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Simultaneous Radioimmunoassay for Corticosterone and Deoxycortisol in Human Serum: Sex Differences in the Mean Serum Concentrations[1])

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Summary: A specific radioimmunoassay is described which allows the simultaneous determination of serum corticosterone and deoxycortisol. The method involves extraction with dichloromethane, purification by paper chromatography in a modified *Bush*-system and quantitation by radioimmunoassay. The normal serum concentration of both steroids were found to be dependent on sex and menstrual cycle. Mean concentrations (± S. D.) in males, females (follicular phase) and females (luteal phase) were 4210 ± 2170 ng/l, 2410 + 1480 ng/l and 4390 ± 2320 ng/l for corticosterone and 499 ± 273 ng/l, 207 ± 152 ng/l and 335 ± 182 ng/l for deoxycortisol.

After adrenal stimulation by corticotropin itself or by insulin induced hypoglycemia, the serum concentrations of corticosterone became significantly higher than those of deoxycortisol. After oral administration of dexamethasone serum concentrations of both steroids were suppressed to levels below the limit of the normal range. One hour after oral metyrapone administration at midnight, serum corticosterone decreased, while serum deoxycortisol increased by a factor of about five. After eight hours serum concentrations of both steroids were increased considerably. Corticosterone attained levels slightly higher than the normal range and deoxycortisol rose to levels which were higher than the normal mean concentrations by a factor of about 500.

Simultaner Radioimmunoassay für Corticosteron und Desoxycortisol in Humanserum: Geschlechtsdifferenzen der mittleren Konzentration im Serum

Zusammenfassung: Es wird ein spezifischer Radioimmunoassay beschrieben, der die simultane Bestimmung von Corticosteron und Desoxycortisol im menschlichen Serum ermöglicht. Die einzelnen methodischen Schritte bestehen aus einer Extraktion mit Methylenchlorid, einer papierchromatographischen Reinigung in einem modifizierten *Bush*-System, sowie der quantitativen, radioimmunologischen Bestimmung. Die normalen Serumkonzentrationen beider Steroide sind abhängig vom Geschlecht und Menstruationszyklus. Die Normalwerte für Männer, Frauen in der follikulären Phase und Frauen in der lutealen Phase betragen 4210 ± 2170 ng/l, 2410 ± 1480 ng/l und 4390 ± 2320 ng/l für Corticosteron und 499 ± 273 ng/l, 207 ± 152 ng/l und 335 ± 182 ng/l für Desoxycortisol. Nach Stimulierung der Nebennierenrinde durch Corticotropin oder insulininduzierte Hypoglykämie stiegen die Serumkonzentrationen von Corticosteron signifikant höher als die von Desoxycortisol. Nach oraler Gabe von Dexamethason wurden die Serumkonzentrationen beider Hormone auf ein Niveau supprimiert, das weit unterhalb des Normalbereiches lag. Eine Stunde nach oraler Gabe von Metopiron um Mitternacht fiel die Serumkonzentration von Corticosteron ab, während die von Desoxycortisol um einen Faktor von etwa 5 anstieg. Nach 8 Stunden waren die Serumkonzentrationen beider Steroide beträchtlich angestiegen. Corticosteron erreichte Konzentrationsspiegel, die leicht über dem Normalwert lagen. Dexoxycortisol stieg an bis auf Werte, die um den Faktor von etwa 500 über dem Normalwert lagen.

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Introduction

Over the past few years several methods allowing the simultaneous determination of adrenal steroids have been published. The advantages of a simultaneous estimation of steroids for clinical purposes have been discussed elsewhere (1–4). In all the methods using competitive protein binding techniques as a final quantitation step, steroids have to be separated from the majority of other compounds prior to the radioimmunoassay. Methods for the separation of corticosterone and deoxycortisol reported hitherto are limited to thin-layer (4) or LH-20-Sephadex column chromatography (2). Separation of these two steroids by paper chromatography has not yet been reported.

