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Radioimmunoassay of 11β-Hydroxyandrostenedione in Laboratory Diagnostics of Selected Endocrine Disorders

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Summary: A specific radioimmunoassay of 11β-hydroxy-4-androstene-3,17-dione, an androgen of exclusively adrenal origin, is described and evaluated. The antiserum was raised in rabbits using 6β,11β-dihydroxy-4-androstene-3,17-dione-6β-hemisuccinate:bovine serumalbumin conjugate as an immunogen. The method has been used for determination of the plasma levels of the hormone in healthy subjects, including children, with regard to the possibility its use as a laboratory marker for monitoring corticoid therapy in patients with congenital adrenal hyperplasia and in patients with various endocrine disorders, under basal conditions and after dynamic tests. The plasma levels of 11β-hydroxyandrostenedione (means ± s.d.) in healthy persons at 8 a.m. were 8.69 ± 2.88 (men), 7.72 ± 2.85 (women), 8.73 ± 5.13 (boys) and 7.88 ± 5.23 (girls) nmol/l, respectively. The hormone concentrations followed the pattern of circadian rhythm of cortisol, they were increased markedly after corticotropin and suppressed by dexamethasone.

Introduction

11β-Hydroxy-4-androstene-3,17-dione (11β-hydroxyandrostenedione) is believed to be an androgen of exclusively adrenal origin, measurement of which could serve as an indicator of adrenal contribution to an overall androgen production (1–3). This is sustained by the observations that 11β-hydroxyandrostenedione levels follow the circadian rhythm of cortisol, its production is stimulated by both corticotropin and corticoliberin but not by human chorionic gonadotropin and supresses markedly by dexamethasone, and its pattern of production is independent of the menstrual cycle in women (2, 3). It originates from adrenal 11β-hydroxylation of androstenedione rather than from side chain cleavage of cortisol (4). In the study of its dynamics, its metabolic clearance rate, the main distribution volumes and both blood levels and urinary secretion rates were determined (5). Values close to those of its precursor androstenedione were obtained, the blood production rate being however lower than that of androstenedione, in agreement with the fact that a portion of the latter originates from the gonads.

In this paper a radioimmunoassay is described, using a highly specific antiserum raised with an immunogen prepared by a more effective technique than previously reported (4). The method has been used for determination of 11β-hydroxyandrostenedione in healthy subjects, including children (with a special regard to the possibility of laboratory monitoring of glucocorticoid therapy of children suffering from congenital adrenal hyperplasia, and in various endocrine disorders.

Materials and Methods

Steroids and reagents

[1,2,6,7-^{3}H]11β-Hydroxy-4-androstene-3,17-dione, specific radioactivity 3.81 TBq (103 Ci)/mmol, was prepared by sodium bismuthate oxidation of [^{3}H]cortisol (Radiochemical Centre, Amersham, U.K.) (6) as follows: To a solution of [^{3}H]cortisol (3.7 MBq = 100 μCi), dissolved in 1 ml of 50% acetic acid, 0.5 g of sodium bismuthate was added and mixed by a magnetic stirrer for 30 min. The solid was removed by centrifugation, washed repeatedly with acetic acid and following centrifugation the collected supernatants were diluted with water (2 ml). Approximately 10 mg of sodium metabisulphite was then added and the mixture was extracted with dichloromethane. The organic extract was washed with 0.1 mol/l sodium hydroxide and water, dried over sodium sulphate and after evaporation of the solvent, chromatographed on DC-Alufolien Kieselgel 60/Kieselgur F-254 (Merck, F.R.G.) in the system dichloromethane — methanol 97 + 3 (by vol.) together with an authentic 11β-hydroxyandrostenedione. The radioactivity corresponding to the standard (R_f = 0.25) was detected by scanning, then eluted with ethanol. The yields of the product depended on the purity of the starting [^{3}H]cortisol and averaged usually 60—80%.

