Journal of Steroid Biochemistry, 1977, Vol 8, pp. 995–1009 Pergamon Press Printed in Great Britain

SIMULTANEOUS DETERMINATION OF EIGHT ADRENAL STEROIDS IN HUMAN SERUM BY RADIOIMMUNOASSAY*

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(*Received* 14 *September* 1976)

SUMMARY

A radioimmunological method for estimation of progesterone, 17-OH-progesterone, deoxycorticosterone (DOC), corticosterone, 11-deoxycortisol, 18-OH-deoxycorticosterone (18-OH-DOC), aldosterone and cortisol in one sample of human serum is described. The procedure involves separation of the steroids into four fractions by liquid–liquid partition, purification by paper chromatography and quantitation by radioimmunoassay. The parameters precision, accuracy, sensitivity and specificity of the present method are shown to be equivalent to those of single steroid radioimmunoassays.

Mean serum concentrations in a series of normal males were as follows: progesterone, 17.9 ng/100 ml; 17-OH-progesterone, 179 ng/100 ml; DOC, 6.6 ng/100 ml; corticosterone, 421 ng/100 ml; 11-deoxycortisol, 49 ng/100 ml; 18-OH-DOC, 20 ng/100 ml; aldosterone, 12.4 ng/100 ml and cortisol, 14.4 μg/100 ml The percentage increment of steroid serum concentration after adrenocortical stimulation by ACTH or insulin induced hypoglycemia differed markedly between the individual steroids. The changes of serum steroid concentrations after oral administration of metyrapone are shown. Angiotensin II infusion caused a significant increase of serum aldosterone and a significant fall of serum cortisol, while serum concentrations of the other steroids remained unaltered.

INTRODUCTION

In most diseases involving the adrenal cortex, the estimation of a single adrenal steroid, e.g. aldosterone or cortisol, provides a sufficient screening method for diagnostic purposes. However, studies on the role of the human adrenal cortex in physiological and pharmacological mechanisms or in pathological situations, such as adrenocortical enzymatic deficiencies, hypercorticoidism or "low-renin" hypertension, require reliable methods allowing the determination of as many components of the adrenocortical steroid secretion pattern as possible. Moreover, kinetic studies on regulation of adrenal activity in man or in animals, e.g. episodic secretion of adrenal steroid hormone, require frequent analyses over long periods of time and thus, they are only feasible if methods are available, which allow the simultaneous estimation of multiple steroids in a single serum sample of low volume. The development of such methods has been the aim of several investigators [1–7]. In this field the simultaneous estimation of five adrenal steroids in one serum sample presented by West *et al.* [1] represents the most extensive version reported up to the present.

The present paper describes a radioimmunoassay (RIA) for the simultaneous determination of the adrenal steroids progesterone, 17-OH-progesterone, DOC, corticosterone, 11-deoxycortisol, 18-OH-DOC, aldosterone and cortisol from a single serum sample. Paper chromatography is used as separation technique. Furthermore, normal serum concentrations in males and changes after adrenal stimulation for all steroids estimated will be demonstrated.

MATERIAL AND METHODS

Serum samples. Blood was drawn from a cubital vein into plastic tubes. After clotting, the sample was centrifuged and serum stored at 20° until analysis. If not otherwise described, samples were taken between 8 a.m. and 11 a.m.

Solvents and reagents. All organic solvents were of analytical grade and purchased from Merck, Darmstadt. Before use two-fold distilled water, benzene, *n*-hexane, ethanol (absolute) and methanol were purified by shaking with 25 mg charcoal/l solvent for half an hour, subsequent sedimentation and filtration. Methylene chloride and ethylene glycol were used without further purification. The scintillation mixture (Insta gel) was supplied from Packard Instruments. The buffer solution used throughout all radioimmunoassays consisted of 0.1 M borate buffer of pH 8 (Merck, Darmstadt), 0.6% human-γ-globulin (Kabi) and 2% ethylene glycol. Mixtures of 6.25 g Norit A (Serva, Heidelberg) in 100 ml buffer and 125 mg Dextran (Dextran-70, Pharmacia, Upsala) in 100 ml buffer were set up every two weeks. For separation procedures the charcoal mixture was diluted 1:5 with Dextran solution. Pipettes and counting vials were of the disposable type. Semiautomatic Hamilton syringes were used for the assay pipetting procedures.

* Supported by a grant of the Deutsche Forschungsgemeinschaft.

Conical extraction tubes were washed with diluted detergent for several hours and thoroughly rinsed with distilled water followed by two washings with methylene chloride. Glass vials (Fa. Packard Instruments) were washed with methylene chloride and methanol immediately before being used for collecting eluates of chromatography paper strips. Whatman-2-paper strips (1.8 × 45 cm) were extracted chromatographically with methanol for at least 24 h. Tritium was measured in a liquid scintillation spectrometer (Packard Instruments, model 2480).

Non-radioactive steroids. Progesterone, 17-OH-progesterone, DOC, corticosterone, 11-deoxycortisol, cortisol and aldosterone were of analytical grade and purchased from Merck, Darmstadt. 18-OH-DOC was purchased from Steraloids (Pawling, U.S.A.). The purity of all steroids was checked by t.l.c. in the system: chloroform/methanol (9:1). 2 ng Of each steroid were dissolved in 200 ml of ethanol. The concentration of this stock solution was checked photometrically at 240 nm using a Beckmann photometer. Aliquots of this solution were then further diluted with ethanol to a concentration of 40 ng/100 μl and stored at −20 .

Radioactive steroids. [1,2-^{3}H]-Progesterone (48 Ci mmol), [1,2-^{3}H]-17-OH-progesterone (44.8 Ci mmol), [1,2-^{3}H]-DOC (45.9 Ci/mmol), [1,2-^{3}H]-corticosterone (54.5 Ci mmol), [1,2-^{3}H]-11-deoxycortisol (43 Ci mmol), [1,2-^{3}H]-cortisol (40 Ci/mmol) and [1,2-^{3}H]-aldosterone (57 Ci/mmol) were purchased from New England Nuclear Comp. (Dreieichenhain, Frankfurt Main). [1,2-^{3}H]-18-OH-DOC (51 Ci mmol) was purchased from Amersham–Buchler (Braunschweig) All labelled steroids were purified by paper chromatography fortnightly, dissolved in ethanol and stored at 4 .

Antisera. The antibody against deoxycorticosterone raised in rabbits by immunization with DOC-3-carboxy-methoxime-bovine serum albumin (BSA) conjugate was kindly provided by Dr. R. Fraser, MRC Blood Pressure Unit, Glasgow. Antibodies against progesterone, 11-deoxycortisol, corticosterone and cortisol were raised in rabbits by immunization with the corresponding 21-hemisuccinate–BSA conjugates. They were kindly provided by Dr. P. Vecsei, Institute of Pharmacology, Heidelberg. The 11-deoxycortisol antiserum exhibited complete cross reaction with 17-OH-progesterone and was used for the RIA of the latter one. The antiserum against corticosterone containing antibody fractions with marked binding affinities to 18-OH-DOC [8] was used for the RIA of 18-OH-DOC. The antibody against aldosterone raised in a sheep by immunization with aldosterone-21-hemisuccinate–BSA conjugate was a gift from the National Institute of Health, Bethesda, MD

METHODS

Figure 1 shows the general outline of the complete assay consisting of three main steps: liquid–liquid partition into four fractions, purification by paper chromatography and quantitation by RIA.

