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Plasma 21-deoxycortisol: comparison of a time-resolved fluoroimmunoassay using a biotinylated tracer with a radioimmunossay using ^{125}iodine

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Abstract

Plasma 21-deoxycortisol (21DF) is an excellent marker of 21-hydroxylase deficiency. Currently, it is the only marker able to detect heterozygous carriers with 21-hydroxylase deficiency after ACTH stimulation. We have already developed radioimmunoassays for 21DF using first tritiated, then ^{125}I-21DF which had a ten-fold higher sensitivity. However, because the lifespan of ^{125}I-21DF is short, the tracer needs to be reprepared every two months and this multiplies the risk of contamination by radioactive ^{125}I vapours. We therefore developed a non-isotopic 21DF assay that uses a 21DF–biotin conjugate with a original bridge, a diaminopropyl arm, linking the steroid to biotin. The 21DF–biotin conjugate was measured by time-resolved fluorescence after adding streptavidin-europium to the microtitration wells. The analytical qualities of this assay were very similar to those of the radioimmunoassay using ^{125}I-21DF as tracer. The results obtained by the two methods, in either normal subjects or patients with 21-hydroxylase deficiency, were virtually the same.

Keywords: Biotinylated tracer; 21-Deoxycortisol; Fluoroimmunoassay

1. Introduction

21-Deoxycortisol (21DF) is one of the best markers for diagnosing 21-hydroxylase deficiency [1–6]. In 21-hydroxylase deficiency, 17-hydroxy-progesterone (17-OHP) is not hydroxylated into 11-deoxycortisol and cortisol synthesis is decreased. ACTH secretion and blood 17-OHP consequently increase. The increasing amounts of 17-OHP produced are hydroxylated into 21DF in the adrenals and this metabolic pathway, which is minor in normal subjects, becomes major in patients with 21-hydroxylase deficiency [7]. High plasma 21DF and 17-OHP levels are thus used to diagnose this disorder.

However, heterozygote subjects with 21-hydroxylase deficiency are often clinically asymptomatic. We and others have shown that ACTH provokes much higher plasma 21DF levels in these subjects than in normal subjects [1–3,8,9]. The highest induced 21DF values in normal subjects overlap less than 10% with the lowest induced values in patients whereas the corresponding overlap in 17-OHP plasma levels between normal subjects and patients is from 20 to 80% [10]. Measuring 21DF plasma levels after ACTH stimulation could thus be a means of detecting potential heterozygous carriers of 21-hydroxylase deficiency among the general population.

The first published 21DF radioimmunoassays (RIA) used ^{3}H-21DF as tracer [1–4] and could only detect

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21DF amounts above 6 pg/tube. Since then, assay sensitivity has been increased 10 times by using ^{125}I-21DF [5] but, although analytical results are satisfactory, the tracer's lifespan is short (less than 2 months). Handling ^{125}I has disadvantages. When labelling 21DF–3CMO–histamine with ^{125}I and purifying by HPLC, the technician runs the slight but real risk of inhaling ^{125}I vapours.

We therefore decided to develop a stable non-isotopic tracer for a new sensitive immunoassay of 21DF. We synthesized a 21DF–biotin conjugate by coupling 21DF to biotinylamidopropyl-ammonium, the synthesis of which we have described previously [11]. The 21DF–biotin is detected by fluorometry after binding to a streptavidin-europium reagent. In this paper, we report the development of a solid-phase time-resolved fluoroimmunoassay (TR-FIA) of plasma or serum 21DF and compare the results obtained by this method in normal subjects and patients with late-onset 21-hydroxylase deficiency with those obtained in the same individuals by RIA using an iodinated tracer. The same anti-21DF antibody was used in both methods.

2. Materials and methods

2.1. Biological material

Blood samples were drawn from:

1. Normal men ($n = 32$) and normally menstruating premenopausal women in the follicular ($n = 31$) or luteal ($n = 30$) phase between 8 and 9 a.m. just before, and one hour after, iv injection of 0.25 mg Synacthen Immediat® (Novartis Pharma, 92500 Rueil Malmaison).
2. So-called 'heterozygote' women ($n = 23$), some suffering from hirsutism and/or acne, known to have abnormally high post-Synacthen plasma 21DF levels (0.70 > 21DF < 2 ng/ml by RIA). Blood was drawn under the same conditions as above.
3. Women ($n = 12$) with late-onset 21-hydroxylase deficiency.

All sera were stored at −20°C until 21DF assay.