Non-radioactive steroids were from Koch and Light (U.K.) or Steraloids (Pawling, USA). High-purity standard 11β-hydroxyandrostenedione was kindly supplied by M.R.C. (U.K.). Sephadex G-25 and Dextran T-70 were from Pharmacia (Sweden), active charcoal (Norit A) from Serva (F.R.G.). The scintillation fluid contained 4 g PPO, 50 mg POPOP (Koch + Light) and 20 ml of methanol in 1 liter of toluene. All other chemicals and solvents were of Czechoslovak origin, analytical grade. Diethyl ether for extraction was freed of peroxides and distilled.

Synthesis of the immunogen

6β,11β-Dihydroxy-4-androstene-3,17-dione 6β-hemisuccinate was prepared from cortisol by a novel high-yield photochemical 6β-hydroxylation procedure of *Kasal & Smolíková* (7). It was attached to bovine serum albumin by the mixed-anhydride method of *Erlanger* et al. (8) and purified by Sephadex G-25 gel filtration. The steroid/albumin ratio, assessed from the difference of absorbancies at 254 nm of equimolar solutions of the product and albumin was 18.

Immunization and characterization of antisera

Four female rabbits were immunized with 100—200 μg portions of the immunogen in 1 ml of complete *Freund*'s adjuvant-saline mixture (1 + 1), by multiple intradermal injections on the dorsal area, at three-weeks intervals (9). The working dilution of the antiserum used in the radioimmunoassay was 1 : 20 000. The antisera were lyophilized and stored at −20 °C.

Cross-reactions of steroids with the antisera were estimated by the 50% displacement method (10) under conditions of the assay. The apparent intrinsic association constants were determined at three different temperatures (4, 24 and 37 °C) by a modified (11) *Scatchard* analysis of the standard curves. From the data obtained, the changes of thermodynamic parameters, i.e. the reaction enthalpy and entropy, were calculated according to *Walker & Keane* (12).

Sample preparation and radioimmunoassay

Plasma (100 μl) was extracted with diethyl ether (2 ml) and aliquots of the extracts (200 μl corresponding to 10 μl of plasma) in duplicate were evaporated to dryness in the assay tubes; the residue from this evaporation was processed directly by radioimmunoassay. The extraction recovery of added tracer averaged 96% and thus no correction for extraction losses was made. Extracts of pooled plasma, stripped of endogenous steroids by repeated treatment with charcoal, were used for the standard curve. This contained 0; 1.75; 125 pg/tube of 11β-hydroxyandrostenedione. To each tube (unknown samples and standards) [^{3}H]11β-hydroxyandrostenedione (335 Bq ≙ 10 000 dp. min^{-1}) and antiserum (100 μl each) in the assay buffer were added and the volume was adjusted to 0.5 ml with the buffer. Sodium phosphate 0.05 mol/l, pH 7.4, containing bovine serum-albumin (1 g/l) and sodium chloride (4.5 g/l) was used. After incubation at 4 °C overnight, a charcoal-dextran suspension (2.5 g/l Norit A, 0.25 g/l Dextran T-70, 500 μl) was added. The tubes were mixed briefly by vortexing, incubated for 20 min in an ice bath, then centrifuged at 1500 *g* for 10 min in a refrigerated centrifuge. Aliquots of the supernatants (500 μl) were transferred into the scintillation vials containing scintillation fluid, and after 4 h the radioactivity of tritium was measured in a Beckman LS-1801 liquid scintillation spectrometer. The concentrations of 11β-hydroxyandrostenedione were calculated from the standard curves in weighed log-logit transformation.

Healthy subjects, patients and performance of dynamic tests

The characterization of individual groups of healthy persons and patients with various endocrine disorders under study is given in tables 3 and 4.

Dynamic tests were carried out, if medically indicated, in selected groups of patients, as follows. The patients were hospitalized at least three days, after which blood was collected for determination of the basal hormone levels. On the next day at 7 a.m. they received a corticotropin infusion (Synacthen, Ciba, Switzerland, 0.25 mg in 400 ml of saline), which was repeated on two successive days. Blood was collected on the third day at the end of the infusion. After a 3-day pause, they received dexamethasone (4 × 0.5 mg a day, for the first two days and 4 × 2 mg during the third and fourth day). Blood was sampled after the second and fourth day of the test. Finally they were given a single injection of human chorionic gonadotropin (Praedyn-Spofa, Czechoslovakia, 3000 I.U. i.m.) plus dexamethasone (4 × 2 mg), for two days, after which blood was collected again.