Liquid–liquid partition To serum samples (1–3 ml) placed in a conical glass tube tracer amounts of labelled steroids each dissolved in 50 μl of water were added. The absolute values of labelled steroids are shown in column 1 of Table 1 After intensive stirring for about 1 min, the sample was allowed to stay for 30 min at room temperature. Serum was then extracted twice with *iso*-octane on a horizontal shaker for 15 min. After centrifugation, the aqueous layer was frozen in a methanol–dry ice mixture and the organic phases containing fraction I were decanted into a conical glass tube. The residual serum phase then was extracted twice with 6 ml of methylene chloride. The aqueous phase was discarded The organic phases were collected and evaporated under air in a 30° water bath. The residue was redissolved in 1 ml of 1 M NaCl and extracted with 5 ml of *iso*-octane. The organic phase containing fraction II was separated from the aqueous phase as described above Extraction of the residual aqueous solution with carbon tetrachloride yielded fraction III. After final extraction with methylene chloride, the aqueous phase was discarded and the organic phase containing fraction IV decanted into a conical glass tube.

Chromatography. Fractions I–IV were evaporated under a stream of air in a 30 water bath. The residue of each fraction was redissolved in 200 μl of methylene chloride and chromatographed on paper in the corresponding system indicated in Fig. 1. Each fraction contained one steroid component as marker. The labelled tracer amount of this marker was high enough to be located on the paper strips by radio-

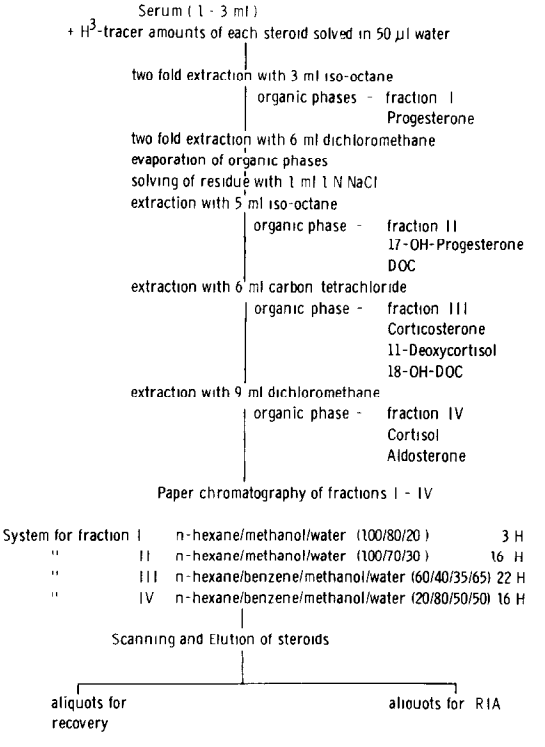

Fig 1 General flow sheet for the overall steroid assay.

Table 1. Data of assay parameters

Steroid	Amount of tracer (10^3 d.p.m.)	Vol. for solving eluate residue (μl)	Aliquot for recovery (μl)	Solvent for labelled and unlabelled steroid	Number and type of 100 μl replicates	Amount of competing labelled steroid (10^3 d.p.m.)	Final dilution of antiserum (in 0.4 ml)
Progesterone	20.8	500	100	ethanol	3 dil.	19.4	50,000
17-OH-Progesterone	33.3	800	200	ethanol	3 dil.	19.4	100,000
DOC	9.7	350	100	ethanol	2 orig.	20.8	20,000
Corticosterone	41.6	1500	500	buffer	3 dil.	26.4	30,000
11-Deoxycortisol	12.4	500	200	buffer	2 orig.	20.8	100,000
18-OH-DOC	22.2	350	100	buffer	2 orig.	27.7	20,000
Aldosterone	11.1	350	100	buffer	2 orig.	19.4	1,200,000
Cortisol	55.5	5000	500	buffer	3 dil.*	18.0	60,000

dil. = original solution was diluted by doubling dilution steps; orig. = original solution; * = before setting up replicates for RIA the original solution was diluted 1:10.

scanning (Packard Instruments, model 7200) such as progesterone itself in fraction I, 17-OH-progesterone in fraction II, corticosterone in fraction III and cortisol in fraction IV. The other steroids with tracer amounts too low to be monitored were identified by use of a ratio R_t, defined as migration of steroid with low tracer amount/migration of marker steroid. Migration is the distance in cm from the origin to the midpoint of the steroid area. The ratio R_t itself was derived from standard strips which were loaded with higher labelled amounts of each steroid (about 0.2 μCi) and developed in each chromatography together with the sample strips. Each steroid area located by the procedure described above was then eluted with 3 ml of methanol.

Radioimmunoassay. The methanolic eluate of each steroid was evaporated to dryness under a stream of nitrogen at room temperature. The residues of the more polar steroids corticosterone, 11-deoxycortisol, 18-OH-DOC, cortisol and aldosterone were redissolved in borate buffer, those of the steroids progesterone, 17-OH-progesterone and DOC in ethanol (column 4 in Table 1). The absolute volumes for redissolving are presented in column 2 of Table 1. Aliquots, the absolute values of which are indicated in column 3 of Table 1, were pipetted into counting vials for estimating H^3-recovery. Duplicates of 100 μl or threefold dilution aliquots as shown in column 5 were used for RIA. Standard curves were set up in duplicate. Unlabelled steroid amounts dissolved in 100 μl of ethanol or buffer ranged from 3.12–800 pg set up in doubling dilution steps. Because of the high blank values in the 18-OH-DOC radioimmunoassay [8], a special technique for setting up a standard curve of this steroid had to be applied. Similar to the DOC radioimmunoassay described previously [9], buffer solutions of eluates of paper blanks were used as solvent for the 18-OH-DOC standard curve. Competing [H^3]-steroid (column 6 in Table 1) dissolved in 100 μl of ethanol or buffer was added both to the unknown and standard curve samples. After evaporation of the ethanolic samples under nitrogen at room temperature, 400 μl of antiserum buffer solution was added to the tubes. To the buffer samples 200 μl of antiserum buffer solution were added. The final dilutions of antisera in 0.4 ml are as shown in column 7 of Table 1. It must be pointed out that assay conditions, i.e. amounts of labelled steroid and antiserum dilutions, were optimized with respect to a high sensitivity by an approximating computer program [10]. Before incubation at 4° for 16 h all samples were intensively stirred. Separation of free and bound steroid was achieved using the Dextran-coated charcoal method. After centrifugation, the supernatant of up to 150 samples was decanted simultaneously by a semiautomatic apparatus [9]. The H^3-amount of the supernatant representing bound fraction was used for assay evaluation. Evaluation of assay and physiochemical data as well as plotting of standard curves was done with a modular constructed computer program [10]. The "spline-approximation" technique was used as standard curve fitting model [11]. All operating procedures were done with an IBM-1800 computer. Statistical calculations were done by the student's t-test and the paired Wilcoxon test.