2.2. Tracer preparation

The tracer (21-deoxycortisol–3-iminoxymethylcarboxamidopropy1)-biotinylamine, referred to as 21DF–3 carboxymethyloxime–biotin (21DF–3CMO–Biot) was prepared as follows. A solution of 21DF–3CMO (0.2 g, 0.47 mmol) and triethylamine (80 μl, 0.6 mmol) in dioxane (5 ml) was treated at 0°C with isobutylchloroformate (62 μl, 0.45 mmol). The reaction mixture was stirred at 0–5°C for 5 min, added to a solution of biotinylaminopropyl-ammonium trifluoroacetate (0.2 g, 0.47 mmol) and triethylamine (80 μl, 0.6 mmol) in 3 ml DMSO, stirred for 4 h at room temperature (RT), and then diluted in 20 ml cold H_2O. The precipitate was purified by column chromatography using AcOEt/MeOH (99.5/0.5, v/v) as eluent. The TLC of the conjugate showed a single spot after column chromatography and there was no impurity in the ^{1}H-NMR spectrum. Yield: 57%.; mp (iPrOH) = 117–124°C. ^{1}H-NMR ($CDCl_3$) δ 0.95 (*s*, 3H, CH_3); 1.29 and 1.31 (2*s*, total: 3H, 18-CH_3 E and Z); 2.65 (*d*, 1H, CHH–S); 2.85 (*dd*, 1H, CHH–S); 3.23 (*q*, 2H, N–$\underline{CH_2}$–CH_2); 3.35 (*q*, 2H, N–$\underline{CH_2}$–CH_2); 3.40 (*q*, C $\underline{H}$S–$\underline{CH_2}$); 4.32 and 4.45 (2*m*, 2H, 2$\underline{H}$–CN); 4.53 and 4.58 (2*s*, total: 2H, NCH_2CO E and Z).

2.3. Tracer and 21DF solutions

2.3.1. Stock tracer solutions

A concentrated mother solution of 21DF–biotin conjugate in ethanol (10 mg per 100 ml, i.e. 0.015 mmol) was prepared and stored at +4°C. An intermediary solution in ethanol was obtained by diluting the mother solution 1/1000 and also kept at +4°C. Working solutions were prepared extemporaneously by diluting the intermediary solution in gelatin phosphate buffer (0.05 M, pH 7.4).

2.3.2. 21DF solutions

A mother alcoholic solution containing 10 mg 21DF was diluted 1/100 in ethanol and this last intermediary solution was used to obtain a standard solution containing 6 ng 21DF per ml of phosphate gelatin buffer. This standard solution was used to prepare 10 calibrators with the following concentrations (in pg/ml and pmol): 1000 (2.88), 500 (1.44), 250 (0.72), 125 (0.36), 62.50 (0.18), 31.25 (0.09), 15.60 (0.045), 7.80 (0.02). All 21DF solutions were kept at +4°C.

2.4. Preparation of microtitration plates

Microtitration plates (8 × 12 NUNC, ref 1244-660 Wallac) were coated with 250 μl goat antirabbit antibody (4 mg/2 ml) from Biosys (60200 Compiegne France) 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 and then washed three times with Tween 20-containing saline (0.5/1000 ml). Free sites were saturated by adding 300 μl of a 0.15 M BSA solution (5 g BSA (Sigma A-9647) in 1 l phosphate buffer (pH 7.4) (9.53 g Na_2HPO_4, $2H_2O$, 8.77 g NaCl, 1.79 g anhydrous KH_2PO_4, 2 g NaN_3 (Merck, 106–688), Tween 20, 0.5 ml/1000 ml). The plates were covered with sealing tape (Corning 430454) and kept at +4°C.

2.5. Ria of 21DF

The RIA method used to assay serum 21DF comprises two purification steps, an extraction step followed by celite chromatography before final radioimmuno-competition [5]. The chromatography step separates 21DF from interfering plasma steroids. 21DF was eluted with iso-octane/dichloromethane, 53/47 (v/v). The eluted fraction was evaporated and the dried residue was taken up in phosphate–gelatin buffer. 21DF content was measured by RIA (100 μl) using ^{125}I-21DF and the same anti 21DF rabbit antibody as used in the TR-FIA described below. Recovery from the extraction plus chromatography steps was monitored by minute quantities of ^{3}H-21DF [5].

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

Anti-21DF antiserum was diluted from 10^2 to 10^7 times with assay buffer (0.05 M, pH 7.4) in the presence of 2–50 pg (3–75 fmol) per well of 21DF–biotin tracer. A standard binding curve for 0–100 pg (0.28 pmol) of 21DF/well was drawn for each antiserum-tracer combination.

