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ANALYSIS OF NATURAL CORTICOSTEROIDS IN ADRENAL EXTRACTS AND IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A liquid chromatographic procedure is described for the analysis of the principal natural corticosteroids in extracts of adrenal glands. Microparticulate silicic acid columns and gradients of methanol in chloroform are used: conditions are described for the quantitative analysis of the single principal steroidal components of adrenal extracts for pharmaceutical use and of adrenal extracts of rats. In the last case, the use of a 5-μm silica column with the appropriate gradient allows the determination of corticosterone and of 18-hydroxydeoxycorticosterone, which were identified by means of mass spectrometry on their eluates. A single analysis can be performed on the extract of 15 mg of rat adrenal tissue. For the last type of analysis, isocratic conditions on a 10-μm LiChrosorb Diol column are also described.

The application of the gradient elution procedure to the analysis of steroidal compounds in human plasma is also described.

INTRODUCTION

The characteristics of high-performance liquid chromatography (HPLC) such as simple preliminary treatment of the sample, the possibility of achieving very selective separations and comparatively short analysis times, have attracted the attention of workers studying the analysis of natural corticosteroids in adrenal extracts and biological fluids.

Such chromatographic procedures have been described by Wortmann *et al.*[1] and Trefz *et al.*[2] for the analysis of cortisol in plasma and more recently by Loo and co-workers for the analysis of prednisolone[3] and some synthetic and natural corticosteroids[4] in plasma. A similar procedure based on HPLC was described by Schwedt *et al.*[5] for free cortisol in urine and by De Vries *et al.*[6] for the assay of aldosterone in urine.

With regard to the analysis of corticosteroids in adrenal extracts of mammalians used in the preparation of pharmaceutical products, Cavina *et al.*[7] and Can-

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tafora *et al.*[8] described the application of column chromatographic methods under conditions very similar to those of HPLC; most of the methods used in attempts at parallel analysis in adrenal extracts of small animals are based on paper or thin-layer chromatography (TLC). One of the TLC methods[9,10] was later applied to the analysis of a small fragment of human adrenals[11].

The need to obtain analytical data that would be more accurate and reproducible as regards the quantitative analysis of adrenal extracts for pharmaceutical use and more specific and selective for the identification of corticosteroids in adrenal extracts of laboratory animals prompted us to improve our previous chromatographic procedure based on gradient elution in order to obtain a highly selective and reproducible HPLC procedure that would be suitable for the analysis of different types of adrenal and biological extracts.

EXPERIMENTAL

Materials

The following steroids, pure for analytical purposes (E. Merck, Darmstadt, G.F.R., or Ikapharm, Raman Gan, Israël), were used for the preparation of standard solutions: 11-deoxycorticosterone (compound Q), 11-dehydrocorticosterone (A), 11-deoxycortisol (S), corticosterone (B), cortisone (E), aldosterone (Aldo), cortisol (F) and prednisolone (Pred.); 18-hydroxydeoxycorticosterone (18-OH Doc) was obtained through the courtesy of Dr. T. Martinez (Searle and Co., Chicago, Ill., U.S.A.). Each steroid was previously checked by HPLC in order to establish a satisfactory degree of chromatographic purity (less than a 2% area contribution by secondary peaks): 11-dehydrocorticosterone (A) was found to be unsuitable (78% pure) and was purified by liquid chromatography on the milligram scale.

Chloroform (analytical-reagent grade) was washed with water (three 200-ml volumes per 1000 ml) and carefully separated. The solvent, free of ethanol and saturated with water, was kept in brown bottles at ambient temperature; it was prepared fresh weekly. Methanol (analytical-reagent grade) was redistilled. These chromatographic solvents were filtered throught 0.45-μm Millipore filters (fluoropore type).

Ethanol (95%; analytical-reagent grade) was redistilled over sodium hydroxide pellets (referred to as purified ethanol in the text). Methylene dichloride, *n*-heptane, isopropanol, benzene, diethyl ether and *n*-butanol were of analytical-reagent grade and were redistilled when necessary.

Chromatographic procedure

A Hewlett-Packard Model 1084 liquid chromatograph was used, equipped with a variable-volume automatic injector, gradient elution facility and a 254-nm fixed-wavelength detector.

Stainless-steel columns (Brownlee Labs, Calif., U.S.A.) were used, with packing and dimensions as reported in Table I for the silicic acid columns. A similar column (250 × 4.6 mm I.D.) was also used, packed with LiChrosorb Diol (Merck) (10 μm particle diameter).

The mobile phase for gradient elution was (A) chloroform (50% or 100°, water saturated) and (B) 3, 5 or 10% of methanol in solvent A, as reported in Table I. The gradient was linear between 10% and 100% of B in A; the times for the

gradient and for the successive isocratic elution, when used, are reported in Table I. After the gradient, the concentration of B was reversed to the initial 10% in 5 min and held isocratic at this value for 10 min before the next injection. A flow-rate of 1 ml/min was used; the temperature was ambient. For isocratic elution, the mobile phase (A) was water-saturated chloroform–isopropanol (99:1).

Preparation of standard solutions

Each steroid was dissolved in ethanol at a concentration of 1 mg/ml. Aliquots of each solution were combined in a 25-ml tared vessel and adjusted to volume with ethanol, in order to obtain the following composition (w/v): compound Q, 2%; A, 14.4%; S, 5.6%; B, 26.4%; E, 16.0%; Aldo, 3.6%; and F, 32.0%; there was a total amount of 12.5 mg (500 μg/ml) in the solution (solution SM-1).

Prednisolone was used as an internal standard at a concentration of 1 mg/ml in ethanol; a working solution was prepared by 1:10 dilution in the same solvent.

The operating standard mixture was prepared by pipetting 1 ml of SM-1 solution and, when necessary, 1 ml of prednisolone working solution, into a 5-ml conical tube, evaporating the solvent and dissolving the residue in chloroform (1 ml in order to inject 20 μg in 40 μl and 5 ml in order to inject 200 μl); in each instance the amount of prednisolone, when present, was 4 μg.

