PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 563–568

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Plasma 11β-hydroxy-4-androstene-3,17-dione: comparison of a time-resolved fluoroimmunoassay using a biotinylated tracer with a radioimmunoassay using a tritiated tracer

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Received 18 October 2002; accepted 27 January 2003

Abstract

The plasma concentration of 11β-hydroxy-4-androstene-3,17-dione (11β) is very high in 21-hydroxylase deficiency, Cushing's syndrome, and hyperandrogenism of adrenal origin, and very low in congenital 11-hydroxylase deficiency and adrenal insufficiency. Thus, when plasma 4-androstenedione is elevated, it is useful to measure the plasma 11β level in order to determine the adrenal or ovarian origin of the hyperandrogenism.

To eliminate disadvantages related to the 11β radioimmunoassay (RIA), which uses a tritiated tracer, as well as the high cost associated with scintillation proximity assay (SPA), we developed a non-isotopic 11β assay that utilizes an 11β-biotin conjugate synthesized in our laboratory to measure time-resolved fluorescence after addition of streptavidin-europium to microtitration wells.

The analytical qualities of this assay are very similar to those of the radioimmunoassay using a tritiated tracer, and an extraction step followed by celite chromatography (which separates 11β from interfering plasma steroids) prior to a final radioimmuno-competition step. The correlation coefficient between 11β levels measured by time-resolved plasma 11β fluoroimmunoassay (TR-FIA) and RIA was 0.965.

Finally, the TR-FIA technique was more sensitive and of greater precision than the RIA method.

Keywords: 11β; Time-resolved fluoroimmunoassay

1. Introduction

It is difficult to determine the origin of hyperandrogenism in female patients. The adrenal gland is the probable origin in cases of hyperandrogenism with hypercorticism, and ovarian participation is suspected in cases of hyperandrogenism in the presence of large and polycystic ovaries. In the absence of such indicators, it is difficult to identify the source of hyperandrogenism. Localizing the etiology of hyperandrogenism on the basis of plasma 4-androstenedione and testosterone concentrations is impossible, since equivalent quantities of those two steroids are secreted by both the ovaries and adrenal glands.

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEA-S), which are basically of adrenal origin, could also be synthesized by the ovaries in hyperandrogenism [1]. 11β-Hydroxy-4-androstene-3,17-dione (11β) is a steroid considered to be of strictly or mainly adrenal origin (11β-hydroxylase is present in adrenal tissue, but absent in ovarian tissue) [2].

The 11β concentration is greatly increased following Synacthen injection, and decreased after dexamethazone. The plasma 11β concentration is very high in congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency, in Cushing's syndrome, and non-tumoral hyperandrogenism of adrenal origin [3]. It is very low in congenital 11-hydroxylase deficiency and in adrenal insufficiency [4].

On the other hand, the plasma 11β concentration was normal or decreased in patients with polycystic ovaries [3,4], and not elevated following ovarian stimulation by injection of hCG to patients treated by dexamethazone, which does not plead in favor of an ovarian origin for this steroid.

Finally, it is useful to measure the plasma 11β when 4-androstenedione increased, in order to distinguish the adrenal or ovarian origin of hyperandrogenism.

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0960-0760/03/$ – see front matter
doi:10.1016/S0960-0760(03)00077-3

Because of the disadvantages of ^{125}I and ^{3}H-11β, and because of the short lifespan of the ^{125}I-tracer (less than 2 months), we decided to develop a stable non-isotopic tracer for a sensitive new 11β immunoassay.

As previously stated [5], we report the development of a time-resolved plasma 11β fluoroimmunoassay (TR-FIA) and compare the results obtained by this method with results obtained in the same individuals by radioimmunoassay (RIA) using ^{3}H-11β.

The same anti-11β antibody was used in the both methods.

2. Materials and methods

2.1. Biological materials

Blood samples were drawn from laboratory sera and stored at −20 °C until 11β assay.

