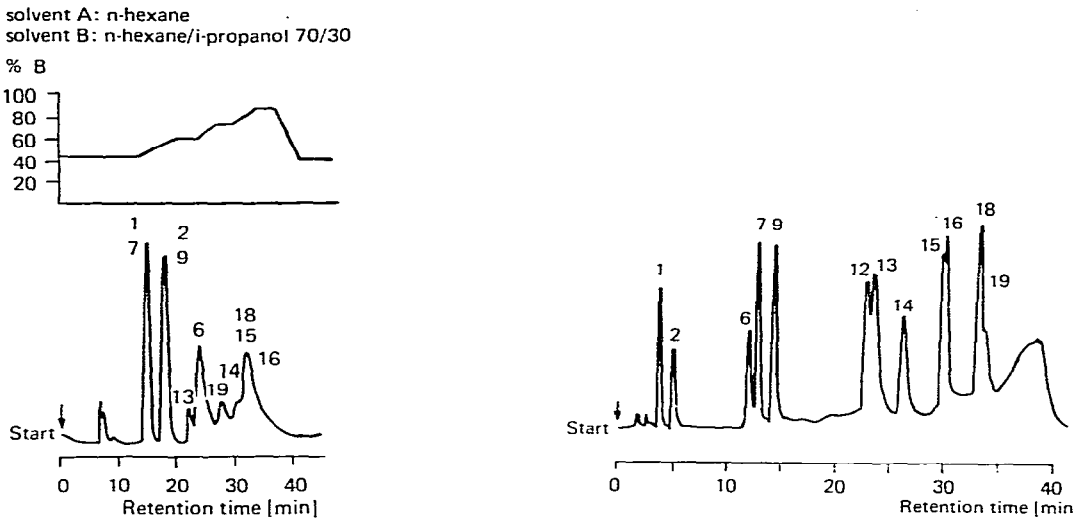

Fig. 4. Separation of adrenal steroids on a CN column using an *n*-hexane—isopropanol gradient (system 7 in Table I) and UV detection.

Fig. 5. Separation of adrenal steroids on a DIOL column using an *n*-hexane—isopropanol gradient (system 2 in Table I) and UV detection. Temperature of column was 40° instead of 30° (see Fig. 2).

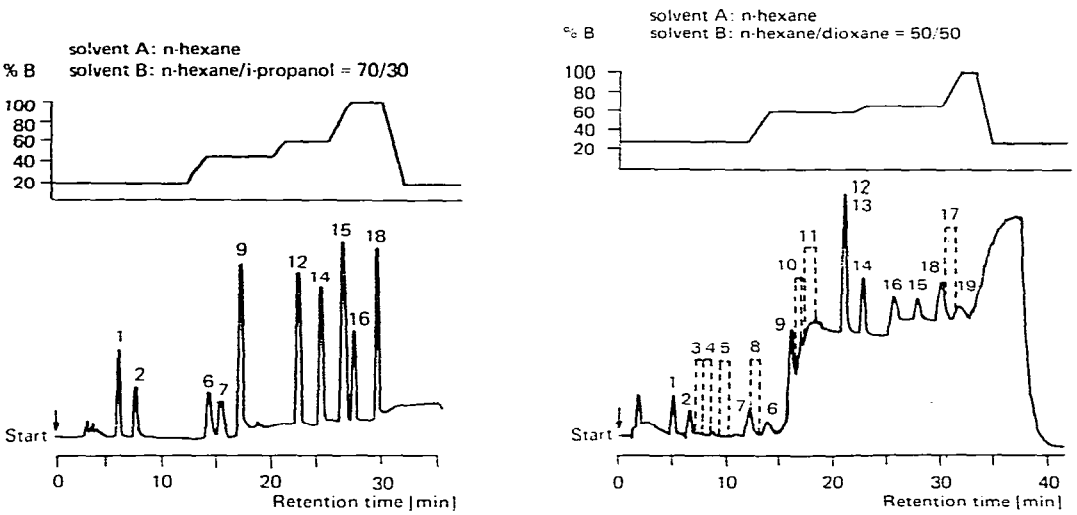

Fig. 6. Separation of adrenal steroids on a DIOL column using an *n*-hexane—isopropanol gradient (system 5 in Table I) and UV detection. Diameter of particles was 5 μm instead of 10 μm.