Preparation of sample solutions

Adrenal extracts for pharmaceutical use. Samples of cortical extracts obtained following the Cartland and Kuizenga procedure[12] were prepared by chloroform extraction as described by Cavina *et al.*[13] and dissolved in ethanol.

A volume of 1 or 2 ml of the ethanol solution (25–50 μg of total corticosteroids) was dried with nitrogen in a small conical tube, the residue dissolved in 400 μl of chloroform and the solution transferred into the injection vessel; the injection volume was 200 μl.

In the external standard procedure, injections of the sample were alternated with injections of the standard mixture (20 μg), with an injection volume of 200 μl. In the internal standard procedure, to the sample and standard mixture were added 4 μg of prednisolone as an internal standard, using 400 μl of a 20 μg/ml chloroform solution of this steroid for redissolving the sample residue. The injection volume was 200 μl. Calculations were performed in both instances by peak-area measurements using the integrator facility of the instrument.

Rat adrenal extracts. Male albino rats, of Wistar strain, mean weight 150–350 g were used; adrenal glands were pooled in groups of eight or sixteen, depending on the mean weight of the glands, in order to obtain about 250 mg of tissue.

The tissue was triturated with fine quartz sand and extracted with chloroform–methanol (2:1) (25–30 ml per 250 mg) in a graduated cylinder with occasional shaking for about 1 h. The extracts were filtered and washed with water, following the Folch *et al.* procedure[14]. The washed extracts were evaporated to dryness under reduced pressure and the residue was partitioned between 25 ml of purified ethanol diluted to 90% and three 5-ml volumes of *n*-heptane in a 50-ml separating funnel. The *n*-heptane layers were discarded, the ethanolic layer was dried under reduced pressure, adding in the last steps small amounts of purified ethanol; the residue was dissolved in this solvent, calculating 0.1 ml of the extract as corresponding to 15 mg of the fresh tissue.

This solution (E_s), stored in a refrigerator, was used for the chromatographic analysis. A 150-μl volume of solution E_s was transferred into the injector vial of the instrument, evaporated to dryness with nitrogen and the residue dissolved in 150 μl of water-saturated chloroform, just before the analysis; the injection volume was 100 μl. In the external procedure the injections of the sample were alternated with injections of a standard mixture, without modifying the volume setting of the injector. The standard mixture was prepared by dissolving in 1 ml of purified ethanol, 15 μg of corticosterone, 5.22 μg of 18-OH Doc and 5 μg of aldosterone. For the injections a volume of 250 or 500 μl was transferred into the injection vial, evaporated to dryness and the residue dissolved in 500 μl of water-saturated chloroform; the injection volume was 100 μl. Calculations in the external standard procedure were performed by peak-area measurements using the integrator facility of the instrument.

Human plasma. The extraction of human plasma was performed in accordance with the procedure described by Trefz *et al.*[2], using purified ethanol, and dichloromethane (Merck; analytical-reagent grade) that had been redistilled twice before use. A check of all the reagents to be used for the extraction procedure was performed using a blank extraction; on the liquid chromatogram there must be no significant peaks after a retention time of about 15 min.

Prednisolone was used as an internal standard, as described by Trefz *et al.*[2], but the amount added was 300 ng as 200 μl of a 150 mg per 100 ml ethanolic solution. For the injection, the residue from the dichloromethane extraction was transferred into a small conical tube with 5 ml of dichloromethane, evaporated to dryness and the residue dissolved in 200 μl of the same solvent. The injection volume was 100 μl.

Calculations were performed by peak-area measurements, applying the external standard procedure. A calibration graph was plotted with increasing amounts of cortisol from 50 to 300 ng; recoveries were calculated on the peak due to 300 ng of the internal standard prednisolone by comparison with an unextracted prednisolone chromatographed standard.

Identification of steroids in liquid chromatographic peaks

By means of liquid chromatography of rat adrenal extracts on 5-μm LiChrosorb columns it was possible to isolate fractions with retention times between 17 and 18 min and between 20.7 and 22 min. Fractions 1 and 2 corresponded to two peaks with retention times of 17.3 and 17.8 min; these peaks were considered to be corticosterone and 18-OH Doc, respectively. Fraction 3 corresponded to a small peak with a retention time of 21.4 min, believed to be aldosterone. The eluates were evaporated to dryness with nitrogen and the residues were dissolved in 150 μl of purified ethanol (solution S_F).

For the identification of the main components of the fractions, the following techniques were applied.

(a) Mass spectrometry with the direct insertion technique was employed, using small quartz tubes with 200–400 ng of steroid for the electron-impact system in an LKB 9000-S instrument at an ionization voltage of 20 and 70 eV, a source temperature of 250° and a sample temperature of 80–150°. This procedure is suitable for the fractions 1 and 2 or for the eluates from the HPTLC plates described below in (c).

(b) Liquid chromatography on a LiChrosorb Diol column was carried out as

described under *Chromatographic procedure*, with the mobile phase for isocratic elution at a flow-rate of 1 ml/min, with a sample size of 100 μl of solution S_F. This procedure allows a clear separation of corticosterone and 18-OH Doc and is suitable for fractions 1 and 2.

(c) Thin-layer chromatography on 10 × 10 cm micro-particle plates (HPTLC) (Merck) by the 90° two-dimensional technique was carried out with chloroform–methanol–water (90:10:0.5) as the solvent in the first direction and benzene–diethyl ether–*n*-butanol (water-saturated)–methanol–water (8:83:6:2:1) as the solvent in the second direction. For spotting, 100 μl of solution S_F were evaporated to dryness in a small conical tube, the residue was dissolved in a few microlitres of chloroform–methanol (9:1) and the solution was applied to the plate. After development, the plate was examined under UV light and sprayed with alkaline blue tetrazolium[13]. This procedure can be applied to all of the fractions; for fraction 3 a pool of fractions must be applied in order to achieve the detection sensitivity of the procedure.

