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Highly sensitive and specific time-resolved fluoroimmunoassay (TR-FIA) of cyproterone acetate and free cyproterone

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Abstract

We have developed a non-isotopic TR-FIA for Cyproterone acetate and Cyproterone in plasma. Synthesis of the biotinylated tracers, biotinylated Cyproterone acetate, and Cyproterone, as well as the preparation of anti–Cyproterone acetate and anti-Cyproterone antisera are reported. The specificity of anti-Cyproterone acetate antiserum resulting from the coupling of link bridge (link bridge between steroid and BSA), on the 3-position on the steroid skeleton, allowed to carry out the Cyproterone acetate assay directly on extracted plasma (without chromatography). On the other hand Cyproterone assays require a purification step, including extraction plus chromatography, because the plasma Cyproterone acetate concentrations in Cyproterone acetate-treated women are 200 times higher than for Cyproterone. Theses plasma TR-FIA of Cyproterone acetate and Cyproterone presented the advantage of needing only small doses of radioactivity for recovery controls, and better praticability related to the only existing RIA described to date.

Keywords: Cyproterone acetate; Cyproterone; Time-resolved-fluoroimmunoassay

1. Introduction

Cyproterone acetate (CPA) is a powerful antiandrogen, used alone or in combination with ethinylestradiol or percutaneous estradiol, in the treatment of women suffering from disorders associated with androgenization, such as hirsutism and acne. Equally, in elderly men, it is used for the therapy of prostate carcinoma.

A few rare plasma assays of CPA have been published, such as radioimmunoassays [1–4]. In fact, the same anti CPA antiserum was used in these different reports. Comparatively, two non-immunoassays were described, an HPLC assay [3] and a gas chromatography/mass spectrometry assay [2].

We have developed two non-isotopic immunoassays for CPA, and its free form, Cyproterone (C), in plasma of CPA-treated women suffering from hirsutism or/and acne. We specifically studied the specificity and the sensitivity of these two assays.

2. Materials and methods

2.1. Steroids

All steroids used except Cyproterone (C), were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, Cedex, France) or from Steraloids Inc. (Wilton, NH). C **1b** was obtained from CPA **1a** by saponification in the presence of sodium perchlorate [5].

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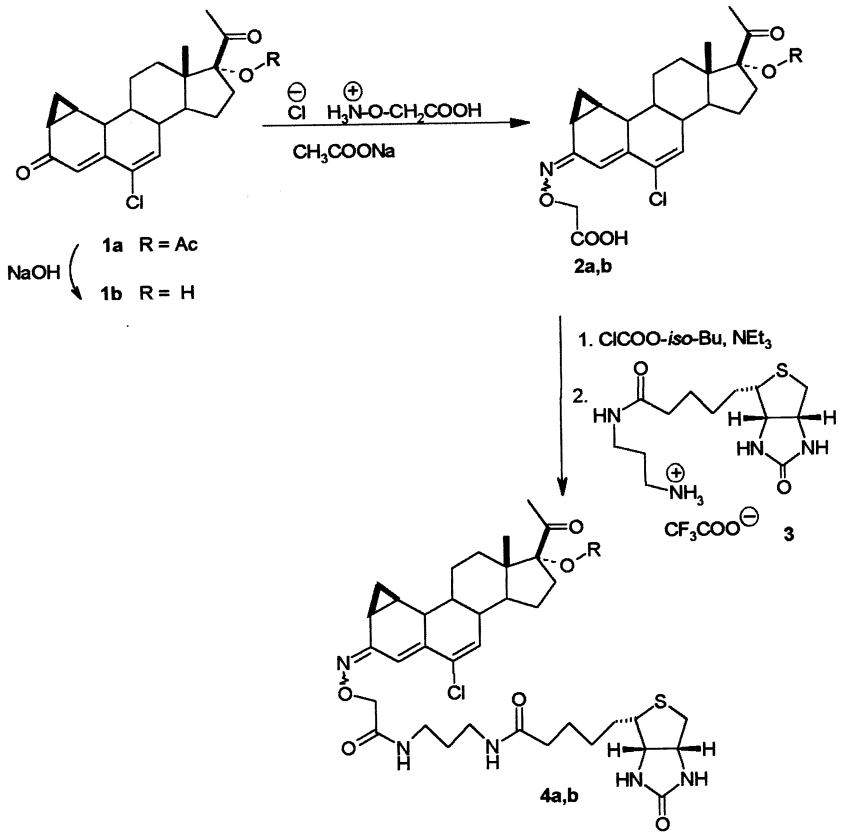