Mean plasma concentrations of corticosterone and especially of deoxycortisol in normal subjects exhibit a considerable range of variability (range for corticosterone: 4–10 μg/l; range for deoxycortisol: 0.6–10 μg/l). Little information is available concerning the sex differences of these two steroid hormones. In the present paper a sensitive and specific radioimmunoassay for corticosterone and deoxycortisol is described using paper chromatography as separation technique. Furthermore, we report the normal ranges, sex differences and changes after adrenal stimulation and suppression, for the serum concentrations of both steroids.

Materials and Methods

Solvents and reagents

Analytical grade chemicals were used throughout. Two-fold distilled water, benzene, n-hexane and methanol were purified by charcoal extraction. Borate buffer (pH 8) containing 20 g/l ethylene glycol and 6 g/l human γ-globulin (Kabi) was used for incubation. Dextran (Dextran-70, Pharmacia, Upsala) and Norit A (Serva, Heidelberg) were used for the separation procedure. The scintillation mixture "Insta Gel" was supplied from Packard Instruments. Tritium was measured in a liquid scintillation spectrometer (Packard Instruments, model 3385).

Pipettes and counting vials were of the disposable type. Semi-automatic Hamilton syringes were used for the assay pipetting procedures. Conical extraction tubes were washed with diluted detergent for several hours and thoroughly rinsed with distilled water, followed by two washings with dichloromethane. Whatman-2-paper strips were extracted chromatographically with methanol for at least 24 hours.

The unlabelled corticosterone (21, 11-dihydroxy-4-pregnene-3, 20-dione) and deoxycortisol (21, 17-dihydroxy-4-pregnene-3, 20-dione) were purchased from E. Merck (Darmstadt). [^{3}H]-corticosterone (S. A. 54 Ci/mmol) and [^{3}H]deoxycortisol (S. A. 43 Ci/mmol) were obtained from New England Nuclear Corporation. Steroid standards were dissolved in ethanol and stored at − 20 °C. The labelled steroids were purified by paper chromatography fortnightly, dissolved in ethanol and stored at 4 °C.

Antisera for corticosterone and deoxycortisol were raised in rabbits, immunized with corticosterone- and deoxycortisol-21-hemisuccinate-bovine serum albumin conjugate, respectively (5).

The final antiserum dilutions (in 0.4 ml) were 1:30 000 for the corticosterone assay and 1:100 000 for the deoxycortisol assay.

Sample preparation

Serum and plasma samples

Blood was drawn from the cubital vein into plastic tubes and allowed to stand for two hours at room temperature for the preparation of serum samples and about 15 minutes for the preparation of plasma samples. After centrifugation the serum fraction was separated and stored at −20 °C until used for analysis. Samples were taken between 8 a. m. and 11 a. m.

Extraction and chromatographic separation

Known quantities of [^{3}H]corticosterone (about 444 Bq (12 nCi)) and [^{3}H]deoxycortisol (about 133 Bq (3.6 nCi)) were mixed with a serum sample (1–3 ml) in conical glass tubes. After extraction with 8 ml of dichloromethane for 10 minutes and centrifugation at 3000 rev./min the organic phases were evaporated to dryness under a stream of nitrogen at 35 °C. The residue was redissolved in 150 μl of dichloromethane and chromatographed on paper in a modified *Bush* system, consisting of n-hexane/benzene/methanol/water (60 + 40 + 35 + 65 by vol.). The [^{3}H]corticosterone area was located on paper strips by radio scanning (Packard Instruments, model 7200). As the amount of [^{3}H]deoxycortisol was too low to be monitored, the deoxycortisol area was identified by the R_B ratio, defined as migration of deoxycortisol/migration of corticosterone. Migration is the distance in cm from the origin to the midpoint of the steroid area. This ratio R_B was derived from standard strips which were loaded with a higher activity of each steroid (about 7400 Bq (0.2 μCi)) and developed in each chromatographic run together with the sample strips. The corticosterone and deoxycortisol areas were eluted with 3 ml of methanol.