RESULTS AND DISCUSSION

Operating conditions

In previous work[7,8], the separation of the principal steroids was performed by silicic acid column chromatography, that is, by the normal phase procedure; this is in accordance with most of the procedures followed by other workers in similar studies[2–6]. Using this work as a basis, we first studied the chromatographic conditions in order to optimize the separation of the principal corticosteroids present in adrenal extracts. The columns tested and the eluents used are indicated in Table I; in this test we used a reference mixture with seven corticosteroids as described under *Preparation of standard solutions*.

The results in Table I indicate that the best operating conditions with respect to resolution, efficiency and separation time, require the use of a 5-μm LiChrosorb Si 100 column (250 × 4.6 mm I.D.) and a linear gradient of methanol in chloroform, from 0.5% to 5% in 20 min, followed by isocratic elution at the final concentration.

In addition, we found that, for the usual analytical purposes, a 10-μm column of the same material can give good results, and subsequent analyses were therefore performed using both of these columns. We also examined a 150 × 4.6 mm I.D. column packed with 5-μm Spherosil Normaton X0A-600 (Rhone-Poulenc, Paris, France) and obtained satisfactory results [number of plates (N) = 21.170, and resolution between 11-dehydrocorticosterone and 11-deoxycortisol peaks (R_s) = 5.57], but only in a limited number of experiments.

Accuracy and precision of the chromatographic procedure

These parameters were established by testing the chromatographic procedure with a standard mixture of seven corticosteroids. The composition of the mixture was chosen in accordance with the results obtained in the analysis of most of the cortical extracts for pharmaceutical use as demonstrated previously[7,8]. Three different levels of the mixture (15, 20 and 25 μg in an injection volume of 40 μl) were analysed and the chromatographic analysis was repeated five times for each amount.

Calculations were performed by peak-area measurements, using the integrator of the instrument and an internal standard procedure with prednisolone as the internal standard.

TABLE I

EVALUATION OF THE OPERATING CONDITIONS FOR THE SEPARATION OF A STANDARD MIXTURE OF SEVEN CORTICOSTEROIDS

Column packing and dimensions	*Eluent A*	*Eluent B (% of methanol in eluent A)*	*Gradient No., concentration of B in A (%) and time*	*Isocratic elution (100% B) : time (min)*	*Resolution*	*No. of plates per column§§§*
Zorbax Sil* (250 × 2.1 mm)	100% $CHCl_3$ sat. with water	3	G-1, 10–100%, 30 min	—	1.31*** 1.46§	2280
LiChrosorb Si 100**, 30 μm (500 × 2.1 mm)	100% $CHCl_3$ sat. with water	10	G-2, 10–100%, 30 min	—	1.21*** 1.70§	1340
Lichrosorb Si 100**, 10 μm (250 × 4.6 mm)	100% $CHCl_3$ sat. with water	5	G-3, 10–100%, 20 min	10	3.00*** 2.37§§	7640
Lichrosorb Si 100**, 5 μm (250 × 4.6 mm)	100% $CHCl_3$ sat. with water	5	G-3, 10–100%, 20 min	10	4.10*** 3.44§§	20,260

* DuPont, Wilmington, Del., U.S.A.
** E. Merck, Darmstadt, G.F.R.
*** Peaks: 11-dehydrocorticosterone–11-deoxycortisol.
§ Peaks: aldosterone–cortisol.
§§ Peaks: cortisol–prednisolone.
§§§ For the cortisol peak.

TABLE II

EVALUATION OF ACCURACY AND PRECISION IN THE HPLC ANALYSIS OF SEVEN NATURAL CORTICOSTEROIDS IN A MIXTURE, USING A REFERENCE SOLUTION AT THREE DIFFERENT CONCENTRATIONS

Column, Lichrosorb Si 100, 10 μm; gradient, G-3. Calculations: internal standard procedure by peak-area measurements. Values are the means of 5 replicate analyses with standard deviations.

Amount of reference mixture chromatographed	*Amount of the single steroid recovered in the corresponding peak***														*Total corticosteroid content*
	Deoxycorticosterone		*11-Dehydrocorticosterone*		*11-Deoxycortisol*		*Corticosterone*		*Cortisone*		*Aldosterone*		*Cortisol*		
*(μg per 40 μl)**	*μg/ml****	*%*	*μg/ml*	*%*	*μg/ml*	*%*	*μg/ml*	*%*	*μg/ml*	*%*	*μg/ml*	*%*	*μg/ml*	*%*	*(μg/ml)*
15	6.94	1.85	53.32 ± 0.82	14.22	20.57 ± 0.34	5.49	98.49 ± 0.87	26.26	59.66 ± 0.21	15.91	12.70 ± 0.54	3.39	119.54 ± 0.88	31.88	367.06
	(7.5)	(2.0)	(54.0)	(14.4)	(21.0)	(5.6)	(99.0)	(26.4)	(60.0)	(16.0)	(13.5)	(3.6)	(120.0)	(32.0)	(375.0)
20	10.67	2.13	71.64 ± 0.87	14.33	27.70 ± 0.31	5.54	132.45 ± 2.46	26.49	80.44 ± 1.63	16.09	18.23 ± 1.15	3.65	161.70 ± 3.02	32.43	496.44
	(10.0)	(2.0)	(72.0)	(14.4)	(28.0)	(5.6)	(132.0)	(26.4)	(80.0)	(16.0)	(18.0)	(3.6)	(160.0)	(32.0)	(500.0)
25	11.62	1.86	86.00 ± 2.53	13.76	33.37 ± 0.80	5.34	159.33 ± 3.30	25.49	97.74 ± 0.78	15.64	22.38 ± 1.12	3.58	194.24 ± 2.17	31.08	597.71
	(12.5)	(2.0)	(90.0)	(14.4)	(35.0)	(5.6)	(165.0)	(26.4)	(100.0)	(16.0)	(22.5)	(3.6)	(200.0)	(32.0)	(625.0)

* Plus 4 μg of internal standard (prednisolone).

** Concentrations are referred to the injected solutions as μg/ml from μg per 40 ml. Percentages are referred to the total amount chromatographed. Calculated amounts are reported in parentheses.