RESULTS

Liquid–liquid partition

The distribution of each steroid in the course of the liquid–liquid partition procedures was studied by adding a particular labelled steroid to one 3 ml serum sample and extraction in the way described above. The percentage recoveries of each steroid in the different fractions are shown in Table 2. The results reveal that steroids with similar polarity are accumulated in particular fractions, such as progesterone in fraction I, DOC and 17-OH-progesterone in fraction II, corticosterone, 11-deoxycortisol and 18-OH-DOC in fraction III and cortisol, aldosterone in fraction IV.

Paper chromatography

The chromatographic properties of the steroids in the systems applied for paper chromatography are shown in Fig. 2. In these chromatographic runs all steroids measured in the method were chromato-

Table 2 Percentage recovery of steroids in fractions I to IV and total recovery after liquid liquid partition

Fraction	Progesterone	17-OH-Progesterone	DOC	Corticosterone	11-Deoxy-cortisol	18-OH-DOC	Aldosterone	Cortisol
I	**72.4***	4.9	9 1	0 2	0 8	2 6	0.4	0 1
II	13.8	**86.8**	**83.5**	24.4	35.0	27 8	3 6	6 8
III	0.2	0 6	0 4	**54.0**	**46.3**	**59.5**	20 7	9 7
IV	0 1	0 1	0 2	7 7	3 2	5 3	**59.9**	**65.1**
Total	86.5	92 4	93 2	86 3	85 3	95 2	84 6	81 7

* Values of recovery accumulated in particular fractions are printed in bold type

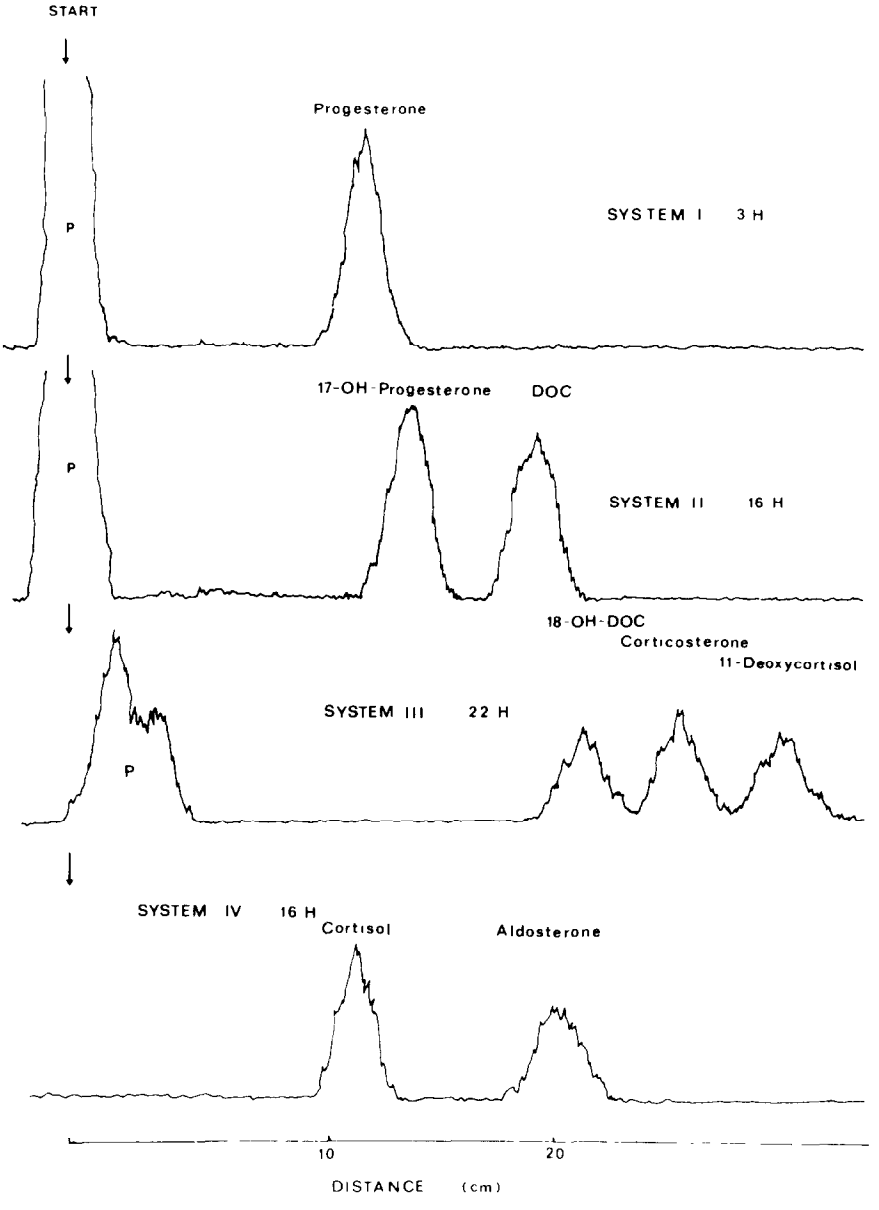

Fig. 2. Paper-chromatograms of corticosteroids, Peaks were monitored by radioscanning of ^{3}H-labelled steroids. Activity of each steroid was about 0.2 μCi. In each chromatogram P indicates area of the more polar steroids: system I: P = all steroids apart from progesterone, system II P = corticosterone, 11-deoxycortisol, 18-OH-DOC, aldosterone and cortisol; system III: P = aldosterone and cortisol. Steroids not monitored in the particular chromatograms were run from the paper.

Table 3. Percentage recovery of steroids after chromatography (column 1), the ratio of steroids with low tracer amounts/marker steroids (column 2), the absolute migration of marker steroids (column 3), and the residues of tracer in a 100 µl aliquot of the eluate expressed as percentage of the activity used for RIA (column 4)

Steroid	Recovery (%) mean ± S.D.	N	Ratio R_i mean ± S.D.	N	Migration of marker steroid (cm) mean ± S.D.	N	Residue of tracer (%)
Progesterone	37.3 ± 4.4	20			20.3 ± 2.9	8	7.9
17-OH-progesterone	49.0 ± 10.6	18			16.0 ± 4.1	10	10.5
			$R_{17\text{-OH-P}}$				
DOC	54.7 ± 10.2	14	1.36 ± 0.02	14			7.1
Corticosterone	39.3 ± 3.7	18			28.4 ± 4.8	9	4.0
			R_B				
11-Deoxycortisol	39.7 ± 4.7	13	1.16 ± 0.01	13			4.6
			R_B				
18-OH-DOC	27.4 ± 3.7	13	0.82 ± 0.01	11			6.1
			R_F				
Aldosterone	36.7 ± 4.0	16	1.48 ± 0.31	22			5.8
Cortisol	39.1 ± 5.5	19			14.8 ± 1.9	12	0.2

17-OH-P = 17-OH-progesterone; B = corticosterone; F = cortisol.

graphed in each system. From the profiles of the chromatograms it becomes evident (1) that steroids of higher polarity (P) than the "main" steroids the particular chromatographic system was established for are located at the starting point of the chromatograms, (2) that the steroids less polar than the "main" ones are run from the paper and (3) that the steroids of similar polarity are separated adequately in the special systems. A negligible overlap occurs between 18-OH-DOC, corticosterone and 11-deoxycortisol in system III. In order to minimize this potential error, these steroids, therefore, were eluted from the middle zones of the peaks only, ignoring the overlapping border regions. The mean migrations of steroids used as marker, the ratios R_i of steroids with low tracer amounts as well as the percentage recovery of each steroid after chromatography are demonstrated in Table 3. From all these results the adequate separation of all steroids is apparent. Considering the recovery values after extraction (Table 2) and after chromatography, it becomes obvious that the loss of 18-OH-DOC due to the chromatographic step is markedly higher than those of other steroids. This phenomenon is explained by the known instability of this steroid.