Radioimmunoassay

The procedure for radioimmunoassay was essentially the same for both steroids. The methanolic eluates were evaporated to dryness under a stream of nitrogen at room temperature. The residues were redissolved in borate buffer (1500 μl for corticosterone and 500 μl for deoxycortisol). Aliquots (500 μl for corticosterone and 200 μl for deoxycortisol) were pipetted into counting vials for estimating [^{3}H]recovery. Three 100 μl aliquots representing three-fold dilution samples of the original buffer solution were used for radioimmunoassay. Standard curves were set up in triplicate. The quantities of unlabelled steroid dissolved in 100 μl borate buffer ranged from 3.12 to 800 pg in steps of two fold dilution. Competing [^{3}H]steroid (370 Bq (10 nCi) for corticosterone and 259 Bq (7 nCi) for deoxycortisol) dissolved in 100 μl borate buffer and 200 μl of antiserum buffer solution were added both to the unknown and standard samples. After incubation at 4 °C for 16 h separation of free and bound steroid was achieved using the dextran-coated charcoal method.

The radioimmunoassay data were evaluated and the standard curves were plotted with a modular constructed computer program (6). The "spline-function"-method was used as standard curve fitting model for the standard curve (7). All operating procedures were done with an IBM-1800 computer.

Results

Paper chromatography

The chromatographic properties of the two steroids in the modified *Bush* system are shown in figure 1. The distance of migration after 20 h was about 23 cm for corticosterone. The R_B ratio was found to be 1.16 ± 0.01 (S. D.) in 20 different assays.

Antisera

Table 1 demonstrates the physicochemical parameters of the corticosterone- and deoxycortisol-antibody reactions.

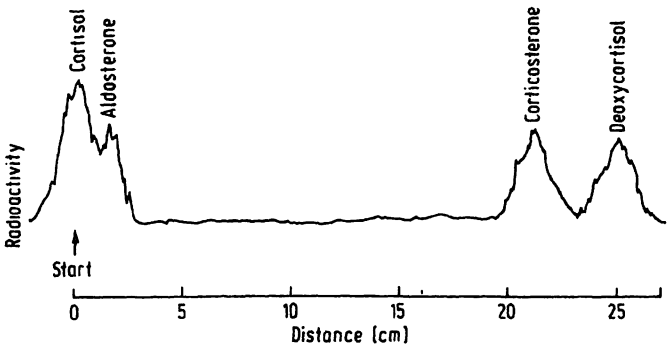

Fig. 1. Radiochromatogram of a steroid paper chromatography. System: n-hexane/benzene/methanol/water (60 + 40 + 35 + 65 by vol.). Time of equilibration: 20 hours. Time of chromatography: 20 hours.

Tab. 1. Physicochemical parameters of steroid-antibody complexes.

Steroid	Mean Affinity Constant [l/mol]	Capacity of Binding Equivalents [mol/l]	Dissociation Rate [s^{-1}] r_1	r_2	Half-Life Time [min] $\tau^1_{1/2}$	$\tau^2_{1/2}$
Corticosterone	$2.9 \cdot 10^9$	$2.3 \cdot 10^{-6}$	$5.2 \cdot 10^{-4}$	$3.8 \cdot 10^{-5}$	22	300
Deoxycortisol	$3.3 \cdot 10^9$	$1.6 \cdot 10^{-5}$	$6.3 \cdot 10^{-4}$	$6.5 \cdot 10^{-5}$	18	177