*** Values are the means of two results only.

In Table II the results are expressed as micrograms of the single corticosteroids per millilitre of extract (mean and standard deviation and as a percentage of the amount injected, calculated on the mean value. The precision of the procedure is shown by the standard deviations; the average coefficient of variations is less than 3%. Aldosterone, which is present in the mixture in the smallest amount (3.6%) gives a coefficient of variation about twice as high. Regarding the accuracy and precision, the results are equivalent for the three levels of concentration analysed. Thus, there is a linear response among the concentration levels chromatographed for each steroid. Figs. 1 and 2 show chromatograms obtained by analysing the same standard mixture on the different types of columns.

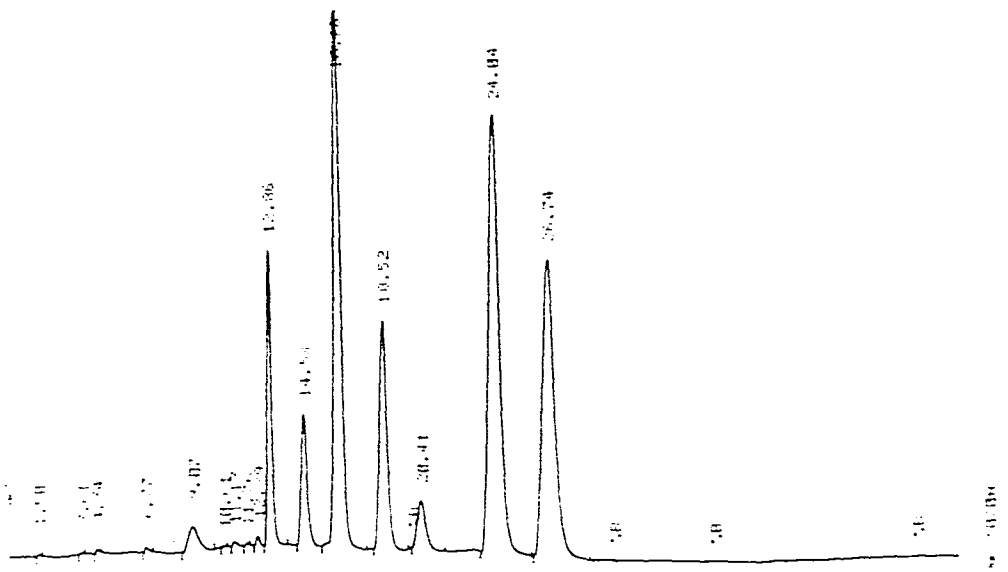

Fig. 1. Separation of standard mixture (20 μg) and prednisolone (4 μg). Column, LiChrosorb Si 100 (10 μm); flow-rate, 1 ml/min; gradient, see text; chart speed, 0.5 cm/min; attenuation, 2^8:1 cm = 256 · 10^{-4} a.u. Peaks (the numbers indicate retention times in minutes): 9.07 = deoxycorticosterone; 12.86 = 11-dehydrocorticosterone; 14.58 = 11-deoxycortisol; 16.28 = corticosterone; 18.52 = cortisone; 20.41 = aldosterone; 24.04 = cortisol; 26.74 = prednisolone.

Application to the analysis of cortical extracts for pharmaceutical use

In Table III some data are reported as examples of the analysis of various cortical extracts for pharmaceutical use. The results for the contents of the single steroids are clearly reproducible in replicate analyses, as was previously found for the standard mixture; it is important to note the possibility of evaluating the composition of a cortical extract on the basis of the effective content of each identifiable UV-absorbing steroid. As an indication, in the last column the percentage recovery is reported as the sum of the individual corticoid contents, each evaluated on the basis of its specific absorbance, and referred to the total corticosteroid content, expressed in terms of hydrocortisone on the basis of UV measurement at 240 nm. In this manner foreign UV-absorbing substances may be excluded, giving a more accurate result for the corticosteroid content (see results for sample EC-3).

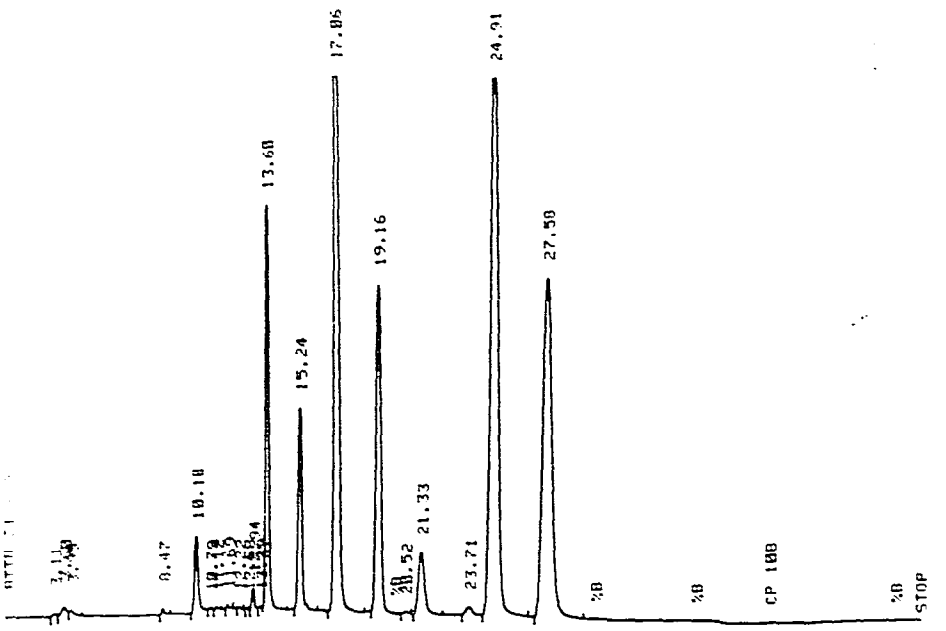

Fig. 2. Separation of standard mixture (20 μg) and prednisolone (4 μg). Column, LiChrosorb Si 100 (5 μm); other conditions as in Fig. 1. Peaks (the numbers indicate retention times in minutes): 10.10 = deoxycorticosterone; 13.60 = 11-dehydrocorticosterone; 15.24 = deoxycortisol; 17.06 = corticosterone; 19.16 = cortisone; 21.33 = aldosterone; 24.91 = cortisol; 27.58 = prednisolone.