2.7. TR-FIA of 21DF

After washing the microtitration plate wells, the following reagents were added to each well: 50 μl of 21DF standards or of the same eluates as used for 21DF RIA, then 50 μl of the appropriately diluted conjugate, 21DF–biotin tracer, and finally 100 μl of anti 21DF rabbit antiserum. The plates were shaken at 350 rpm at RT for 3 h. The assay was stopped by washing the wells 3 times (using a Model 1296-024 Platewash device (EG and G Wallac, 91047 Evry, France). A 200 μl aliquot of a solution of europium-labelled streptavidin, previously prepared by diluting (1/1000) 2.5 ml (0.1 mg/ml) of reagent (ref 1244-360, Wallac) in buffer (5 g BSA, 4 ml Tween 20, 1 g NaN_3 dissolved in 1000 ml saline adjusted to pH 7.8 with Tris–HCl), was then added to each well. The plates were shaken for 20 min, washed three times, removed from the machine, turned upside down, and washed again twice. The europium was dissociated by adding 200 μl of enhancement solution to each well, then agitated at 350 rpm for 20 min. Time-resolved fluorescence was measured with a 1234 Delfia fluorimeter (EG and G Wallac, 91047 Evry, France).

3. Results

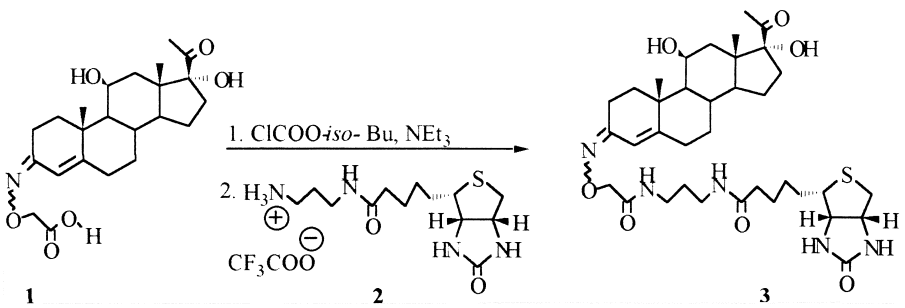
3.1. Validation of the TR-FIA for plasma 21DF

The 21DF–3CMO–biotin tracer was prepared by acylation of biotinylaminopropyl-ammonium trifluoroacetate with 21DF–3CMO using the mixed anhydride method (Scheme 1). The conjugate was easily purified by column chromatography.

In the TR-FIA, the best sensitivity all along the 21DF standard curve (see Fig. 1) was obtained with a 1/70000 anti-21DF-antiserum dilution (under 50 μl)and 7.5 pg of tracer (under 50 μl). In Fig. 1, the binding of each calibrator, expressed in pg (pmol)/well, i.e., 0.78 (0.002); 1.56 (0.004); 3.12 (0.009); 6.24 (0.018); 12.50 (0.036); 25 (0.072); 50 (0.114); 100 (0.288) represents 93; 88; 78; 63; 47; 32; 21; 14%, respectively, of the binding of standard zero.

The lowest 21DF amount that was significantly different from the zero value in the wells of the microtitration plates, calculated from 10 consecutive assays, was 0.39 pg (1.11 fmol) compared to a lowest detectable amount of 0.29 pg (0.86 fmol) by RIA [5]. The quantities of 21DF that displaced 20, 50, and 80% of the tracer were 2.85, 11 and 54 pg (0.008, 0.031 and 0.155 pmol) per well, respectively. The corresponding values for a RIA using ^{125}I-tracer were 0.97, 8 and 40 pg (0.0027, 0.023 and 0.115 pmol) [5].

The 21DF antibody cross-reacted (relative 50% dis-



Scheme 1.