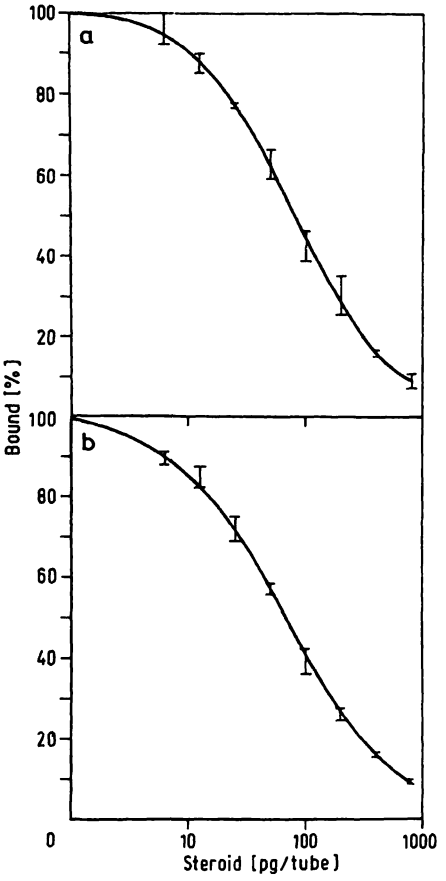

Fig. 2. Representative standard curves of corticosterone (a) and deoxycortisol (b) radioimmunoassay. Calculation and plotting was done by the "spline-function"-model (7).

Equilibrium constants as well as binding capacities were determined by antibody saturation. The resulting saturation curves were evaluated by a *Scatchard* plot.

Rates of dissociation of the ^{3}H-labelled steroid-antibody-complexes at 4 °C were determined by preincubation for 20 h followed by the addition of 4000 pg of unlabelled steroid. ^{3}H-measurement of the supernatant as a function of time permitted the estimation of the dissociation rate. The semilogarithmic plot of the dissociation of both steroid complexes yielded a curve indicating the existence of at least two antibody species with different physicochemical behaviour. The values of the two different rates were obtained by extrapolation of the two asymptotes of the hyperbolic curve.

Standard curve parameters

Figure 2 represents the computer plots of a standard curve for corticosterone and deoxycortisol, respectively. The mean sensitivites (2 standard deviations of the zero point) were 5.8 ± 3.7 (S. D.) pg for corticosterone and 7.4 ± 9.2 (S. D.) pg for deoxycortisol. These values corresponded to 59 ng/l for corticosterone and to 25 ng/l for deoxycortisol if 3 ml of serum were used. The amount of unlabelled steroid displacing 50% of the labelled steroid from the antibody was 111.4 ± 15.7 (S. D.) pg for corticosterone and 92.4 ± 11.8 (S. D.) pg for deoxycortisol, corresponding to 1142 ng/l and 920 ng/l, respectively, for a 3 ml sample.

Assay parameters

The *overall specificity* of the present assay technique is an expression of the specificity of the antibody together with that of the chromatographic separation.

The applied chromatographic system provides a high degree of separation of corticosterone and deoxycortisol from the major C_{21}-steroids. The cross reactions of the antibodies with certain other dihydroxylated progesterone derivatives, that exhibit chromatographic behaviour similar to that of corticosterone and deoxycortisol, were negligible (5).

Recovery of added [^{3}H]corticosterone and [^{3}H]deoxycortisol after chromatography averaged 65.6 ± 10.4 (S. D.) % and 48.8 ± 10.1 (S. D.) %, respectively.

Assay blanks were studied in distilled water and "charcoal-stripped" serum. The blanks arising in 3 ml water were 44 ± 59 (S. D.) ng/l (n = 9) for corticosterone and 31 ± 18 (S. D.) ng/l (n = 7) for deoxycortisol. The blank