Determination of other steroid hormones

All hormonal steroids were determined by radioimmunoassay, using antisera prepared in our laboratories, and tritiated radioligands. The reagents and procedures for determination of testosterone (13), dehydroepiandrosterone (14), androstenedione (15) and cortisol (16) were described previously. 17α-Hydroxyprogesterone was estimated by the method of *Hubl* et al. (17).

Results

Characterization of the antiserum

Specificity

The specificity of the antiserum used for the radioimmunoassay in terms of cross-reactions with 16 steroids is shown in table 1.

Tab. 1. Cross-reactions of sixteen steroids with the antiserum to 6β,11β-dihydroxy-4-androstene-3,17-dione-6β-hemisuccinate: bovine serum albumin.

Steroid	Cross-reaction (%)
11β-Hydroxy-4-androstene-3,17-dione	100
6β,11β-Dihydroxy-4-androstene-3,17-dione	69.4
11β-Hydroxy-5α-androstane-3,17-dione	30.9
4-Androstene-2,17-dione	3.20
5α-Androstane-3,17-dione	2.98
6β-Hydroxy-4-androstene-3,17-dione	1.94
3α,11β-Dihydroxy-5α-androstan-17-one	0.85
5β-Androstane-3,17-dione	0.71
11β,17β-Dihydroxy-4-androsten-3-one	0.70
4-Androstene-3,11,17-trione	0.58
3β-Hydroxy-5-androsten-17-one	0.29
3α,11β-Dihydroxy-5β-androstan-17-one	0.11
Testosterone (17β-hydroxy-4-androsten-3-one)	0.09
Cortisol	0.07
11β-Hydroxy-4-pregnene-3,20-dione	0.07
Progesterone	0.03
Estrone	0.007

Standard curve parameters and thermodynamic characteristics

The characteristics of the standard curves (specific binding, 50% intercepts), the apparent intrinsic association constants and derived thermodynamic parameters (i. e. the reaction enthalpy and entropy), measured at three assay temperatures (4, 24 and 37 °C) are given in table 2.

Tab. 2. Parameters of the standard curves and derived thermodynamic parameters at three assay temperature.

Temperature (°C)	Specific binding (%)	50% Intercept (pg/tube)	$K_{ass} \cdot 10^{-10}$ ($l \cdot mol^{-1}$)	ΔS (kJ/mol · K)	ΔH kJ/mol
4	33.2	33	7.3		
24	37.6	43	2.7	+ 0.092	−33.47
37	37.0	55	1.6		

Evaluation of the method

Precision

The intra-assay coefficient of variation for a control sample (concentration 4.90 nmol/l) was 6.3% (n = 15); the respective inter-assay coefficient of variation was 8.8% (n = 11).

Sensitivity

The least amount of 11β-hydroxyandrostenedione distinguishable from zero at 95% confidence level was 0.8 pg/tube, corresponding to 0.26 nmol/l.

Accuracy

The average recovery of 20 and 40 pg/tube of 11β-hydroxyandrostenedione added to the above control sample of plasma was 101%. Statistically undistinguishable results were obtained by the analyses of low, normal and high concentration of 11β-hydroxyandrostenedione (3.94, 5.01 and 9.89 nmol/l), irrespective of whether the thin layer chromatographic step (DC-Alufolien developed with dichloromethane — methanol 97 + 3 (by vol.)) was involved. In the latter case correction for recovery of added tracer (approximately 34 Bq of the tracer per tube) was performed.

11β-Hydroxyandrostenedione levels in plasma

Normal subjects

The concentrations of 11β-hydroxyandrostenedione in plasma of healthy men, women and children are summarized in table 3. A group of young healthy men (23—33 years) with idiopathic oligozoospermia (sperm count < $20 \cdot 10^9$/l) but without any endocrine disorder is also included.