2.2. Tracer preparation

2.2.1. Synthesis of the biotinylated tracer (Scheme 1)

Isobutylchloroformate (35 μl, 0.22 mmol) was added to a cold (5 °C) solution of 11β-hydroxy-4-androstene-3,17-dione-3-carboxymethyloxime **1** (0.1 g, 0.27 mmol) and triethylamine (40 μl, 0.3 mmol) in dioxane. After 5 min of stirring, this solution was added to a solution of biotinyl-aminopropyl-ammonium trifluoroacetate (0.1 g, 0.25 mmol) and triethylamine (50 μl, 0.45 mmol) in 2 ml DMSO. After 4 h of stirring, the mixture was diluted in 10 ml cold water. Tracer **2**, which precipitated, was filtered and washed with 2 ml cold water and purified by column chromatography, using AcOEt:MeOH (98/2 (v/v)) as the eluent. Yield 65%; mp = 137–140 °C. ^{1}H NMR ($CDCl_3$): 1.05 (s, 3H, 19-CH_3); 1.37 (s, 3H, 18-CH_3); 2.65 and 2.75 (d and dd, 2H, CH_2–CH); 3.10 and 3.40 (2H, 2m CH_2–S), 3.45 (m, 1H, CHS), 4.40 (t, 1H, H-11); 4.50 (2H, s, CH_2, *E* and *Z* CMO); 5.65 and 6.30 (2s, 1H, H-4); 6.10 and 6.75 (2t, 2 NH, 2 CH_2-NH).

Acylation of biotinylaminopropyl-ammonium [6–8] with 11β-hydroxy-4-androstenedione-3-CMO afforded the biotinylated tracer in 65% yield. This product was shown by ^{1}H NMR to be a 55:45 mixture of *E*/*Z* stereoisomers.

2.3. Tracer and 11β solutions

2.3.1. Stock tracer solutions

A concentrated mother solution of 11β-biotin conjugate in ethanol was prepared and stored at +4 °C. An intermediate ethanol solution was obtained by diluting the mother solution 1/1000, and also kept at +4 °C.

Working solutions were prepared extemporaneously by diluting 30 μl of the intermediate solution at 224 ng/ml in 5 ml phosphate gelatine buffer (0.05 M, pH 7.4).

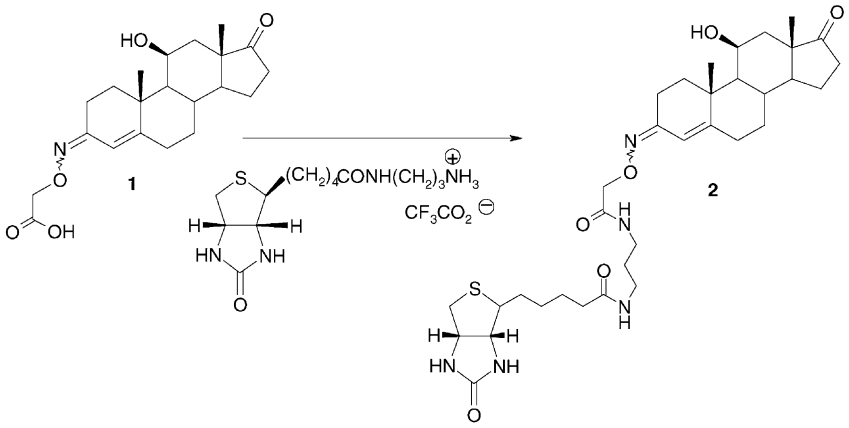
2.3.2. 11β standard solutions

An alcoholic mother solution containing 10 mg 11β in 100 ml ethanol was diluted 1/100 in ethanol. This intermediate solution was used to obtain an aqueous solution containing 12 ng 11β per milliliter of phosphate gelatine buffer. This standard solution was used to prepare seven calibrators with the following concentrations (in nmol/L): 19.84, 9.92, 4.96, 2.48, 1.24, 0.62 and 0.31. All 11β solutions were kept at +4 °C.