Fig. 7. Separation of adrenal and gonadal steroids on a DIOL column using an *n*-hexane—dioxane gradient (system 3 in Table I). Steroids were detected by UV absorption or radioactivity.

with system 2. However, 18-hydroxylated steroids such as 18-OH-DOC and 18-OH B, were obviously damaged on this column packing, as even 5-μg amounts of these steroids remained undetectable in terms of discrete peaks.

Influence of solvents on separation

Fig. 7 shows the chromatogram of a DIOL column using dioxane as polar component of the eluent (system 3 in Table I). Due to the relatively high absorbance of dioxane itself, the baseline changes markedly with the increasing gradient. On the other hand, it is well demonstrated by this profile that the maximum gradient of 100% is completely abolished if the column is washed with the initial composition of the gradient for at least 5 min. As to the resolution of individual steroids, it is obvious that this system provides excellent separation of all the steroids studied. Apart from PL and DHT, and 18-OH-DOC and S, all steroids are separated distinctly and no steroid was damaged.

If dichloromethane is used as polar component of the gradient mixture (system 4 in Table I) an elution pattern as shown in Fig. 8 can be observed. Apart from the reversal behaviour of the steroids DHT and PL, DOC and DHEA, or B and 18-OH-DOC, no marked differences are apparent compared with the chromatogram obtained using system 2. As with system 4, the 18-hydroxylated steroid 18-OH-B was not detectable as an absorbing fraction in this system.

Reproducibility of retention times

A high degree of reproducibility of retention times is one of the most important requirements if steroid amounts not detectable by absorbance have to be fractionated prior to immunoassay. This parameter was studied in detail for systems 2—4 (Figs. 2, 7 and 8) by evaluating several chromatographic runs each followed immediately by the start of the next one, thus simulating conditions of automatic analyses of series of samples. The results are listed in Table III. Highest values of absolute standard deviations were 1 min in system

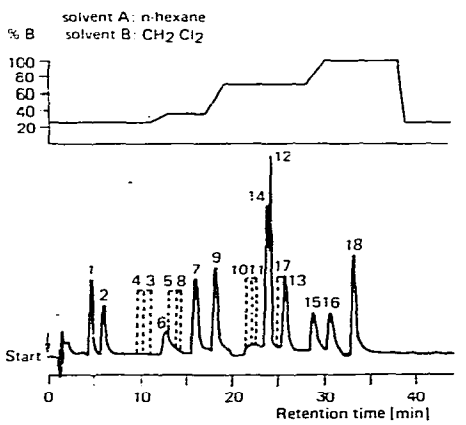

Fig. 8. Separation of adrenal and gonadal steroids on a DIOL column using an *n*-hexane—dichloromethane gradient (system 4 in Table I). Steroids were detected by UV absorption or radioactivity.

2, 1.01 min in system 4 and only 0.49 min in system 3. Coefficients of variation ranged from 6% in system 2, to 8.16% in system 4 and to 3.96% in system 3. A more precise analysis of the data reveals that in all systems reproducibility of retention times in terms of coefficient of variation is relatively low in the initial part of the run and is continuously impaired during the isocratic parts of the gradient.

Reproducibility of the retention times in the chromatography of organic extracts of urine was studied with the dichloromethane extracts of ten different samples of urine, which were diluted 1:20 with water (see extraction procedure 1 in the experimental part) and to which 1 μg cortisol had been added (Fig. 9). It must be pointed out that the gradient profile chosen provided the elution of cortisol at the end of the slowly increasing linear gradient of the run. The resulting parameters of reproducibility were mean retention time $\bar{x}$ = 7.55 min, S.D. = 0.03 min, CV = 0.4%; and the range 7.52—7.61 min.