Tab. 3. Plasma 11β-hydroxyandrostenedione levels in normal subjects (nmol/l).

Group (age, years)	n	11β-Hydroxyandrostenedione	
		mean ± s. d.	(range)
Men (19—65)	44	8.69 ± 2.88	(5.09—14.14)
Women (20—48)	107	7.72 ± 2.85	(3.25—12.4)
Boys (2—15)	21	8.73 ± 5.14	(0.3 —19.1)
Girls (0.5—16)	18	7.88 ± 5.23	(1.2 —17.2)
Men with idiopathic oligozoospermia (23—33)	30	8.84 ± 3.75	(3.57—15.7)

The daily profile of 11β-hydroxyandrostenedione and cortisol concentrations in a healthy women (32 years) is shown in figure 1.

Patients with endocrinopathies

The characteristic hormonal parameters (except of 11β-hydroxyandrostenedione) of patients under study and of the control group are given in table 4. Plasma levels of 11β-hydroxyandrostenedione in these patients under basal conditions and following stimulatory and suppression tests are shown in table 5.

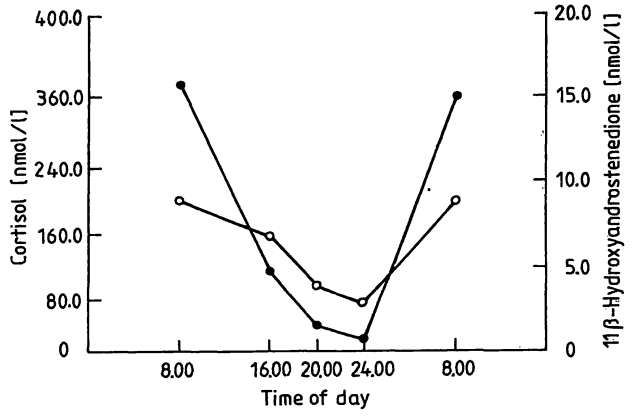

Fig. 1. Diurnal profile of cortisol and 11β-hydroxyandrostenedione (nmol/l) in a healthy women (32 years). Full circles: cortisol, open circles: 11β-hydroxyandrostenedione.

Children with congenital adrenal hyperplasia

Together with other hormonal steroids (i. e. testosterone, dehydroepiandrosterone sulphate, androstenedione and 17α-hydroxyprogesterone) 11β-hydroxyandrostenedione was measured in a group of 37 children (0.5—14 years) with various degrees of congenital adrenal hyperplasia due to the block of 21-hydroxylase. With one exception (11β-hydroxyandrostenedione concentration = 35 nmol/l) they all were under therapy with corticoids (hydrocortisone + fludrocortisone). The overall 11β-hydroxyandrostenedione levels in this group were 12.7 ± 10.2 nmol/l. No difference was found between adequately and unsufficiently treated children, as controlled by clinical features, i. e. mineral homeostasis, the degree of bone maturation corresponding to the chronological age, and absence of precocious secondary sexual development. 11β-Hydroxyandrostenedione levels in the former group (n = 9) averaged 14.4 ± 7.2 nmol/l as compared with 12.1 ± 11.1 nmol/l in the latter one (n = 27).

Tab. 4. Hormonal characteristics of patients with various endocrine disorders and of controls. Steroid concentrations are in nmol/l, means ± s. d. are given.

Group	n	Testosterone	Androstenedione	DHEA	17α-Hydroxy-progesterone
Healthy women (follicular phase)	30	1.90 ± 0.45	3.60 ± 0.90	16.1 ± 7.90	1.24 ± 0.27
Idiopathic hirsutism	45	2.11 ± 1.01	4.88 ± 1.80	50.9 ± 34.9	1.74 ± 2.21
Polycystic ovaries	19	2.41 ± 0.60	6.07 ± 2.40	48.9 ± 26.3	2.13 ± 1.73
Adrenal hyperplasia late manifestation	3	3.20 ± 0.38	20.2 ± 11.1	60.8 ± 2.62	above 50
Virilizing ovarian tumour	1	above 56	10.6	14.0	0.80

DHEA = unconjugated dehydroepiandrosterone

Tab. 5. 11β-Hydroxyandrostenedione levels in patients with various endocrine disorders under basal conditions and following dynamic tests (nmol/l). Means ± s. d. are given.