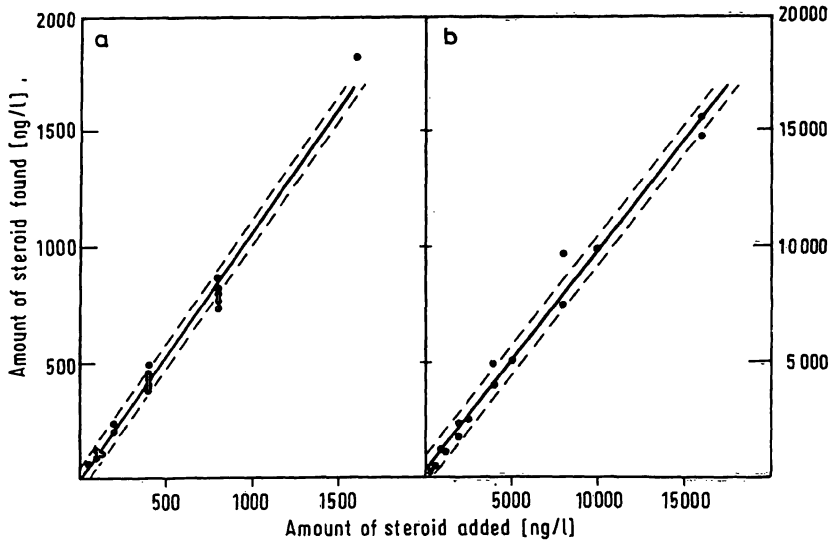

Fig. 3. Correlation between steroid amounts added and estimated. Broken lines indicate standard deviations of regression lines.
a) Deoxycortisol: equation of regression: $y = -0.6 + 1.07 \cdot x$; coefficient of correlation: 0.992.
b) Corticosterone: equation of regression: $y = 26.1 + 0.95 \cdot x$; coefficient of correlation: 0.992.

values in 3 ml "charcoal-stripped" serum amounted to 77.3 ± 72 (S. D.) ng/l (n = 14) for corticosterone and to 56 ± 59 (S. D.) ng/l (n = 8) for deoxycortisol. Paper blanks were lower than the sensitivity of the standard curve.

Accuracy was studied by the estimation of added known quantities of steroid, corresponding to endogenous levels. Regression analysis for the relationship between the amounts added and measured were computed (fig. 3). The best-fit straight lines have slopes which are not significantly different from the theoretically expected ones (b = 1).

The *intraassay and interassay variabilities* of both assays at different concentration levels are shown in table 2. The concentrations studied correspond to endogenous levels. The coefficient values indicate a satisfactory precision.

Figure 4 demonstrates the *correlations* of steroid concentrations found in *plasma and serum*. The statistical

Tab. 2. Intraassay and interassay variation of corticosterone and deoxycortisol radioimmunoassay.

Steroid	Intraassay Variation			Interassay Variation		
	Mean [ng/l]	N	Coefficient of Variation [%]	Mean [ng/l]	N	Coefficient of Variation [%]
Corticosterone	780	12	12.9	680	10	13.9
	1460	11	10.0	1290	9	10.6
	5360	10	9.0	5080	6	9.4
	9749	11	14.9	10190	9	13.0
	12880	11	10.2			
Deoxycortisol	172	9	11.8	194	7	7.6
	340	5	11.0	467	7	18.4
	377	10	8.6	838	5	15.9
	587	10	8.3			
	1397	10	9.7			

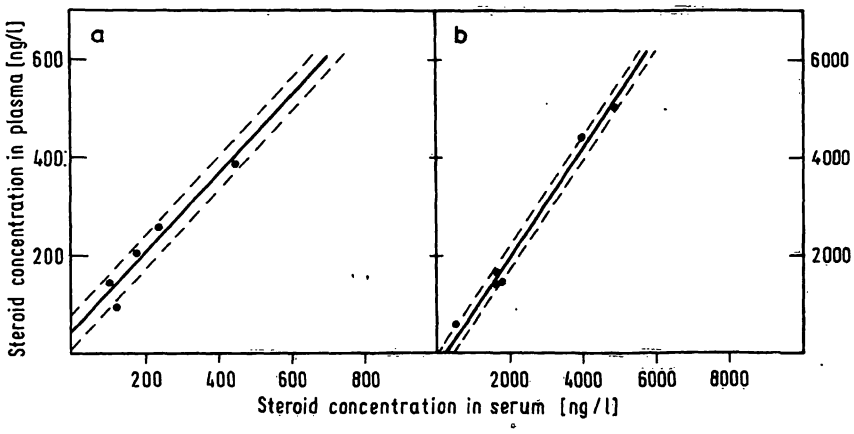

Fig. 4. Correlation between serum and plasma concentrations for deoxycortisol (a) and corticosterone (b). Broken lines indicate standard deviations of regression lines. Equation of regression: $y = 3.7 + 0.81 \cdot x$ for deoxycortisol and $Y = -21.8 + 1.1 \cdot x$ for corticosterone. Coefficients of correlation: 0.964 for deoxycortisol and 0.993 for corticosterone.

evaluation exhibited no significant difference between plasma and serum concentrations both of corticosterone and deoxycortisol.