Extracts of serum and urine

Chromatograms representative of the HPLC of ether extracts of urine and serum (see extraction procedure 2 in the experimental part) are shown in Fig. 10. Comparison with the chromatogram of the mixture of pure steroids (each peak corresponded to about 500 ng of steroid) documents quite well that apart from cortisol in the serum chromatogram (about 80 ng) all other steroids

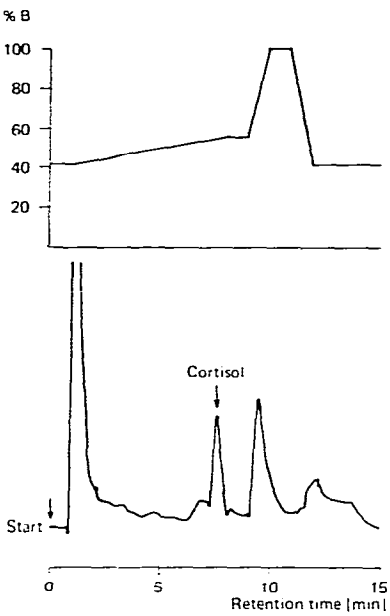

Fig. 9. Chromatogram of a dichloromethane extract of 100 μl urine diluted 1:20 with water. Cortisol (1 μg) had been added to the sample. Conditions of chromatography were similar to those in system 2 (see Table I).

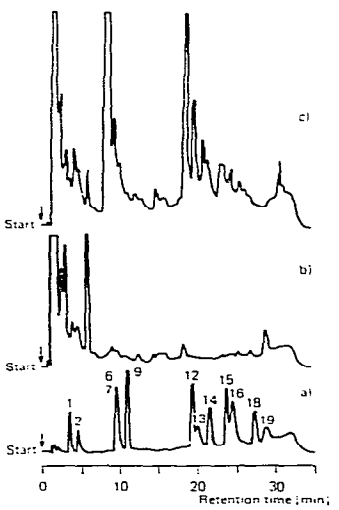

Fig. 10. Representative chromatograms of ether extracts of 2 ml urine (c) and 1 ml serum (b) compared with that of a mixture of pure steroids (a). The amount of each steroid was about 500 ng in chromatogram a. The DIOL column, an *n*-hexane—isopropanol gradient (system 2 in Table I) and UV detection were used.

TABLE III

REPRODUCIBILITY OF RETENTION TIMES OF STEROID HORMONES SEPARATED BY
See systems 2—4 in Table I.

Steroid	System 2 (n = 18)			
	$\bar{x}$ (min)	S.D. (min)	CV (%)	Range (min)
Progesterone	3.28	0.06	1.88	3.16—3.39
Androstedione	4.11	0.10	2.60	3.88—4.29
Deoxycorticosterone	8.02	0.31	3.96	7.33—8.60
Testosterone	8.44	0.40	4.75	7.89—9.20
17-OH-Progesterone	10.49	0.69	6.63	9.36—11.58
Deoxycortisol	18.96	0.66	3.51	17.98—19.92
18-OH-DOC	19.88	0.59	3.00	19.12—20.77
Corticosterone	21.98	0.70	3.20	21.13—22.96
Cortisone	25.29	0.88	3.49	24.29—26.75
Aldosterone	26.52	1.00	3.78	25.14—28.02
Cortisol	31.55	0.35	1.12	31.12—32.11
18-OH-Corticosterone	32.09	0.37	1.15	31.64—32.67

exhibiting rather low physiological concentrations in these biological fluids are not detectable by absorbance if 1—2-ml samples are assayed. Furthermore, the peak areas of the individual steroids are overlapped — this is especially pronounced in the chromatogram of the urine sample — by unspecific components exhibiting much stronger absorbance than the steroids of interest.