2.4. Preparation of microtitration plates

Microtitration plates (8 × 12 NUNC, Wallac reference 1244-660) were coated with 250 μl goat anti-rabbit antibody (4 mg/2 ml), that had been diluted 1/1000 in 0.05 M coating buffer (1.55 g anhydrous Na_2CO_3, 2.97 g $NaHCO_3$ qsp 1000 ml distilled H_2O, pH 9.6).

They were kept at room temperature overnight, then washed three times with Tween 20-containing saline (0.5/1000 ml). Free sites were saturated by adding 300 μl of the saturation solution. The plates were covered with sealing tape and kept at +4 °C.



Scheme 1. Synthesis of the biotinylated tracer.

Table 1
11β levels obtained by the direct RIA method and RIA method after chromatography

RIA	Group (women)	Reference	Normal (nmol/l)	Normal (ng/ml)
Direct	$n = 107$	[9]	7.72 ± 2.85	2.33 ± 0.86
	$n = 30$	[10]	5.2 ± 1.9	1.57 ± 0.57
Extraction	$n = 27$	[11]	3.5 ± 2.3	1.06 ± 0.70
+ chromatography	$n = 26$	[12]	4.0 ± 0.23	1.21 ± 0.07

2.5. 11β RIA

11β levels obtained by the direct method were higher than those of RIA methods carried out after chromatography, as reported in (Table 1). In view of this, and in order to obtain the most precise 11β levels, we chose to compare the 11β levels obtained by TR-FIA with levels obtained by the RIA method, which includes two purification steps: extraction and celite chromatography, prior to final radioimmuno-competition. The chromatography step separates 11β from interfering plasma steroids, particularly 4-androstenedione, which was eluted with pure isooctane (an apolar solvent), while 11β was eluted with isooctane/dichloromethane (70/30 (v/v)) (polar solvent).

The eluted fraction was evaporated and the dried residue was taken up by phosphate gelatine buffer. The 11β content was measured by RIA, using ^{3}H-11β and scintillation proximity assay (SPA) reagent [3], and the same anti-11β rabbit antibody was used in the TR-FIA described below.

2.6. Determination of the optimal antibody dilution and tracer concentration for TR-FIA

Anti-11β antiserum that had been kept at −20 °C at a dilution of 1/20, was diluted at 1/100 in assay buffer (0.05 M, pH 7.4) in the presence of 67.2 pg/well of biotinylated 11β tracer.

2.7. 11β TR-FIA

After adding minute doses of tritiated 11β to each plasma sample to monitor losses occurring during the extraction and chromatographic steps, 10 ml of cyclohexane/ethyl acetate (50/50 (v/v)) was added, and after vortexing, the phases were allowed to separate, the aqueous phase frozen, and the upper organic layer transferred for evaporation. The dried residue was redissolved in pure isooctane (1.5 ml) and introduced into a siliconized 5 ml glass pipette filled with celite + ethylene glycol for chromatography [3]. Finally, the 11β was eluted with isooctane + dichloromethane (70/30 (v/v)). 11β was simultaneously assayed (in duplicate) in this elution fraction by TR-FIA and RIA.

After washing the microtitration plate wells, the following reagents were added to each well: 50 μl of 11β standards, or of the same elutes as used for 11β RIA, then 50 μl of the diluted conjugate, 11β-biotin tracer, and finally 100 μl of anti-11β rabbit antiserum.

The plates were shaken at 350 rpm at room temperature for 3 h. The assay was stopped by washing the wells three times. A 200 μl aliquot of the solution of europium-labelled streptavidin that had been previously prepared by diluting (1/1000) the reagent in buffer (5 g bovine serum albumin (BSA), 4 ml Tween 20, 1 g NaN_3 dissolved in 1000 ml saline adjusted to pH 7.8 Tris–HCl) was then added to each well. The plates were shaken for 30 min, washed three times, removed from the machine, and turned upside-down. The europium was dissociated by adding 200 μl of enhancement solution to each well, then shaken at 350 rpm for 20 min.