The mean amounts of tracer activity remaining in a 100 µl aliquot of eluate used for RIA are shown in column 4 of Table 3. Values are expressed as percentage of competing RIA activity. Since residual tracer amounts cannot be considered to be negligible, these errors were corrected approximately for each sample by computer evaluation [10].

Characterization of antisera

Physicochemical parameters of antibody–steroid reactions are summarized in Table 4. Affinity constants of steroid–antibody complexes and capacities of binding equivalents were evaluated by the method of Scatchard [12]. Saturation of antibody was achieved by a portion of labelled steroid and increasing amounts of unlabelled steroid [10]. For all steroid antibodies the plot of bound/free against bound revealed a hyperbolic form particularly pronounced in the region of high steroid concentrations. This indicates that antisera contain at least two antibody fractions with different physicochemical behaviour. The results of the corresponding two antibody species were calculated by a computerized approximation technique [10] based on the graphical evaluation method of Berson [13]. Rates of dissociation of

Table 4. Physicochemical parameters of steroid-antibody reactions

Steroid	Affinity constants [l/nmol]		Capacities of binding equivalents [µmol/l]		Free energy change [kcal/mol]		Dissociation rates [s^{-1}]	
	K_1	K_2	q_1	q_2	ΔG_1	ΔG_2	r_1	r_2
Progesterone	1.8	0.12	10	26	−11.7	−10.2	$3.0 \cdot 10^{-5}$	$1.1 \cdot 10^{-3}$
17-OH-Progesterone	4.8	0.2	36	41	−12.2	−10.6	$2.8 \cdot 10^{-5}$	$3.2 \cdot 10^{-4}$
DOC	4.0	0.17	3.7	7.1	−12.1	−10.4	$2.4 \cdot 10^{-5}$	$4.8 \cdot 10^{-4}$
Corticosterone	2.5	0.26	4	4.2	−11.9	−10.7	$3.8 \cdot 10^{-5}$	$5.2 \cdot 10^{-4}$
11-Deoxycortisol	10.0	0.27	14	22	−12.7	−10.7	$6.5 \cdot 10^{-5}$	$6.3 \cdot 10^{-4}$
18-OH-DOC	2.6	0.06	0.58	0.86	−11.8	− 9.8	$2.8 \cdot 10^{-4}$	$3.4 \cdot 10^{-3}$
Aldosterone	11.0	0.18	110	260	−12.8	−10.5	$4.6 \cdot 10^{-5}$	$5.5 \cdot 10^{-4}$
Cortisol	3.3	0.19	3.7	8.8	−12.0	−10.5	$5.8 \cdot 10^{-5}$	$6.0 \cdot 10^{-4}$

Table 5. Means of standard curve parameters arising from several steroid radioimmunoassays, as well as competing labelled steroid, antibody saturation and 50% displacement values both calculated and found in one steroid standard curve using antibody dilutions as indicated in Table 1

Steroid	Bound at zero dose mean ± S.D. (%)	Sensitivity mean ± S.D. (pg)	Sensitivity calculated for a 3 ml serum sample (ng/100 ml)	50% Displacement mean ± S.D. (pg)	Amount of labelled steroid (c.p.m.)	Amount of labelled steroid (pg)	Saturation of antibody at zero dose (%)	50% Displacement (pg)	
								Found	Calculated
Progesterone	13.9 ± 3.1	6.3 ± 5.6	1.05 ± 0.9	99.8 ± 18.1	9800	78.7	49.0	92.7	113.0
17-OH-Progesterone	19.7 ± 5.9	3.2 ± 4	1.7 ± 7.1	67.7 ± 18.0	8200	77.9	63.1	92.8	134.7
DOC	16.7 ± 3.4	2.9 ± 1.4	0.62 ± 0.3	68 ± 9.4	7150	59.0	57.9	65.7	107.7
Corticosterone	13.7 ± 3.1	7.1 ± 6.3	9.1 ± 8.0	112.1 ± 10.5	9900	107.8	63.6	148.2	173.0
11-Deoxycortisol	13.1 ± 2.8	7.0 ± 9.2	2.9 ± 3.8	92.4 ± 11.8	5800	58.0	75.8	86.6	79.3
18-OH-DOC	8.6 ± 3.1	7.4 ± 8.7	3.1 ± 3.7	138.7 ± 34	5000	28.9	19.8	177.0	204.0
Aldosterone	16.0 ± 2.7	4.2 ± 2.2	1.2 ± 0.6	57.6 ± 11.4	7100	60.4	79.5	79.4	77.5
Cortisol	14.2 ± 3.2	4.8 ± 2.8	20.5 ± 11.9	99.2 ± 8.9	6150	52.3	50.0	89.0	109.4

H^3-labelled steroid–antibody complexes at 4° were determined by preincubation of labelled steroid and antiserum for 20 h followed by the addition of 4000 pg of unlabelled steroid. H^3-measurement of the supernatant as a function of time permitted the estimation of dissociation rates. The semilogarithmic plot of bound steroid against time exhibited a hyperbolic form for all steroid antisera indicating the existence of at least two different antibody species. This was in agreement with the findings of Scatchard plot studies. The values of the two different rates were obtained by graphic extrapolation of the asymptotes of the hyperbolic curve.

Standard curve parameters

The main parameters of the standard curve characteristics are shown in Table 5. The low percentage activity at zero dose ranging from 8.6% for 18-OH-DOC to 19.7% for 17-OH-progesterone, differs markedly from those commonly reported and theoretically established [14]. However, experiments with higher levels of zero bound activity yielded lower sensitivities thus supporting the results of the computerized optimization technique applied in the present radioimmunoassays. The mean sensitivities (2 standard deviations of the zero point) ranged from 2.9 pg for DOC to 7.4 for 18-OH-DOC (column 2 in Table 5).

As a further measure of sensitivity, the amount of unlabelled steroid displacing 50% of the labelled steroid from the antibody was used. The mean values of each steroid RIA are summarized in column 4 of Table 5.

In the last five columns of Table 5, the assay conditions of a single standard curve of each steroid as well as the 50% displacement values theoretically calculated and practically found are summarized. The results reveal that 50% displacement of activity requires a higher amount of unlabelled steroid than the mass of labelled steroid at zero dose (columns 6 and 8). This phenomenon can be explained by the fact that at zero dose antibodies are not yet saturated (column 7). The calculated and measured values of 50% displacement (column 8 and 9) exhibit a good agreement.