Most of the analyses were performed by using the external standard procedure, using a reference steroid mixture, in order to obtain the specific area measurements for each steroid, which were stored in the memory of the calculator–integrator of the instrument. Fig. 3 shows the chromatogram of a cortical extract, as obtained with our analytical procedure.

Application to the analysis of rat adrenal extracts

Small laboratory animal adrenal extracts or incubates have been an important subject of analysis by chromatographic procedures for many years. Using paper chromatography[15–17], TLC[18,19] or classical column chromatography[20], many workers have identified and determined in such extracts many important components of corticosteroid metabolism.

In rat adrenals, the steroid mainly secreted is known to be corticosterone[21,22] followed by aldosterone[20], 11-dehydrocorticosterone[23] and deoxycorticosterone[22]. More recently, some 18-hydroxylated corticosteroids have been found in such glands or in their incubates[16,22,24]. The presence of 17-oxygenated corticosteroids is considered to be doubtful[22].

We have previously reported some data on the corticosterone content of rat adrenals[9]. Using a multiple TLC procedure we were able to determine a corticosterone content of about 3–4 μg per 100 mg of fresh tissue. In this work we applied our HPLC procedure to the chloroform–methanol (2:1) extracts of rat adrenals, simply purified by partition between 90% ethanol and *n*-heptane; Fig. 4 shows the chromatographic profile of a rat adrenal extract obtained using a 5-μm column packing.

TABLE III

RESULTS OF QUANTITATIVE ANALYSIS OF CORTICAL EXTRACTS FOR PHARMACEUTICAL USE

Calculations: external standard procedure with reference mixture by peak-area measurements.

Sample	*Amount chromatographed (μg)* *	*Amounts of the individual steroids determined in the corresponding peaks (μg)* **							*Sum of amounts of individual steroids (μg)* ***
		Deoxycorticosterone	*11-Dehydrocorticosterone*	*11-Deoxycorticosterone*	*Corticosterone*	*Cortisone*	*Aldosterone*	*Cortisol*	
EC-2	12.06	0.02	1.84	0.68	2.67	1.55	0.15	3.81	10.72
EC-2	12.06	0.02	1.95	0.69	2.75	1.59	0.14	3.88	11.02
		(0.16)	(15.67)	(5.64)	(22.47)	(13.02)	(1.16)	(31.84)	(89.96)
EC-3	16.57	—	3.28	0.82	2.50	1.73	0.24	3.10	11.68
EC-3	16.57	—	3.21	0.83	2.47	1.74	0.30	2.78	11.33
EC-3	16.57	—	3.15	0.81	2.41	1.65	0.16	2.86	11.05
		—	(19.37)	(4.95)	(15.03)	(10.32)	(1.39)	(17.56)	(68.62)
EC-5	22.74	—	3.63	0.83	5.45	3.75	0.52	7.31	21.49
EC-5	22.74	—	3.65	0.83	5.45	3.71	0.52	7.38	21.54
EC-5	22.74	—	3.71	0.84	5.50	3.78	0.52	7.37	21,72
		—	(16.09)	(3.64)	(24.05)	(16.49)	(2.28)	(32.32)	(94.87)
EC-6	23.14	—	3.20	1.11	5.85	3.40	0.49	7.55	21.60
EC-6	23.14	—	3.14	1.08	5.72	3.37	0.49	7.35	21.15
		—	(13.69)	(4.71)	(24.98)	(14.60)	(2.11)	(32.19)	(92.28)

* Amounts are conventionally expressed in cortisol by UV absorption measurement.

** Percentages referred to the amount injected, calculated from the mean values, are reported in parentheses.

*** Percentage total recovery is reported in parentheses.

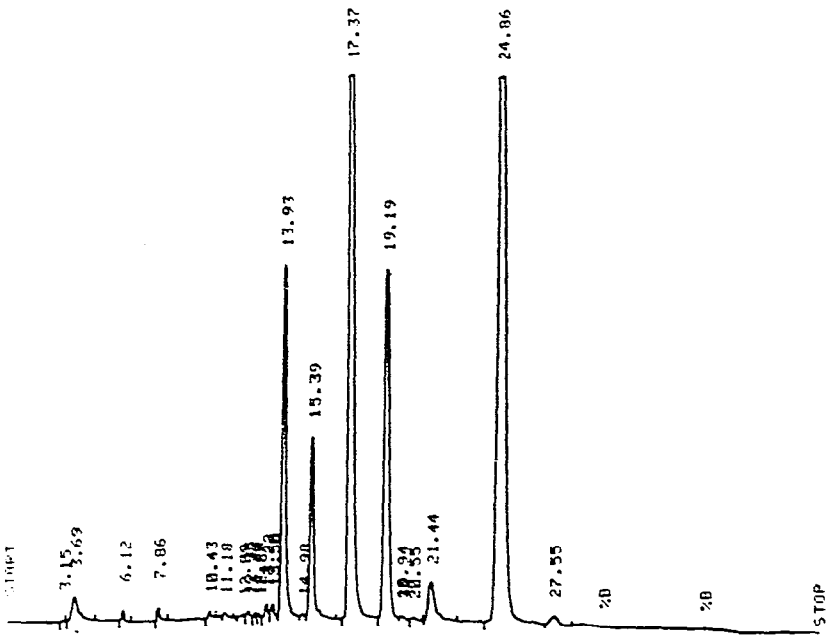

Fig. 3. Separation of 23.14 μg of cortical extract No. 6. Conditions as in Fig. 2. Peaks (the numbers indicate retention times in minutes): 13.93 = 11-dehydrocorticosterone; 15.39 = 11-deoxycortisol; 17.37 = corticosterone; 19.19 = cortisone; 21.44 = aldosterone; 24.86 = cortisol.