Normal values

The serum concentrations of corticosterone and deoxycortisol were measured in a series of normal males, and in normal females in the follicular and luteal phase. All the females studied had a normal menstrual cycle. The mean concentration, number of subjects studied and range of each group as well as statistical values of comparison are demonstrated in table 3.

Tab. 3. Normal serum concentrations of corticosterone and deoxycortisol in normal subjects and statistical comparison of mean values. NS = not significant.

Steroid		Males ♂	Females (follicular) ♀ F	Females (luteal) ♀ L
Corticosterone	Mean [ng/l]	4210	2410	4390
	N	31	16	15
	range [ng/l]	850–9150	575–5400	1750–10270
	Comparison: ♂ – ♀F		$p < 0.01$	
	♂ – ♀L		NS	
	♀F – ♀L		$p < 0.01$	
Deoxycortisol	Mean [ng/l]	499	207	335
	N	18	14	12
	range [ng/l]	152–1036	46–504	137–734
	Comparison: ♂ – ♀F		$p < 0.002$	
	♂ – ♀L		NS	
	♀F – ♀L		NS	

Significant differences between males and females in the follicular phase were found for both steroids, while differences between males and females in the luteal phase were not significant. Mean serum concentrations were significantly different between females in the follicular phase and females in the luteal phase for corticosterone, but the corresponding mean values for deoxycortisol were not significantly different.

Changes after stimulation and suppression of adrenal cortex

Corticotropin was administered to normal subjects between 8 a. m. and 9 a. m.. Figure 5 shows a marked increase of both steroids. After 90 min, the mean level of 5 subjects rose from 4060 ng/l to 31 440 ng/l for corticosterone and from 970 ng/l to 3596 ng/l for deoxycortisol. The percentage increase of serum corticosterone was significantly higher than that of deoxycortisol ($p < 0.05$). The effects of insulin-induced hypoglycemia on serum corticosterone and deoxycortisol were studied in normal subjects. Figure 6 demonstrates the considerable increase of both steroids, which begins after about 30 min. In

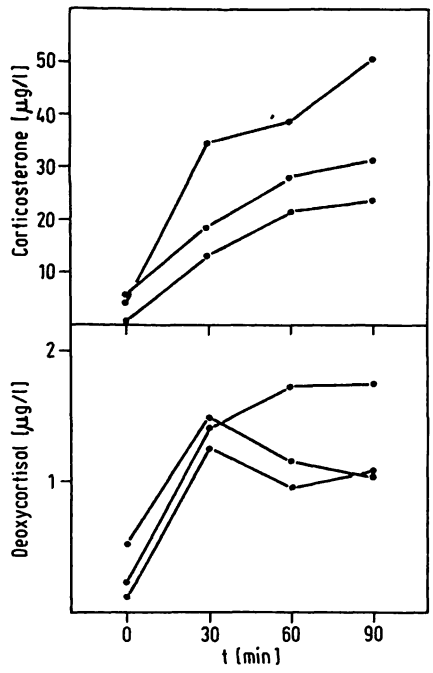

Fig. 5. Changes in serum concentrations of corticosterone and deoxycortisol after i. v. injection of 250 µg corticotropin between 8 a. m. and 9 a. m.

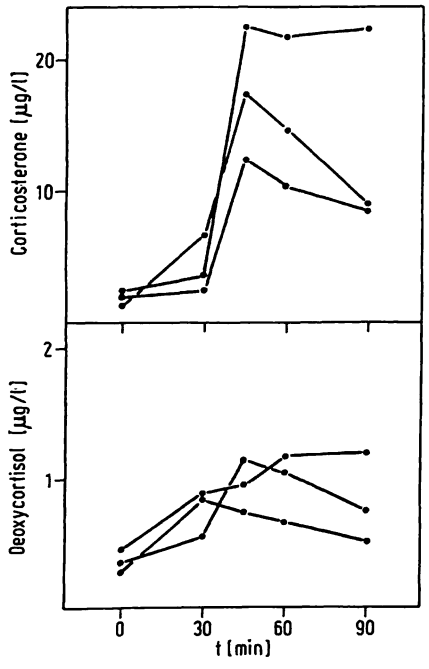

Fig. 6. Changes in serum concentrations of corticosterone and deoxycortisol after i. v. injection of 0.1 IE/kg body weight of insulin between 8 a. m. and 9 a. m.