Assay parameters

Specificity. The overall specificity of the present multiple steroid RIA depends on two main factors: (1) specificity of antisera and (2) the removal of interfering steroids by paper chromatography. The effective separation of steroids with different polarity is shown in Fig. 2. The per cent cross-reactions between the antisera and steroids with chromatographic behaviour similar to the specific steroids are shown in Table 6. The method of Abraham was used for calculation of per cent cross-reactions [15]. The data indicate that, with the exception of the 18-OH-DOC assay, cross-reactions with steroids of similar chromatographic behaviour are negligible. The relatively unspecific characteristics of the 18-OH-DOC antiserum described in detail elsewhere [8] requires an unequivocal separation from the other steroids, if a reliable 18-OH-DOC estimation shall be warranted.

Serum blanks. Assay blanks were studied in "charcoal-stripped" serum and included in all batches of analyses. The mean blanks arising in 3 ml of this serum are summarized in Table 7. In almost all assays, blanks arose to a slightly higher magnitude than assay sensitivity. Paper blanks, also included in all batches of analysis, yielded almost undetectable values (Table 7). In the 18-OH-DOC assay, blank free conditions only could be established, if eluate of paper blanks was used as solvent for setting up the standard curve.

Precision. Within assay precision was determined from replicate measurements in the same assay on

Table 6. Cross reactions of specific antibodies with steroids exhibiting similar chromatographic behaviour. R_i indicates the chromatographic ratio of unspecific steroid/specific steroid measured in the system used for the specific steroid

Steroid of specific antibody	Unspecific steroids	R_i	Cross reaction %
Progesterone	Pregnenolone	0.78	0.5
17-OH-Progesterone	DOC	1.36	10.2
	Testosterone	1.43	5.0
DOC	17-OH-Progesterone	0.73	0.03
	Testosterone	1.05	<0.01
Corticosterone	18-OH-DOC	0.82	12.5
	11-Deoxycortisol	1.16	5.4
	Cortisone	0.17	0.6
11-Deoxycortisol	Corticosterone	0.86	1.0
	18-OH-DOC	0.70	1.5
	Cortisone	0.14	10
18-OH-DOC	Corticosterone	1.22	131
	11-Deoxycortisol	1.42	47.6
	Cortisone	0.21	3.9
Aldosterone	Cortisol	0.67	<0.01
	Cortisone	1.22	0.4
Cortisol	Aldosterone	1.48	1.5
	18-OH-Corticosterone	0.75	<0.01

Table 7. Blanks occurring in steroid radioimmunoassays. Values of paper blanks represent amounts arising in 100 μl aliquots of eluates. Values for serum blanks were obtained from 3 ml of serum

	Progesterone	17-OH-Progesterone	DOC	Corticosterone	11-Deoxycortisol	18-OH-DOC	Aldosterone	Cortisol
Paper blank (pg) mean ± S.D.	0.9 ± 1.5	1.18 ± 1.9	0.45 ± 0.8	3.0 ± 2.9	3.3 ± 3.4	2.2 ± 3.2	1.9 ± 2.0	1.6 ± 3
Blank in "charcoal-stripped" serum (ng/100 ml) mean ± S.D.	2.5 ± 2.2	2.8 ± 1.3	0.43 ± 0.08	6.8 ± 4.1	1.5 ± 1.12	1.0 ± 0.95	1.2 ± 0.7	13.7 ± 17

Table 8. Intra- and interassay variability of steroid radioimmunoassays

Steroid	Intraassay variation			Interassay variation		
	Mean (ng/100 ml)	N	Coefficient of variation (%)	Mean (ng/100 ml)	N	Coefficient of variation (%)
Progesterone	16.1	5	13.8	10.3	8	10.3
	45.6	11	4.81	16.2	8	8.9
	106.0	9	12.6	30.4	7	12.4
17-OH-Progesterone	46.1	10	11.4	50.9	6	17.8
	73.3	9	7.5	110.3	14	17.1
	161.7	12	8.6	205.0	7	14.2
DOC	6.4	10	8.6	6.7	10	19.5
	13.2	9	10.4	7.4	6	21.0
	24.0	10	17.8	14.8	5	21.8
Corticosterone	74	8	13.9	70.6	7	12.1
	549	8	8.3	217.0	5	11.3
	1255	7	10.6	483.8	5	10.9
11-Deoxycortisol	14.7	6	14.1	12.7	4	15.6
	37.9	8	9.5	17.0	8	19.1
	142	8	8.3	43.7	4	16.4
18-OH-DOC	19.8	10	17.9	7.5	5	25.9
	30.3	8	11.2	33.2	5	13.9
	64.7	10	15.5	67.1	5	11.1
Aldosterone	5.8	10	7.7	3.5	5	25.1
	10.0	9	10.1	6.1	6	10.0
	19.9	10	13.9	17.9	13	12.1
Cortisol	9850	10	15.7	5900	13	14.4
	4900	10	16.0	10600	8	19.4
	13700	12	8.2	15800	8	7.6

serum pools with high, intermediate and low levels of steroids within the normal range. Between assay precision was determined from different assays on the same serum pools. In Table 8, the precision is expressed in terms of coefficient of variation at different serum steroid concentrations.

Accuracy. To test the accuracy of the method, known amounts of unlabelled steroids corresponding to endogenous levels were estimated. Fig. 3 shows the regression analyses of steroid amounts added and found. The data indicate that recovery of unlabelled steroid amounts was essentially quantitative and consistent.

Normal values. The serum concentrations of all steroids were measured in a series of 20 normal healthy men, aged between 20 and 50 years. None of the subjects received medication of any kind. The mean concentrations and ranges are summarized in Table 9.

Changes of serum steroid concentrations after stimulation of adrenal cortex

Corticotrophin (ACTH). ACTH (250 μg Synacthen i.v.) was administered to 6 normal subjects at 8 a.m. Figure 4 shows the mean steroid serum concentrations immediately before and 60 min after drug administration in terms of absolute and percentage values, thus setting base levels of each subject at 100%. With the exception of aldosterone the increase of steroid concentrations was highly significant. B levels exhibited the highest rise of all steroids (878% of control).

Insulin. The effect of insulin induced hypoglycemia on serum levels of the adrenal steroids was studied in 7 normal subjects. Mean steroid serum concentrations before and 90 min after insulin injection are shown in Fig. 5. The results exhibit a similar pattern to those of ACTH stimulation. Corticosterone increased to 698% of control.

Metyrapone. Figure 6 shows the 8 a.m. serum concentrations of adrenal steroids on two consecutive days before and after administration of 2-methyl-1,2-bis-(3-pyridyl)-1-propanone (metyrapone) at midnight. Due to the metyrapone induced 11-hydroxylase inhibition, steroids localized before 11-hydroxylation within the pathway of adrenocortical steroid biosynthesis rose significantly. Although the adrenal biosynthesis of the 11-hydroxylated steroids should be markedly suppressed by metyrapone, the 8 a.m. serum concentration of these steroids, i.e. corticosterone and cortisol, are relatively high. Furthermore, it must be stressed that the 18-OH-DOC serum levels only rose to about 340%, although the substrate for the 18-hydroxylase, DOC, increased to about 10.000%.