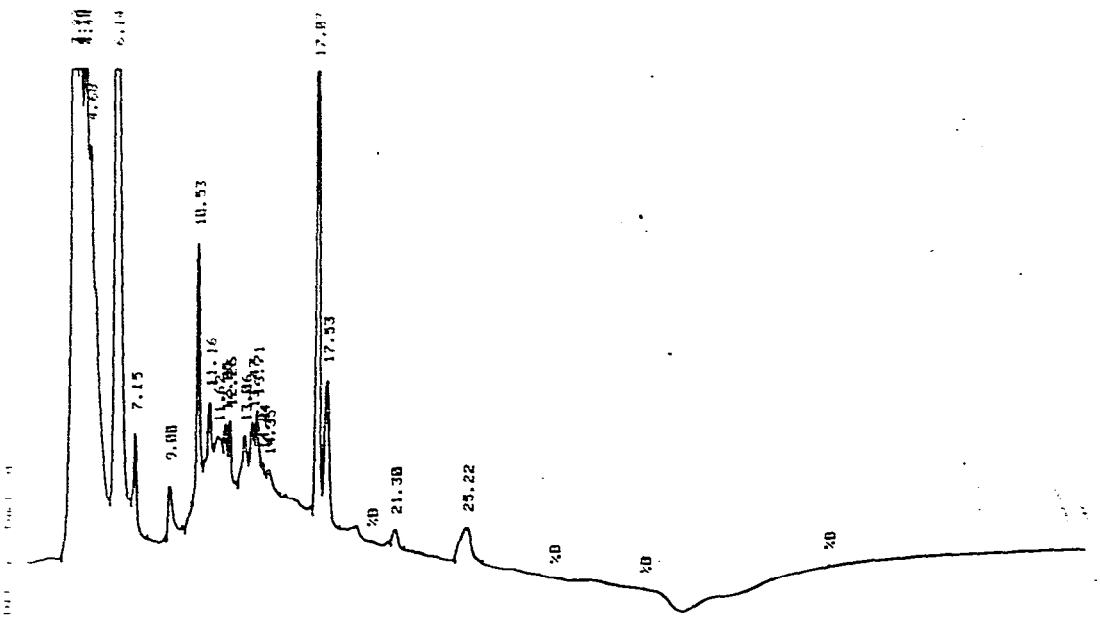

Fig. 4. Separation of 15.75 mg of extracted rat adrenal tissue R-2. Column, LiChrosorb Si 100 (5 ...); flow-rate, 1 ml/min; gradient, see text; chart speed, 0.5 cm/min; attenuation 2^5:1 cm = ... $\cdot 10^{-4}$ a.u. Peaks (the numbers indicate retention times in minutes): 17.03 = corticosterone; ...53 = 18-hydroxy-11-deoxycorticosterone; 21.30 = aldosterone; 25.22 = cortisol (?).

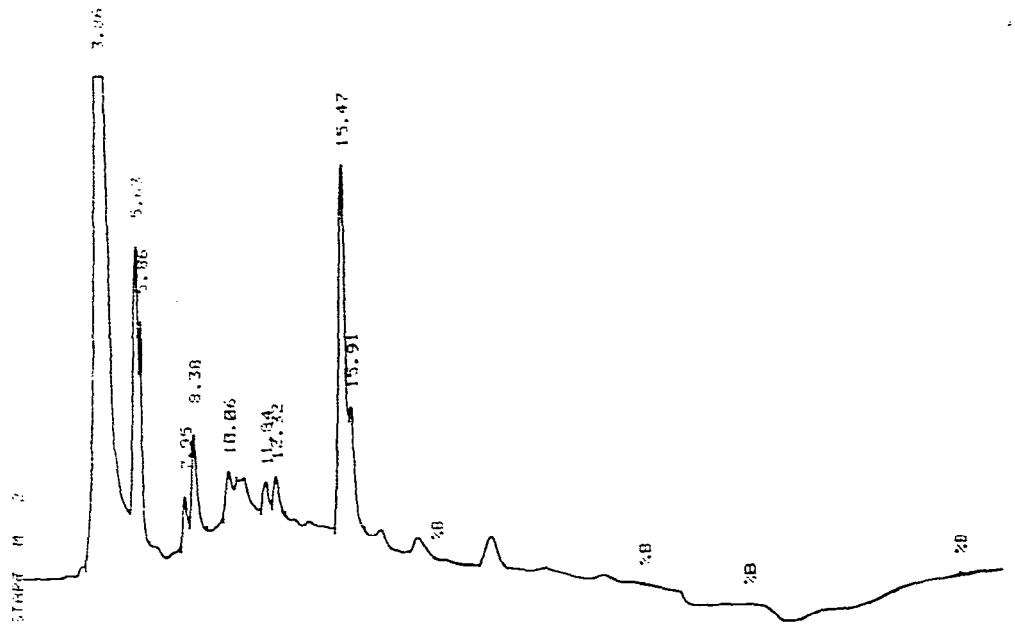

Fig. 5. Separation of 15.75 mg of extracted rat adrenal tissue R-2. Column, LiChrosorb Si 100 (10 μm); other conditions as in Fig. 4. Peaks (the numbers indicate retention times in minutes): 15.47 = corticosterone; 15.91 = 18-hydroxy-11-deoxycorticosterone.

It is interesting that near the corticosterone peak there is another peak that is difficult to distinguish from the former using different conditions for the normal phase chromatography (see Fig. 5, in which a chromatogram of the same extract obtained with a 10-μm column packing is shown).

On the basis of its chromatographic behaviour, this peak could be considered to be 18-hydroxy-11-deoxycorticosterone, which is an important component of the adrenal secretion in the rat[16,17,24] and also in man, being a possible precursor in the biosynthesis of aldosterone and or an adrenocortical factor in hypertension[25,26]. In fact, the peak had the same retention time as an authentic sample of 18-OH Doc in an underivatized form (the latter is an internally emiketalized form)[27]. In addition, the identification was based on the following observations.

(1) After collection of the eluate corresponding to the peaks of corticosterone and of the compound under investigation (fractions 1 and 2 described under *Identification of steroids in liquid chromatographic peaks*), these two compounds could be separated and identified by the two-dimensional HPTLC system described in paragraph (c) of the same section. After TLC, both spots showed a positive UV absorption, but the latter compound gave a negative reaction with blue tetrazolium (BT) reagent, in contrast to corticosterone. This behaviour is in accordance with an 18-hydroxy-20-carbonyl-hemiketal structure, which was described by Roy *et al.*[27] for this type of compound.