6 subjects, mean serum corticosterone rose from 2060 ng/l to 19 535 ng/l after 90 min and mean serum deoxycortisol rose from 570 ng/l to 2063 ng/l after 90 min. As in the corticotropin stimulation, the percentage increase of corticosterone was significantly higher than that of deoxycortisol ($p < 0.01$). Figure 7 shows the

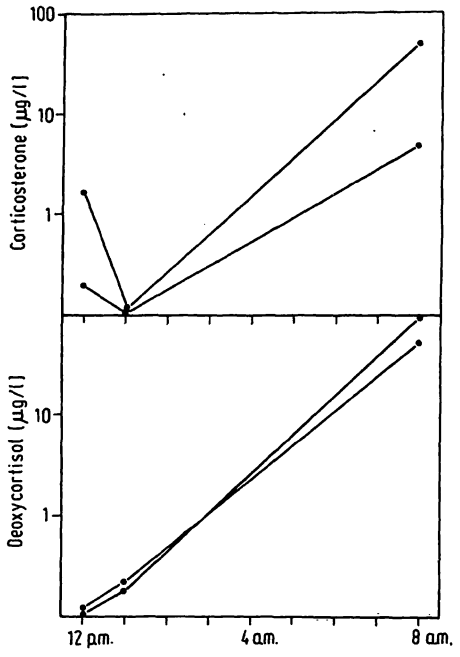

Fig. 7. Serum concentrations of corticosterone and deoxycortisol before and after oral administration of 30 mg/kg of metyrapone at midnight.

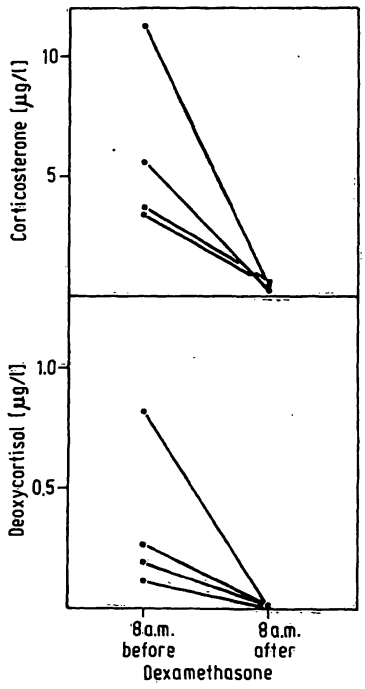

Fig. 8. Serum concentrations of corticosterone and deoxycortisol before and after oral administration of 1 mg dexamethasone.

changes of serum corticosterone and deoxycortisol after oral administration of metyrapone at midnight. One h after administration, serum deoxycortisol increased slightly, while serum corticosterone decreased to the level of the detection limit. These changes are caused by the inhibition of 11-hydroxylase due to metyrapone. Up to 8 a. m. deoxycortisol increased markedly, and corticosterone rose to levels slightly higher than the upper end of the normal range, although enzyme blockade obviously was still effective.

Figure 8 shows changes of serum corticosterone and deoxycortisol before and after oral administration of 1 mg dexamethasone. Mean serum concentrations of both steroids decreased below the lower limit of the normal range.

Discussion

A radioimmunoassay procedure has been described for the simultaneous measurement of the serum concentrations of corticosterone and deoxycortisol. A few methods have been published for the simultaneous estimation of these two steroids (1–4), but in no procedure paper chromatography was used for the separation which is necessary for every simultaneous steroid assessment. Paper chromatography was preferred to other techniques, because paper can be easily and reliably cleaned by solvent extraction, overnight development can be used and positive location of each sample is simple and non-destructive if radioactive tracer is included.