Angiotensin II. The effect of prolonged angiotensin II infusion (6 ng/kg/min) on adrenal steroid secretion was studied in two normal men [16]. Subjects received a diet containing 135–140 mEq of sodium and 70–80 mEq of potassium. The 9 a.m. serum steroid concentrations of two pre-treatment days and of three days during angiotensin II infusion, are shown in Fig. 7. Values are expressed in absolute and per cent dimensions. A significant increase only was observed for aldosterone serum levels. Percentage

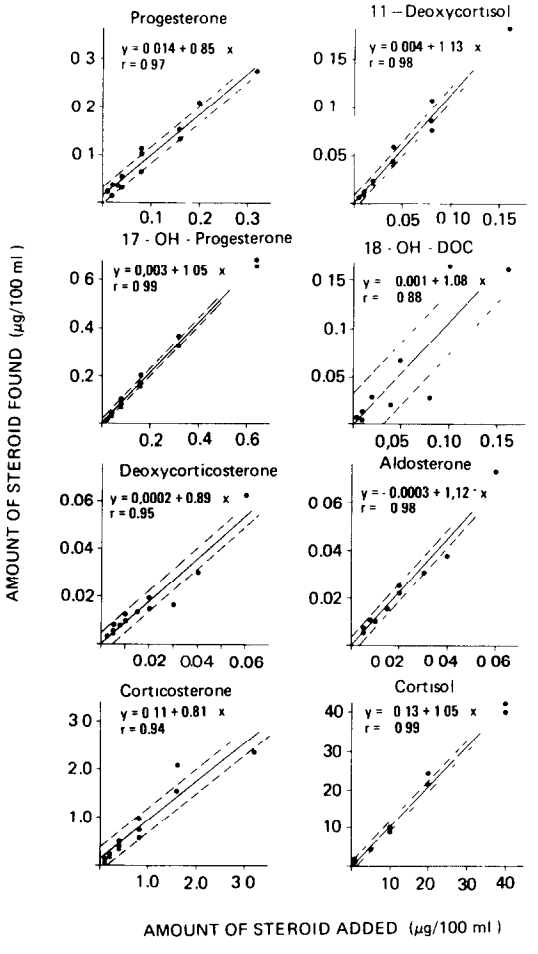

Fig. 3 Correlations between unlabelled steroid amounts added and estimated. r = Coefficient of correlation. Dotted lines indicate standard deviations of regression lines.

18-OH-DOC serum levels rose—though not significantly—to 150%. Serum cortisol levels exhibited a significant fall. All other serum steroid concentrations remained nearly unaltered.

DISCUSSION

Adequate studies on the physiology of adrenal steroid secretion in man or on pharmacological effects on adrenal steroid biosynthesis often require designs where frequent steroid estimations in short time intervals are necessary. Such purposes may only be feasible by the use of methods allowing the simultaneous estimation of multiple steroids from a single serum sample. Moreover, multiple steroid determinations are of obvious advantage in situations such as adrenocortical enzymatic defects in pregnancy or in hypersecretion states, such as Conn's or Cushing's syndrome. The present multiple steroid method represents the most extensive version reported hitherto concerning the main adrenal steroids.

Methodological aspects

The problems of establishing an assay for the simultaneous estimation of several serum steroids may be divided into three categories: effective separation, sufficient purification and sensitive quantitation of the steroids The adrenal steroids estimated by the present method vary considerably by polarity ranging from progesterone to its trihydroxylated derivative, cortisol, thus representing a considerable challenge for a reliable separation of these steroids. In none of the methods for multiple steroid estimation described hitherto, an adequate separation of these steroids in a single chromatographic system has been presented. The celite technique introduced and extensively applied for the separation of estrogens and gestagens by Abraham involves several, different solvents of increasing polarity [17].

In the present method, paper chromatography was chosen as separation and, in addition, as purification technique, because paper is easy to handle and blanks may be reliably eliminated by solvent extraction. As an adequate separation of the adrenal steroids on one paper strip is not realizable, a rough fractionation by liquid–liquid partition, therefore, had to precede the final chromatographic steps. By this technique, a high degree of separation is provided. Moreover, the direct monitoring of the higher labelled marker steroids and the strongly reproducible chromatographic ratios of the steroids allow a positive location of each steroid. The known difficult separation of corticosterone and 11-deoxycortisol by means of paper chromatography [7] was achieved by use of a more polar stationary phase in the chromatographic system III.

The method as described takes about two weeks to produce approximately 320 steroid values of 40 serum samples, if handled by one technician. This represents a much shorter operating time, if steroids would be estimated in single assay technique, but is more time consuming than the gas liquid chromatography technique [7]. At this moment, the method, therefore, only should be limited for scientific purposes. On the other hand, the method is very flexible, allowing the simultaneous estimation of additional adrenal steroids, e.g. androgens, 18-OH-corticosterone or cortisone.

The specificity of the present method is achieved by the chromatographic separation and the specificity of the individual antisera applied. With the exception

Table 9. Serum levels of adrenal steroids in healthy men. All subjects were in upright posture and on *ad lib.* diet

Steroid	Mean ± S.D ng/100 ml	Range ng/100 ml
Progesterone	17.9 ± 10	5–47
17-OH-Progesterone	179 ± 61	73–317
DOC	6.6 ± 2.6	3.1–13.7
Corticosterone	421 ± 217	85–915
11-Deoxycortisol	49 ± 27	15–103
18-OH-DOC	20 ± 11	9–51
Aldosterone	12 4 ± 5.0	5.3–24.4
Cortisol	14500 ± 4900	6000–22800