(2) Fractions 1 and 2 (see above) were re-chromatographed on HPTLC plates as described for complete fractionation of both peaks; the eluates obtained from TLC were used for recording the mass spectra characteristic of each compound.

With the 18-OH Doc fraction, it was possible to identify peaks of *m/e* 315 and 299; the latter is very intense and is characteristic of this compound. With the corticosterone fraction, peaks of *m/e* 269, 227 and 315 were clearly identified; these findings are in accordance with the spectra described by Genard *et al.*[28].

(3) The eluate containing the adjacent peaks of corticosterone and 18-OH Doc can be evaporated to a small volume and re-chromatographed on a different LC column. The LiChrosorb Diol column, with the isocratic eluent described under *Chromatographic procedure*, gave a clear separation of the adjacent peaks, as is shown in Fig. 6. This LC procedure can be applied directly to the rat adrenal extract but it cannot be applied if the purpose is the isolation of 18-OH Doc for further analytical work. In fact, this compound cannot be recovered unchanged in the eluate from this column. By re-chromatography, the concentrated eluate also in presence of triethylamine, which is a stabilizer of the emiketalized structure[27], again showed the presence of a different compound with a very short retention time.

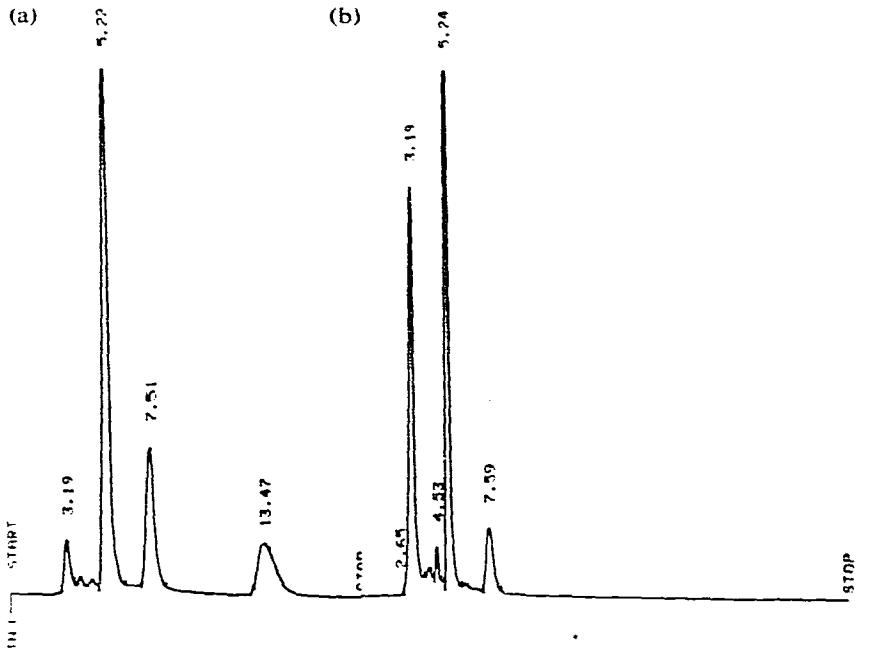

Fig. 6. (a) Separation of 1.5 μg of corticosterone (retention time 5.22 min), 0.5 μg of 18-hydroxydeoxycosterone (7.51 min) and 0.25 μg cortisol (13.47 min). Column, LiChrosorb Diol (10 μm); eluent, water-saturated chloroform–isopropanol (99:1); flow-rate, 1 ml/min; chart speed, 0.5 cm/min; attenuation, 2^5:1 cm = 32 · 10^{-4} a.u. (b) Separation of adrenal extract R-5 corresponding to 15 mg of fresh tissue. Peaks (the numbers indicate retention times in minutes): 5.24 = corticosterone; 7.59 = 18-hydroxydeoxycorticosterone. Conditions as in (a).

For analytical purposes, this LC procedure compared favourably with that recently described by Chan *et al.*[29], avoiding the use of their proposed 240-cm column without decreasing the resolution.

Table IV shows the results obtained in the analysis of some extracts of different pools of rat adrenals; the sensitivity, selectivity and reproducibility are good.

TABLE IV

RESULTS OF QUANTITATIVE ANALYSIS OF RAT ADRENAL EXTRACTS

Calculations: external standard procedure with reference mixture by peak-area measurements.

Sample	*Amount chromatographed (expressed as fresh tissue in mg)*	*Amounts of the individual steroids determined in the corresponding peaks (calculated as μg per 100 mg of fresh tissue)*	
		*Corticosterone**	*18-Hydroxy-11-deoxycorticosterone**
R-1	14.04	3.68 ± 0.06 (3)	1.49 ± 0.06 (3)
R-2	15.75	3.35**	—
R-2	15.75	2.39 ± 0.04 (3)	0.84 ± 0.07 (3)
R-3	15.00	9.58 ± 0.21 (4)	2.44 ± 0.36 (4)
R-5	15.00	8.66 ± 0.38 (6)	3.02 ± 0.23 (6)
R-6	15.00	4.77 ± 0.24 (5)	1.48 ± 0.10 (5)

* Mean ± standard deviation. The number of replicate determinations is given in parentheses.

** Evaluated as unresolved peak of corticosterone plus 18-hydroxy-11-deoxycorticosterone on a 10-μm column packing.

In addition, in all of the extracts we found a peak with the same retention time as that of aldosterone; tentative attempts to quantitate this peak on samples R-5 and R-6 reported in Table IV gave values of 46 and 20 ng, respectively. The identity of the aldosterone peak was confirmed by two-dimensional HPTLC on a pool of eluates from six liquid chromatographic separations. A single spot was obtained, corresponding to crossing position of the aldosterone reference spots, with a positive BT reaction.