DISCUSSION

The HPLC of steroid hormones recently reviewed by Fitzpatrick [15] has been intensively investigated during the last five years and has been shown to represent an excellent technique for the separation of steroid mixtures. However, photometric quantitation in addition to separation of steroid hormones from urine or serum samples by HPLC is limited to steroids occurring in relatively high amounts in biological fluids, such as cortisol in serum [16, 17] and estriol in urine [18]. Due to their low physiological concentrations and to the strong interference of unspecifically absorbing compounds, as well illustrated in the chromatograms of Fig. 10, other steroids of biological interest will hardly be assayable by HPLC alone [15]. On the other hand, the immunoassay technique widely used for steroid estimation lacks sufficient specificity in many cases [19]. Thus, one may conclude that HPLC and immunoassay in conjunction represents quite an attractive means of determining single and particularly multiple steroids in small samples of biological fluids [20].

The suitability of HPLC in this field depends on several criteria:

(1) adequate separation of steroids,

(2) good reproducibility of retention times and independence of chromatographic behaviour from individual sample background,

HPLC USING THE DIOL COLUMN AND VARIOUS ORGANIC ELUENTS

System 3 (n = 18)				System 4 (n = 15)			
$\bar{x}$ (min)	S.D. (min)	CV (%)	Range (min)	$\bar{x}$ (min)	S.D. (min)	CV (%)	Range (min)
4.36	0.08	1.92	4.26—4.63	4.79	0.36	7.67	4.30—5.40
5.52	0.11	2.13	5.43—5.92	6.07	0.46	7.66	5.47—6.98
10.88	0.38	3.50	10.35—11.95	12.49	1.01	8.16	11.42—14.80
9.44	0.31	3.31	9.03—10.43	15.79	0.58	3.69	15.12—17.05
12.49	0.49	3.96	11.71—13.92	17.93	0.59	3.33	17.26—19.27
19.59	0.19	0.99	19.25—19.97	23.91	0.18	0.76	23.48—24.16
19.59	0.19	0.99	19.25—19.97	25.61	0.25	0.97	24.85—25.94
21.10	0.19	0.94	20.73—21.51	23.61	0.17	0.74	23.23—23.85
26.06	0.20	0.79	25.68—26.45	28.92	0.37	1.29	27.74—29.35
23.68	0.22	0.95	23.24—24.12	30.56	0.39	1.30	29.62—31.13
28.12	0.32	1.14	27.66—28.97	33.22	0.33	0.99	32.07—33.53
29.50	0.29	1.00	29.04—30.06	—	—	—	—

(3) easy evaporation of the eluent,

(4) complete elimination of polar or non-polar steroids from the column after each run and rapid reequilibration,

(5) automation of sample loading and of fractionation.

(1) As regards the systems studied, adequate separation of steroids is achieved by the reversed-phase system (system 1) and by the DIOL systems. The quality of the reversed-phase system measured in this study confirms the findings reported by other authors [11, 13]. The good suitability of the DIOL systems for steroid separation has not been reported hitherto. This finding, however, is not astonishing, as the properties of this stationary phase are comparable to those of the widely used Celite columns which were introduced by Abraham and Odell several years ago [21] and have been shown to provide excellent separations of steroids [19]. Of the eluents studied, the n-hexane—dioxane mixture provides the best resolution (Fig. 7). An equivalent level of separation is achieved by the isopropanol system if particles of 5 μm in diameter are used (Fig. 6). However, this system is disqualified as 18-hydroxylated steroids are damaged. A possible explanation for this latter effect may be the instability of these steroids on silica gels as observed in thin-layer chromatography [22].

(2) The best reproducibility of retention times measured in this study is provided by system 7. However, the problem of reproducibility is obviously independent of the nature of the eluent, which is illustrated by comparing the chromatogram obtained using system 7 with that using the DIOL system (Fig. 9). The quality of reproducibility seems to be better if steroids are eluted by a gradient (see reproducibility of cortisol in Fig. 9). Impaired reproducibilities of the initial period of each run (see Table III) should generally be improvable if the steroid of interest is eluted during a gradient part of a chromatographic

run. No, or negligible, influence of the individual sample background could be demonstrated in the chromatography of the extracts of diluted urine samples (Fig. 9).