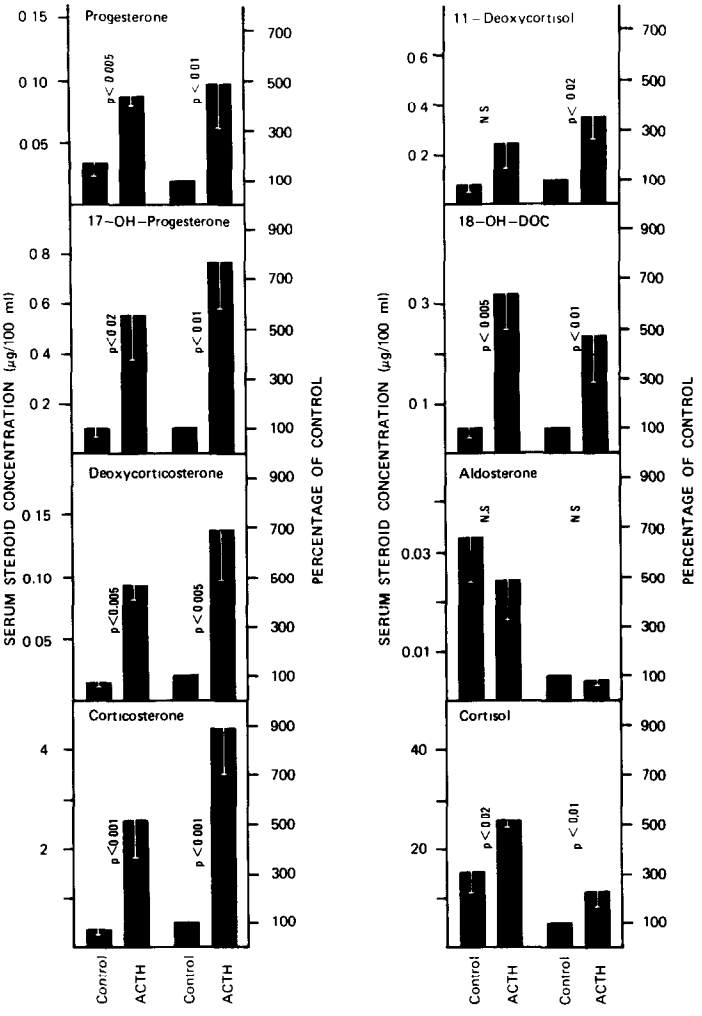

Fig. 4. Serum concentrations of adrenal steroids before (C) and 60 min after i.v. administration of 250 µg ACTH (Synacthen) as well as percentage values of control. Subjects (3 males, 2 females in menopause and 1 female in follicular phase) were on *ad lib.* diet. Control values were taken at 8 a.m. in upright posture and stimulated values after 1 h recumbent posture. NS = not significant on the $P < 0.05$ level.

of 18-OH-DOC, the cross reactions between steroids with similar chromatographic behaviour are negligible. The specificity of the 18-OH-DOC estimation is totally limited to an equivocal chromatographic separation from corticosterone, because the 18-OH-DOC antiserum has been shown to crossreact with corticosterone to the extent of 131%. The amount of [H^3]-corticosterone in the 18-OH-DOC area of chromatographed paper strips was found to be less than 0.5%, thus indicating that 18-OH-DOC estimation somehow represents a problematical step in this assay.

The precision, sensitivity and accuracy of the present multiple assay is within the range normally achieved in single steroid radioimmunoassays.

Normal values

The plasma or serum concentrations of progesterone and 17-OH-progesterone in normal men are exhaustively investigated during the last years. The levels of 17-OH-progesterone found in this study are slightly higher than values reported by other authors [18–23]. Mean serum progesterone concentrations reported in this study are much lower than those found by competitive protein binding methods [24–26], but similar or slightly lower than values reported by Abraham [27], Furuyama *et al.* [28], Youssefnejadian *et al.* [29], Tea *et al.* [22] and Vermeulen *et al.* [23] using radioimmunological techniques. The normal level of serum DOC measured by the present method is within the lower range of values reported by other authors [5, 7, 9, 30–35]. A similar agreement between normal values reported in literature and those measured by the present method was found for corticosterone [1–7, 36–38], cortisol [1–7, 39–41] and aldosterone [4–7, 34, 42–50]. The normal serum 11-deoxycortisol concentrations reported in the present study are the lowest presented in literature hitherto [2, 3, 5, 7, 51–56]. At present, there is relatively little information on the normal

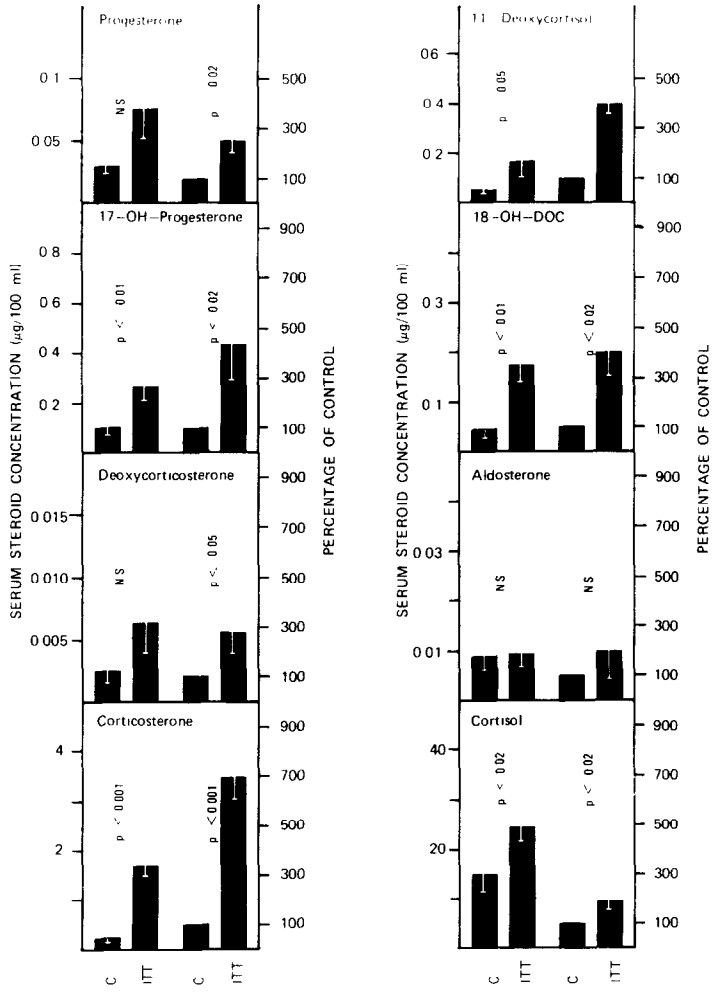

Fig. 5. Serum concentrations of adrenal steroids before (C) and 90 min after i.v. administration of 0.1 IE/kg body weight insulin as well as percentage values of control. Seven subjects (2 males, 2 females in follicular phase and 3 females in menopause) were studied. Conditions were as in the ACTH study. NS = not significant on the $P < 0.05$ level.

levels of 18-OH-DOC in human peripheral plasma. The levels found by the present method are much lower than those reported by Mason and Fraser [7, 57] using a gas liquid chromatography technique, but higher than the values presented by Chandler *et al.* [58] using a radioimmunological technique and those calculated by Melby *et al.* [59] on the basis of secretion and metabolic clearance estimations.

ACTH and insulin induced hypoglycemia cause a marked increase in serum concentrations of all steroids estimated by the present study with the exception of aldosterone. This observation confirms that all these steroids are primarily under anterior pituitary control. The unequivocal response of 17-OH-progesterone to ACTH stimulation indicates that the adrenal cortex is involved in 17-OH-progesterone secretion in males and females. A similar mechanism has to be assumed for progesterone. It is noteworthy that the increase of progesterone, DOC, 17-OH-progesterone, 18-OH-DOC and corticosterone is much more pronounced than that of 11-deoxycortisol and cortisol, if values are expressed as percentage of control. These findings confirm previous observations of an increase of the corticosterone/cortisol ratio after ACTH stimulation [2, 5, 6]. The stimulatory effect of ACTH on aldosterone secretion, meanwhile, is well established [60]. The failure of aldosterone increase after ACTH in the present study may be due to the fact that the control values were taken in upright posture and stimulated values after one hour at recumbent conditions, thus provoking inverse effects of the renin-angiotensin mechanism and the ACTH stimulation.