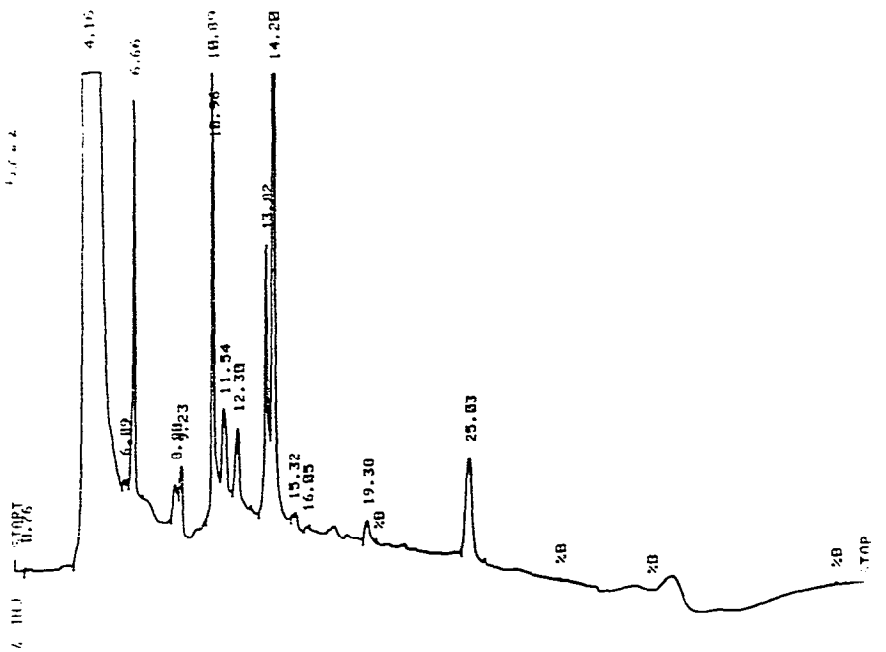

Fig. 7. Separation of 1 ml of extracted human plasma P-2. Conditions as in Fig. 4. The peak at 25.0 min is cortisol.

Application to the analysis of human plasma extracts

The proposed chromatographic procedure can be utilized for the analysis of the steroids of interest when present in a lipid extract of human plasma: under normal conditions only cortisol which is the most abundant corticosteroid in human plasma, can be effectively analysed.

Fig. 7 shows a chromatogram of a plasma extract and Fig. 8 the chromatogram of the same plasma spiked with possible plasma steroids such as cortisone, aldosterone, corticosterone and 11-dehydrocorticosterone. The presence of synthetic corticosteroids such as prednisone, betamethasone and 6α-methylprednisolone in the plasma extracts can be clearly recognized: their retention times are different from that of cortisol.

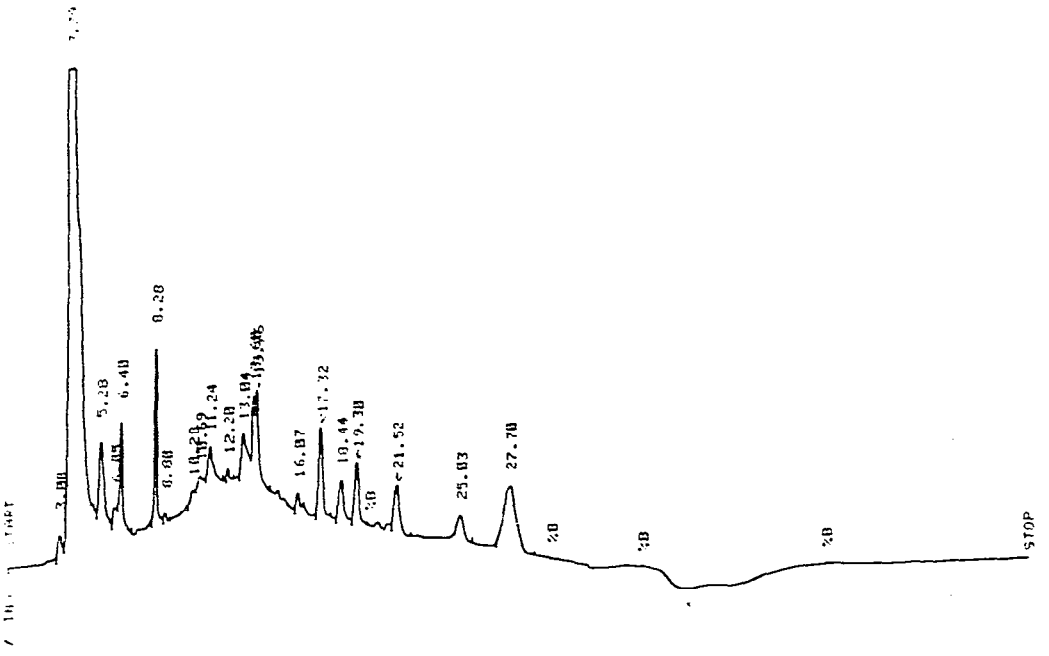

Fig. 8. Separation of 0.25 ml of extracted human plasma P-1 spiked with 200 ng of 11-dehydrocorticosterone (retention time 13.76 min), 200 ng of corticosterone (12.32 min), 200 ng of cortisone (19.30 min) and 200 ng of aldosterone (21.52 min). The peak at 25.03 min is unspiked cortisol. Conditions as in Fig. 4.

The results obtained in the plasma analysis are in accordance with values obtained with similar analytical procedures. Five replicate analyses on two different pools (the first of which was from a case under cortisol therapy) showed mean values and standard deviations of 613.1 $\pm$ 51.4 and of 142.0 $\pm$ 8.9 ng/ml. The recovery was calculated by using the internal standard prednisolone and the mean value was 87.1 $\pm$ 4.3%.

A more detailed description of the application of this chromatographic procedure to the analysis of plasma corticosteroids will be described elsewhere.

CONCLUSION

The chromatographic procedure described here has been demonstrated to be simple and to be reproducible with respect to retention times and quantitative evaluations by peak-area measurements. The analysis of 1 μg of a mixture of seven corticosteroids can be performed, giving a sensitivity of 36 ng for the aldosterone peak.

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