Finally, time-resolved fluorescence was measured using a WALLAC 1232 Delfia fluorimeter.

3. Results

3.1. Validation of the plasma 11β TR-FIA

In Fig. 1, the binding of each calibrator expressed in pg/well (4.69, 9.37, 18.75, 37.5, 75, 150, 300, and 600) represents, respectively, 90, 79, 67, 54, 41, 30, 22, and 16% of the binding of standard zero.

The lowest amount of 11β that was significantly different from the zero value, calculated from 12 consecutive assays, was 1.1 pg/well with 2S.D., and 1.65 pg/well with 3S.D. The quantities of 11β that displaced 20, 50, and 80% of the tracer were 214, 31.7, and 9.2 pg/well, respectively. The corresponding values for RIA using ^{3}H-tracer were 997,182.7, and 35.8 pg/well.

The 11β antibody cross-reacted (relative 50% displacement values) with other steroids (which were not eluted in the 11β elution fraction) as follows: 4-androstenedione, 40%; testosterone, 1.86%; dihydrotestosterone, 0.6%; 5β-androstanedione, 21.7%; and androsterone, 4.3%.

The intra- and inter-assay variation, including the extraction and chromatography steps, were 7.2% and 10%, respectively.

3.2. Comparative determination of plasma 11β by TR-FIA and RIA

Plasma 11β concentrations in 62 consecutive patients measured by TR-FIA and by RIA were, respectively, from 1.98 nmol/l to 18.52 nmol/l and 2.65 nmol/l to 18.85 nmol/l. The correlation coefficient between 11β levels measured by TR-FIA and RIA was 0.965. The regression curve is shown in Fig. 2.

3.3. Accuracy

3.3.1. Recovery experiments

It was studied by adding five known amounts of 11β (1.65, 3.31, 6.62, 9.93, and 13.24 nmol/l) to one pooled serum