Recently, *Mason* et al. (1) demonstrated that corticosterone and deoxycortisol are difficult to separate by means of paper chromatography in the commonly used *Bush* systems. The use of a more polar stationary phase, as described here, provides a sufficient separation and abolishes further purification steps. The use of solvents purified by charcoal and of paper strips intensively washed with methanol eliminated the assay blanks nearly quantitatively as demonstrated by the blank studies in distilled water and on paper. The very low steroid amounts found in "charcoal-stripped" serum probably were due to insufficient stripping procedures.

Accuracy and precision of the method have proved to be adequate and similar to those commonly achieved in radioimmunoassays of individual steroids.

The normal ranges for serum concentrations of corticosterone shown in table 3 are similar to those reported in the literature (1–4, 8–13). Sex differences in serum corticosterone have been reported by *West* et al. (13), who found higher values in females (6550 ng/l) than in males (3960 ng/l). These results cannot be supported by the present study. This discrepancy may be explained by the well-known great variability of corticosterone serum concentrations due to episodic secretion and sensitive dependence on stress. The normal range of serum deoxycortisol concentrations measured by the present method is the lowest hitherto reported in the literature (tab. 4). Only *Oddi* et al. (3) using a double isotope derivative dilution technique reported comparable values (mean serum deoxycortisol: 600 ± 500 ng/l). Using an individual radioimmunoassay for serum deoxy-

Tab. 4. Comparison of plasma or serum deoxycortisol in normal subjects determined by different methods and authors. P = plasma; S = serum; DID = double isotope dilution; GLC = gas-liquid chromatography; CPB = competitive protein binding; PSR = *Porter Silber* reaction; RIA = radioimmunoassay;

Reference	Method	N	Mean ± S.D. [µg/l]	Material	Range [µg/l]
Mason (1)	GLC		4	P	0.4–4.0
Newsome (2)	CC		1.8	P	
Oddie (3)	DID	16	0.6	P	0–20
Kolanowski (4)	CPB	34	1.1	P	
Spark (14)	CPB		8.0	P	
Jubiz (15)	CPB		10.0	P	
Waxmann (16)	PSR		2.0	P	
Vielhauer (17)	RIA		1.41	P	
Lee (18)	RIA		8.0	P	5.6–13.8
Mahajan (19)*)	RIA		2.0	P	
Present study	RIA	18	0.49 ♂	S	0.15–1.0
		26	0.27 ♀**)	S	0.09–0.6

*) Dexamethasone suppressed.
**) Values represent the mean of females in the follicular and luteal phase.

cortisol without chromatographic purification, we found a mean deoxycortisol concentration of 1540 ± 935 (S. D.) ng/l in a series of 24 normal males and 17 normal females. This value is similar to the mean concentration of 1410 ng/l reported by *Vielhauer* et al. (17), who used the same antibody in a radioimmunological method without chromatography. This indicates that the higher normal serum deoxycortisol concentrations may represent over-estimations probably due to blanks or cross reacting compounds. Sex differences in deoxycortisol have not been hitherto reported. In the present study, mean deoxycortisol values were found to be nearly two-fold higher in males than in females in the follicular phase. This sex difference is in agreement with those of other corticotropin regulated adrenocortical steroids, such as cortisol (20), deoxycorticosterone (21) or corticosterone (present study).

Adrenal stimulation by corticotropin itself or by insulin-induced hypoglycemia caused a marked increase of serum concentrations of corticosterone and deoxycortisol. This is explained by the pituitary control of the adrenocortical secretion of these steroids. It is of interest that the increase of corticosterone concentration is significantly greater than that of deoxycortisol. A similar relation has already been observed between corticosterone and cortisol after adrenocortical stimulation (4). Obviously, the secretion profile of deoxycortisol is more correlated to that of cortisol than to that of corticosterone.

The decrease of serum corticosterone and the increase of serum deoxycortisol one hour after metyrapone administration is quite well explained by the inhibition of the 11-hydroxylase. The marked increase of corticosterone 8 hours after metyrapone is not easily understandable. Probably, it is caused by an increasing insufficiency of enzyme blockade.

Acknowledgements

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