Influence of metyrapone on the adrenal steroidogenesis

The changes of the 8 a.m. serum steroid concentrations after midnight metyrapone administration reflect primarily the well established inhibitory effect of this drug on 11-hydroxylation. Apart from the considerable increase of DOC and corticosterone due to the enzyme blockade and additional ACTH stimu-

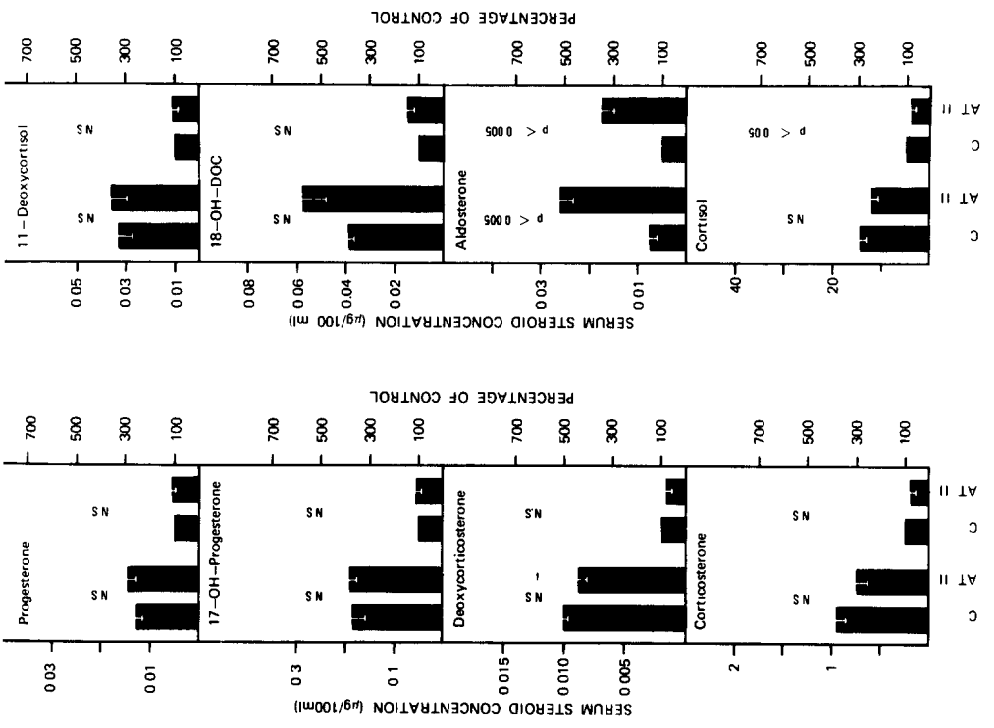

Fig. 7. 9 a.m. Serum concentrations of adrenal steroids before (C) and during angiotensin II (AT II) infusion (6 ng/kg/min) in 2 young men. Control values were obtained as means of two consecutive days, AT II values as means of three consecutive days of the infusion period. NS = not significant on the $P < 0.05$ level.

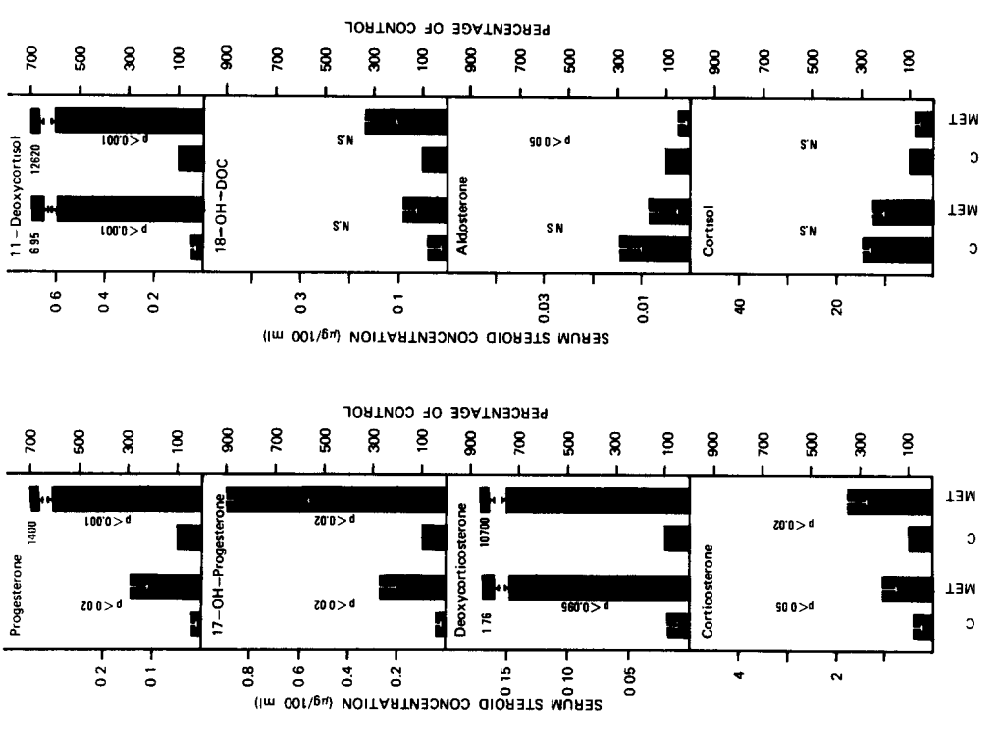

Fig. 6. 8 a.m. Serum concentrations of adrenal steroids before (C) and after oral administration of 30 mg/kg body weight metyrapone at midnight as well as percentage values of control. Subjects (2 males, 4 females in menopause and 2 females in follicular phase) were on *ad lib.* diet. Blood was taken in upright posture. NS = not significant on the $P < 0.05$ level.

lation, the serum concentrations of 17-OH-progesterone and progesterone also increase much more than after exclusive ACTH stimulation (Figs. 4 and 6). This might be explained by accumulation of these steroids caused by the 11-hydroxylase blockade or an additional slight inhibition of the 17- and 21-hydroxylase. The relatively high values of corticosterone and cortisol after 11-hydroxylase inhibition is due to a reduced enzyme blockade eight h after drug administration [61]. The relatively low values of 18-OH-DOC after metyrapone are similar to those of corticosterone, thus indicating an additional 18-hydroxylase inhibitory effect of the drug [62].

Influence of angiotensin II on adrenal steroid concentrations

Angiotensin II infused at sub-pressor doses had no significant effect on serum progesterone, 17-OH-progesterone, corticosterone, 11-deoxycortisol and 18-OH-DOC, while aldosterone levels rose and cortisol levels fell significantly. The significant fall of cortisol is consistent with findings of Oddie *et al.* [5] and Rayyis *et al.* [60].

Acknowledgements—The author is indebted to Prof. Dr. W. Oelkers for helpful discussions, to Prof. Dr. P. Koeppe, Department of Radiology and Nuclear Medicine, for providing computer facilities, to Dr P. Vecsei, Dr. R. Fraser and to the National Institute of Health for the generous gift of steroid antibodies.

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