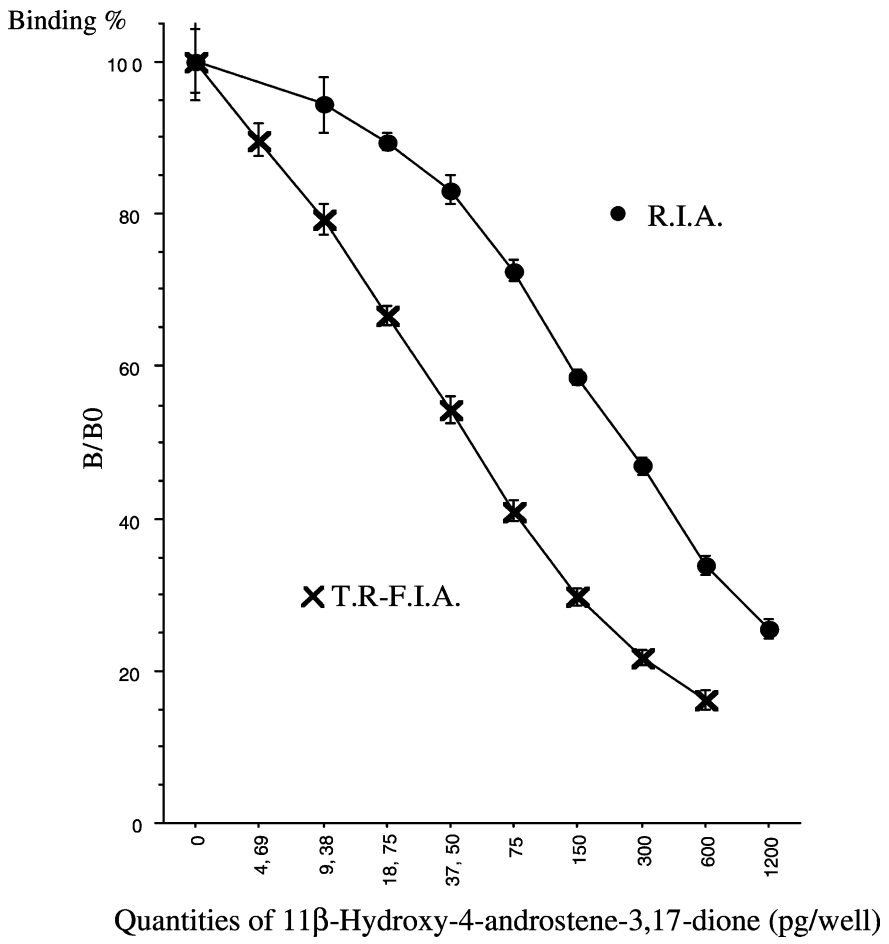


Fig. 1. Dose-response of curves of 11β TR-FIA and RIA. Means ± 2S.D. ($n = 12$) are plotted.

Table 2
11β levels (nmol/l) measured in a pooled serum sample before and after overloading with five different quantities of 11β

11β level			Recovery (%)
Pool (before overloading)	Theoretical (after overloading)	Measured (nmol/l)	
10.88	12.53	12.73/12.57/12.63	101.6/100.3/100.8
		12.57/12.40	100.3/98.9
Mean ± S.D. (CV%)		12.58 ± 0.12 (0.95)	100.40 ± 1 (1)
10.88	14.19	14.12/14.45/13.72	99.5/101.8/96.6
		14.55/14.12	102.5/99.5
Mean ± S.D. (CV%)		14.19 ± 0.33 (2.3)	100 ± 2.3 (2.3)
10.88	17.50	17.26/18.02/18.02	98.6/103/103
		16.96/17.16	96.9/98.1
Mean ± S.D. (CV%)		17.48 ± 0.50 (2.9)	99.9 ± 2.9 (2.9)
10.88	20.81	19.87/20.17/20.40	95.5/96.9/98
		21.10/20.47	101.4/98.4
Mean ± S.D. (CV%)		20.40 ± 0.46 (2.2)	98 ± 2.2 (2.2)
10.88	24.12	23.81/23.38/24.11	98.7/96.9/100
		23.68/23.21	98.2/96.2
Mean ± S.D. (CV%)		23.64 ± 0.35 (1.5)	98 ± 1.5 (1.5)

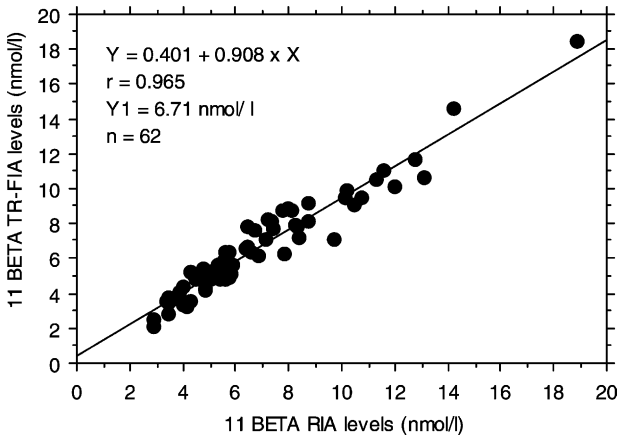


Fig. 2. Comparison of plasma 11β concentrations obtained in 62 patients by TR-FIA and RIA. Regression curve (Y = TR-FIA, X = RIA); correlation coefficient (r); means of serum 11β concentrations according to the method used ($X1$ = RIA, $Y1$ = TR-FIA).