(3) As eluted fractions after chromatography have to be evaporated to dryness prior to immunological quantitation, the volatile nature of the eluents used in systems 2—7 makes the polar-coated stationary phases unequivocally superior to the reversed-phase system which requires time-consuming evaporation of aqueous solutions.

(4) Application of the gradient technique also carries the advantage that polar components of the sample to be chromatographed are easily eliminated from the polar-coated column by increasing the gradient to maximum polarity for a sufficient time, as shown in Fig. 10. As documented by the rather good reproducibility of the initial peaks of each run, sufficient reequilibration of the columns coated with polar groups is already achieved after 5 min.

(5) The problem of automation is of a technical nature and is already or will be solved by manufacturers of HPLC equipment in the near future.

In conclusion the findings of the present study demonstrate quite well that combination of HPLC using stationary phases chemically coated with hydroxyl groups, gradient elution with organic eluents and automated equipment represents a suitable tool for the routine separation of steroid hormones prior to immunoassay.

REFERENCES

1 R.L. Tilden, Clin. Chem., 23 (1977) 211.
2 S. Comoglio and F. Celada, J. Immunol. Methods, 10 (1976) 161.
3 W. Vetter, A. Vetter and W. Siegenthaler, Acta Endocrinol., 74 (1973) 558.
4 E. Nieschlag and E.J. Wickings, Z. Klin. Chem. Klin. Biochem., 13 (1975) 261.
5 M. Schöneshöfer, J. Steroid Biochem., 8 (1977) 995.
6 W.G. Sippell, F. Bidlingmaier, H. Becker, T. Bruenig, H. Doerr, H. Hahn, W. Golder, G. Hollmann and D. Knorr, J. Steroid Biochem., 9 (1978) 63.
7 D.C. Anderson, B.R. Hopper, B.L. Lasley and S.S. Yen, Steroids, 28 (1976) 179.
8 W.F. Beyer and D.D. Gleason, J. Pharm. Sci., 64 (1975) 1557.
9 W.C. Landgraf, J. Pharm. Sci., 62 (1973) 278.
10 J.C. Touchstone and W.Wortmann, J. Chromatogr., 76 (1973) 244.
11 N.W. Tymes, J. Chromatogr. Sci., 15 (1977) 151.
12 P.G. Satyaswaroop, E. Lopez de la Osa and E. Gurpide, Steroids, 30 (1977) 139.
13 M.J. O'Hare, E.C. Nice, R. Magee-Brown and H. Bullman, J. Chromatogr., 125 (1976) 357.
14 J. Breiter and R. Helger, Z. Klin. Chem. Klin. Biochem., 13 (1975) 254.
15 F.A. Fitzpatrick, Advan. Chromatogr., 16 (1978) 37.
16 F.K. Trefz, D.J. Byrd and W. Kochen, J. Chromatogr., 107 (1974) 181.
17 J.H. van den Berg, C.R. Mol, R.S. Deelder and J.H. Thijssen, Clin. Chim. Acta, 78 (1977) 165.
18 R.J. Dolphin and P.J. Pergande, J. Chromatogr., 143 (1977) 267.
19 G.E. Abraham, J. Steroid Biochem., 6 (1975) 261.
20 J. Butler, V. Fantl and C.K. Lim, in P.F. Dixon (Editor), High Pressure Liquid Chromatography in Clinical Chemistry, Academic Press, London, 1976, p. 57.
21 G.E. Abraham and W.D. Odell, in F.G. Peron and B.V. Caldwell (Editors), Immunologic Methods in Steroid Determination, Appleton-Century-Crofts, New York, 1970, p. 87.
22 R.O. Quesenberry and F. Ungar, Anal. Biochem., 8 (1964) 192.