Group	n	Basal levels	Corticotropin	Dexa-methasone 2nd day of test	Dexa-methasone 4th day of test	Dexamethasone + Human chorionic gonadotropin
Idiopathic hirsutism	45	12.6 ± 4.6	34.6 ± 16.0	3.7 ± 2.3	2.7 ± 1.8	2.4 ± 1.4
Polycystic ovaries	19	11.8 ± 5.3	34.0 ± 9.8	4.4 ± 1.9	2.7 ± 1.3	2.9 ± 1.2
Adrenal hyperplasia late manifestation	2	31.3 ± 3.4	65.2 ± 29.5	2.7 ± 1.8	2.1 ± 1.1	2.3 ± 1.3
Virilizing ovary tumour	1	4.0	25.0	3.5	2.9	3.6

Discussion

The 11β-hydroxyandrostenedione radioimmunoassay described here fulfils the common criteria for routine determination of steroids in body fluids. It uses a specific antiserum, which distinguishes the measured hormone from the main potential competitors present in plasma, such as androstenedione, testosterone and their 11β-hydroxy- or 11-oxo- substituted analogues. High cross-reactivity was exhibited only by the 6β-hydroxyderivative of the measured steroid, used for the synthesis of the immunogen, and by the 5α-saturated derivative (11β-hydroxy-5α-androstane-3,17-dione), both present in blood in negligible concentrations.

The 11β-hydroxyandrostenedione levels determinated in healthy subjects were in agreement with values reported by others (1–3). It should be emphasized that all measurements were carried out in the morning, i.e. at the time of the secretory maximum (see fig. 1). The circadian rhythm of 11β-hydroxyandrostanedione was almost identical with that of cortisol. The concentration increase following corticotropin stimulation and the marked decrease after dexamethasone as well as the absence of the response to human chorionic gonadotropin confirm the previous findings and provide evidence for the adrenal origin of 11β-hydroxylated androgens (1–3, 18). Thus 11β-hydroxyandrostendione as well as 11β-hydroxytestosterone may be useful markers of adrenal contribution in hyperandrogenic states.

In two groups of patients studied, i.e. women with idiopathic hirsutism and with polycystic ovaries, somewhat increased levels of 11β-hydroxyandrostenedione were found, indicating the possible contribution of the adrenal cortex to the androgenization of these patients. In one women with virilizing ovarian androblastoma, confirmed by surgery, the normal plasma value of 11β-hydroxyandrostenedione excluded an adrenal origin of hyperandrogenism. In 3 women with adrenal hyperplasia with late manifestation, significantly elevated levels of 11β-hydroxyandrostenedione were observed (31.3 ± 3.4 nmol/l, $p < 0.001$).

Furthermore, 11β-hydroxyandrostenedione was measured in children with congenital adrenal hyperplasia, in the search for another laboratory marker for the accurate follow up the adequacy of corticoid therapy. Increased 11β-hydroxyandrostenedione levels as compared with controls were recorded only in 11% of sufficiently treated patients (for comparison, 17α-hydroxyprogesterone was elevated in 14%). On the other hand, in the second, larger group of unsufficiently treated children, increased 11β-hydroxyandrostenedione concentrations were found in only 19% of patients, in contrast to 17α-hydroxyprogesterone which was increased in 85% of cases (unpublished, in preparation). These preliminary results indicate that 11β-hydroxyandrostenedione is not necessarily more suitable than 17α-hydroxyprogesterone for laboratory monitoring of glucocorticoid substitution therapy, especially when cortisol treatment is to be evaluated. At least a part of 11β-hydroxyandrostenedione may result from direct degradation of exogenous cortisol.

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