Table 3
Dilution test: 11β levels (nmol/l) obtained in pure and diluted sera

Dilution	Serum 1	Serum 2
1/1	15.2	35.1
1/2	15.2	34.8
1/4	15.5	35.1
1/8	14.3	34.1
1/16		35.4
1/32		35.1
Mean ± S.D. (CV%)	15.1 ± 0.5 (3.3)	34.9 ± 0.5 (1.4)

sample containing a mean of 10.88 nmol/l (n = 10) before overloading. The theoretical 11β values after overloading were 12.53, 14.19, 17.50, 20.81, and 24.12 nmol/l. Each overload was assayed five times in duplicate. The percentages of the five overload recoveries were, respectively, 98.9–101.6%; 96.6–102.5%; 96.9–103%; 95.5–101.4%; and 96.2–100%, as seen in (Table 2).

3.3.2. *Dilution tests*

The 11β levels had been measured in undiluted sera and in sera diluted to 1/2, 1/4, 1/8, 1/16, and 1/32 (Table 3).

4. Clinical results

Several simultaneous 11β and 4-androstenedione assays have been used for many years to discriminate among hyperandrogenic states, for which it is useful to assay plasma 11β (theoretically not synthesized in gonads [2], in order to distinguish between adrenal and ovarian origins. (Table 4).

5. Discussion

In recent years, several steroid immunoassays have been developed that use biotinylated steroids as tracers.

Table 4
Simultaneous 11β and 4-androstenedione assays used to discriminate among the hyperandrogenic states

States	4-Androstenedione (nmol/l)	11β (nmol/l)
Normal ranges		
Adult women [3]	1.75–6.98	2.98–8.93
Adult men (n = 15) [13]	2.09–7.33	4.93–13.89
21-hydroxylase deficiency		
(n = 10) [3], (n = 2) [13]	15.68 and 24.09	7.32–32.34
11-hydroxylase deficiency		
(n = 3) [unpublished data; J. Fiet]	52.4, 129.2 and 153.6	0.33, 0.50 and 0.33
(n = 1) [4]		<1.5
Polycystic ovaries		
(n = 32) [4]	9.8 ± 3.1	5 ± 2.1
(n = 19) [9]	6.07 ± 2.40	11.8 ± 5.3
(n = 23) [10]	4.2 ± 1.8	6 ± 1.6
Secreting ovarian tumours		
(n = 6) [unpublished data J. Fiet]	12.2–25.2	6.94–10.91
Cushing's disease		
(n = 2) [13]	8.41 and 21.54	33.40 and 50.26

Biotinylaminopropyl-ammonium trifluoroacetate is a stable, well-crystallized biotinylation reagent. It is more nucleophillic than biotin hydrazides, and is easily acylated under mixed anhydride coupling conditions. Furthermore, isolation of the tracer is particularly simple [6]. The steroid–biotin conjugate is detected with streptavidin-peroxidase [14] or streptavidin-europium [15–18].

It is generally accepted that the linkage position of the bridge on the steroid nucleus should be the same in the tracer and in the immunogen used to raise antibodies. That is why we coupled a biotin derivative on the carboxymethyloxime (CMO) in position 3 of the steroid. It is to this 3CMO chain that bovine serum albumin is coupled in the immunogen. This coupling position has provided immunologically well-recognized biotinylated steroids, as we [15–18] and others [14–19] have previously reported.

We chose to detect the 11β-biotin conjugate with streptavidin-europium, and to measure europium fluorescence by time-resolved fluorescence.

The sensitivity of the TR-FIA was higher than that of the RIA.

The particular advantage of TR-FIA is that the specific activity of the tracer is not affected by storage time, contrary to the radioactive tracer (only a minute dose of ^{3}H-11β was used to monitor the recovery of the extraction + chromatography steps prior to final radioimmunocompetition).

In conclusion, we have developed a non-isotopic 11β immunoassay that is an alternative to RIA, to which its

analytical performance is similar (although more sensitive) and which has the advantage of using a stable tracer rather than a ^{3}H-tracer.

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