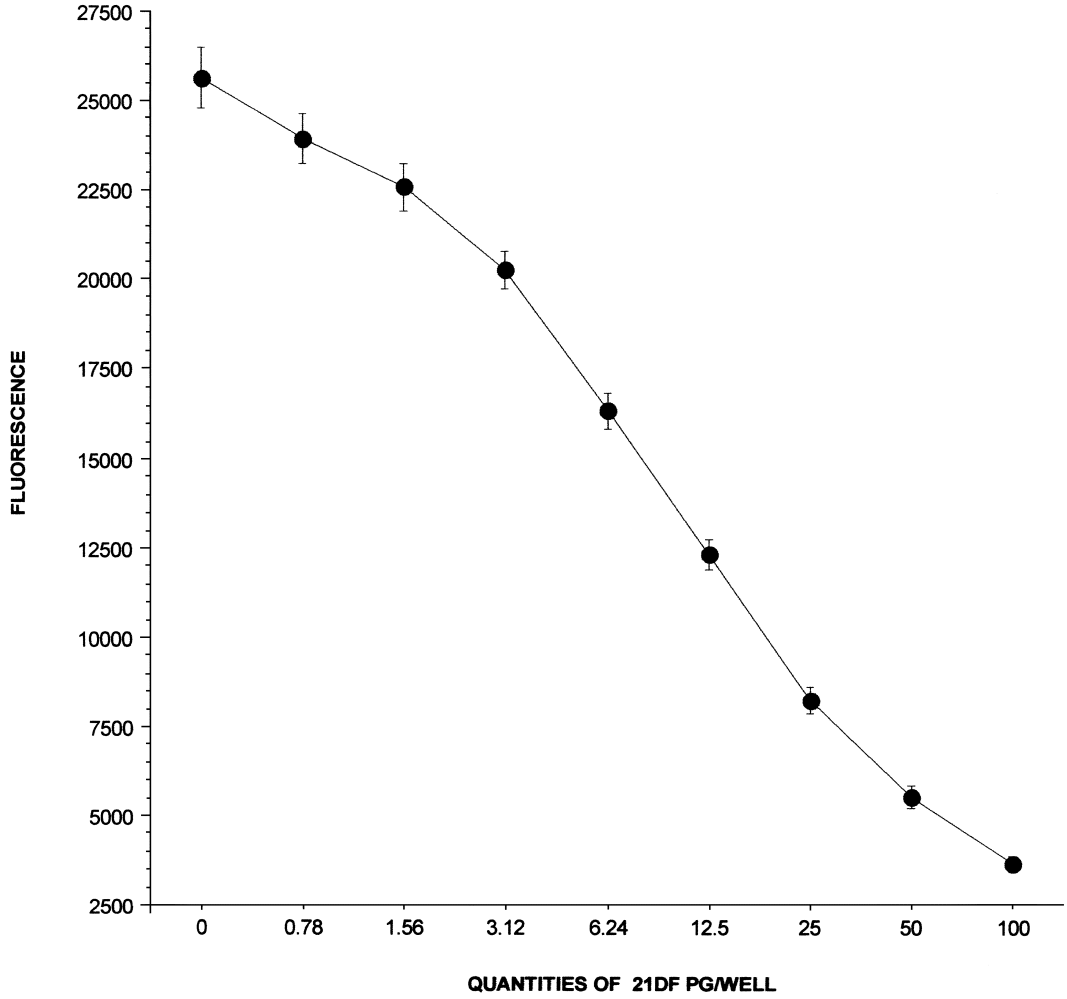


Fig. 1. 21DF calibration curve in arbitary fluorescence units obtained by TR-FIA (means ± 2SD) (n = 10).

placement values) with other steroids as follows: 11-deoxycortisol 0.03%, corticosterone 0.02%, progesterone 0.10%, 11-deoxycorticosterone 0.007%, 17-hydroxyprogesterone 1.15%, cortisol 0.5%, 21-deoxycorticosterone 0.44%, testosterone, Δ4-androstenedione, pregnenolone, 17-hydroxypregnenolone, DHEA, and estradiol $<0.01\%$. Not unexpectedly, these results are very similar to those obtained in the RIA.

Binding repeatability of every calibrator ($n = 10$) of the standard curve was between 0.5 and 4%. Intra-assay variation and interassay variation, inclusive of the extraction and chromatography steps, were 5.5% ($n = 12$) and 7.5% ($n = 12$), respectively. 21DF assay accuracy, as determined, first, on one steroid-free charcoal-stripped pooled serum sample and on three pooled serum samples containing low concentrations of endogenous 21DF and, second, after overloading these four samples by known amounts of 21DF, is indicated in Table 1.

The mother and intermediary tracer solutions have been stored so far for 13 months at $+4^{\circ}C$ without any activity loss.

3.2. Comparative determination of plasma 21DF by TR-FIA and RIA

For both groups of subjects (normal individuals and patients), the correlation coefficient between 21DF levels measured by TR-FIA and RIA was 0.97 or above (see Table 2). When individual levels obtained by the two methods were compared two-by-two in all groups, they were not found to differ significantly. As previously described [3,4], there was no significant difference in basal 21DF values in normal men and in normal women whatever the phase of the menstrual cycle. In addition, plasma 21DF levels in heterozygotes and patients with late-onset congenital adrenal hyperplasia were very similar regardless of whether they were measured by TR-FIA or RIA.

Table 1
Recovery of 21DF in one steroid-free serum and in three normal sera overloaded with increasing amounts of 21DF[a]

Serum 21DF levels before overloading ng (pmol)	Calculated 21DF levels after overloading (pmol)	Measured 21DF levels ng (pmol)	Recoveries (%)
0	0.10 (0.29)	0.09 (0.25)	90
		0.13 (0.37)	130
		0.10 (0.28)	100
		0.10 (0.28)	100
		0.08 (0.23)	80
		0.10 (0.28)	100
0.10 (0.29)	0.30 (0.86)	0.27 (0.77)	90
		0.32 (0.92)	106
		0.31 (0.89)	103
		0.30 (0.86)	100
		0.29 (0.83)	96
		0.32 (0.92)	106
0.15 (0.43)	0.65 (1.87)	0.61 (1.76)	94
		0.71 (2.04)	109
		0.60 (1.73)	92
		0.67 (1.93)	103
		0.68 (1.96)	104
		0.62 (1.78)	95
0.11 (0.30)	1.11 (3.20)	1.08 (3.11)	97
		1.30 (3.75)	117
		1.10 (3.17)	99
		1.11 (3.20)	100
		1.12 (3.23)	101
		1.13 (3.26)	102

[a] Each serum pool was assayed six times.

4. Discussion

In recent years, several steroid immunoassays have been developed using biotinylated steroids as tracers [12–14]. The steroid-biotin conjugate is detected with streptavidin-peroxydase or streptavidin-europium. The position and nature of the bridge between steroid and biotin are crucial for steroid recognition [13]. It is generally accepted that the linkage position of the bridge on the steroid nucleus should be the same in the tracer and immunogen used to raise antibodies. This 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 (BSA) is coupled in the immunogen (21DF–3CMO–BSA) [5]. This coupling position has already provided immunologically well recognized biotinylated steroids such as the 18-oxo-cortisol [12] and cortisol [14] 3CMO-biotinamidocaproyl-hydrazides.

We synthesized a new biotin derivative, a biotinamidopropylamine [11], to be coupled to 21DF–3CMO. Because of the higher nucleophilicity of the amino

Table 2
Comparison of 21DF levels obtained by RIA and TR-FIA

Subjects and patients	Before [1], after [2] synacthen	Correlation coefficient R	RIA mean X_1	TR-FIA mean Y_1	Regression equation
Normal women (FP[a])	Basal levels	0.99	55	56	$Y = 0.96X + 0.48$
$n = 31$	Post ACTH levels	0.97	214	207	$Y = 0.94X + 5.22$
Normal women (LP[b])	Basal levels	0.99	55	54	$Y = 0.94X + 1.88$
$n = 30$	Post ACTH levels	0.96	245	240	$Y = 1.02X - 26.5$
Normal men	Basal levels	0.98	56	53	$Y = 0.98X - 1.37$
$n = 32$	Post ACTH levels	0.99	249	246	$Y = 0.95X + 2.25$
Heterozygotes	Basal levels	0.99	193	190	$Y = 1.03X - 8.96$
$n = 23$	Post ACTH levels	0.99	1267	1226	$Y = 1.07X - 128$
Late onset CAH[c]	Basal levels	0.99	1190	1110	$Y = 0.94 + 36.31$
$n = 12$	Post ACTH levels	1.00	11908	11391	$Y = 0.97X - 204$

[a] FP: follicular phase.
[b] LP: luteal phase.
[c] CAH: congenital adrenal hyperplasia.

group compared to the hydrazide nitrogen [12,14], acylation of 3-biotinamidopropylamine proceeded with a good yield. The tracer was isolated easily and fully characterized by ^{1}H-NMR spectroscopy. We chose to detect the 21DF–biotin conjugate with streptavidin-europium and to measure europium fluorescence by time-resolved fluorescence.

The analytical performances of this first non isotopic 21DF immunoassay were very similar to those of the 21DF RIA [5] in terms of precision, recoveries, and specificity. Both methods used the same antibody and, like other authors [1,4,6], we used an extraction step of serum followed by a chromatography step. The sensitivity of the TR-FIA was slightly lower than that of the RIA but this slight decrease hardly changed the volume of plasma to be extracted. A particular advantage of TR-FIA is that the specific activity of the tracer is not affected by storage time. To date, the tracer has been stored for over a year without any loss in activity.

In conclusion, we have developed a non-isotopic 21DF immunoassay which is an alternative to RIA as it has virtually the same analytical performance and the advantage of using a stable tracer instead of an ^{125}I-tracer.

References

[1] A. Milewicz, P. Vecsei, S. Korth-Schütz, D. Haack, A. Rösler, K. Lichtwald, S. Lewicka, G.V. Mittelstaedt, Development of plasma 21-deoxycortisol radioimmunoassay and application to the diagnosis of patients with 21-hydroxylase deficiency, J. Steroid Biochem. 21 (1984) 185–191.

[2] B. Gueux, J. Fiet, M.T. Pham-Huu-Trung, J.M. Villette, M. Gourmelen, H. Galons, J.L. Brerault, P. Vexiau, R. Julien, Radioimmunoassay of 21-deoxycortisol: clinical applications, Acta Endocrinol. (Copenh.) 108 (1985) 537–544.

[3] J. Fiet, B. Gueux, M. Gourmelen, F. Kutten, P. Vexiau, Ph. Couillin, M.T. Phamm-Huu-Trung, J.M. Villette, M.C. Raux-Demay, H. Galons, R. Julien, Comparison of basal and ACTH-stimulated 21-deoxycortisol and 17-hydroxyprogesterone plasma values as biological markers of late-onset adrenal hyperplasia, J. Clin. Endocrinol. Metab. 66 (1988) 659–667.

[4] K. Nahoul, J. Adeline, J.P. Bercovici, Radioimmunoassay of 21-deoxycortisol, J. Steroid Biochem. 33 (1989) 1167–1172.

[5] J. Fiet, J.M. Villette, H. Galons, Ph. Boudou, J.M. Burthier, N. Hardy, H. Soliman, R. Julien, P. Vexiau, M. Gourmelen, F. Kuttenn, The application of a new, highly sensitive radioimmunoassay for plasma 21-deoxycortisol to the detection of 21-hydroxylase deficiency, Ann. Clin. Biochem. 31 (1994) 56–64.

[6] M. Hill, O. Lapcik, R. Hampl, L. Starka, Z. Putz, Radioimmunoassay of three deoxycorticoids in human plasma following HPLC separation, Steroids 60 (1995) 615–620.

[7] M. Finkelstein, J.M. Shaefer, Inborn errors of steroid biosynthesis, Physiol. Rev. 59 (1979) 353–406.

[8] M. Gourmelen, B. Gueux, M.T. Pham-Huu-Trung, J. Fiet, M.C. Raux-Demay, F. Girard, Detection of heterozygous carriers for 21-hydroxylase deficiency by plasma 21-deoxycortisol measurement, Acta Endocrinol. (Copenh.) 116 (1987) 507–512.

[9] A. Milewicz, A. Afelska, R. Wasikowa, T. Romer, Evaluation of 21-deoxycortisol as a marker for the detection of heterozygous carriers of 21-hydroxylase deficiency, Endokrynol. Pol. 44 (1993) 187–193.

[10] D. Knorr, F. Bidlingmaier, W. Höller, U. Kuhnle, Diagnosis of homozygocity and heterozygocity in congenital adrenal hyperplasia (CAH) and control of treatment, J. Steroid Biochem. 19 (1983) 645.

[11] A. Boudi, J. Fiet, H. Galons, Preparation and acylation of a new biotinylation reagent, Synthetic Commun. 29 (1999) 3137–3141.

[12] C.E. Gomez-Sanchez, J.M. Leon, E.P. Gomez-Sanchez, Biotin-hydrazide derivatives for the development of steroid enzyme-linked immunoassays, J. Steroid Biochem. Molec. Biol. 43 (1992) 523–527.

[13] P. Luppa, C. Brückner, I. Schwab, S. Hauck, S. Schmidmayr, C. Birkmayer, B. Paulus, H. Hauptmann, 7-Biotinylated testosterone derivatives as tracers for a competitive chemiluminescence immunoassay of testosterone in serum, Clin. Chem. 43 (1997) 2345–2352.

[14] R.A. Dressendörfer, C. Kirschbaum, W. Rhode, F. Stahl, C.J. Strasburger, Synthesis of a cortisol-biotin conjugate and evaluation as a tracer in an immunoassay for salivary cortisol measurement, J. Steroid Biochem. Molec. Biol. 43 (1992) 683–692.