Scheme 1.

2.2. 3-Carboxymethyloxycyproterone acetate and 3-carboxymethyloxycyproterone hapten synthesis (Scheme 1)

To a solution of **1a** or **1b** (5 mmol) and of aminoxyacetic acid hemihydrochloride (0.56 g, 5 mmol) in methanol 50 ml was added 15 ml 1 N sodium acetate. The solution was stirred for 12 h at r.t. then concentred in vacuo and acidified to pH 2 with 1N HCl. The haptens were extracted with ethyl acetate 3×50 ml. The organic layer was dried and the CMOs were purified by recrystallization from ethanol. The products **2a** and **2b** obtain as a 2:1 mixture of E and Z stereoisomers.

2a: Yield: 85%, ^{1}H-NMR ($CDCl_3$) δ 0.75 (s, 3H, 18-CH_3), 1.08 (s, 3H, 19-CH_3), 1.98 (s, 3H, CH_3COO); 2.25 (s, 3H, 21-CH_3) 4.60 and 4.65 (s, 2H, Z and E OCH_2CO); 5.98 and 6.12 (d, 1H, $J = 1$ Hz, E and Z 4-H); 6.21 and 6.82 (s, 1H, E and Z 7-H).

2b: Yield: 75%, ^{1}H-NMR ($CDCl_3$) δ 0.78 (s, 3H, 18-CH_3), 1.08 (s, 3H, 19-CH_3), 2.23 (s, 3H, 21-CH_3) 4.60 and 4.65 (s, 2H, Z and E OCH_2CO); 5.98 and 6.10(d, 1H, $J = 1$ Hz, E and Z 4-H); 6.21 and 6.78 (s, 1H, E and Z 7-H).

2.3. Biotinylated conjugates synthesis

The acylation of biotinylaminopropylammonium trifluoroacetate **3** with **2a**, **2b** was achieved as previously described. [6,7]. The tracers **4** were isolated by column chromatography using CH_2Cl_2:MeOH (95:5) as eluent (Scheme 1).

4a: Yield: 54%, ^{1}H-NMR ($CDCl_3$) δ 0.62 (s, 3H, 18-CH_3), 1.08 (s, 3H, 19-CH_3), 1.98 (s, 3H, CH_3COO); 2.08 (s, 3H, 21-CH_3), 4.22 and 4.45 (2m, 2H, 2CH-N), 4.60 and 4.65 (s, 2H, Z and E OCH_2CO), 5.94 and 6.09 (d, 1H, $J = 1$ Hz, E and Z 4-H), 6.21 and 6.78 (s, 1H, E and Z 7-H), 6.52 and 6.70 (2m, 2H, 2N<u>H</u>$COCH_2$).

4b: Yield: 66%, ^{1}H-NMR ($CDCl_3$) δ 0.68 (s, 3H, 18-CH_3), 1.04 (s, 3H, 19-CH_3), 2.12 (s, 3H, 21-CH_3), 4.22 and 4.45 (2m, 2H, 2CH-N), 4.46 and 4.52 (s, 2H, Z and E OCH_2CO); 5.97 and 6.25 (d, 1H, $J = 1$ Hz, E and Z 4-H); 6.21 and 6.78 (s, 1H, E and Z 7-H), 6.67 and 6.92 (2m, 2H, 2N<u>H</u>$COCH_2$).

2.4. Preparation of CPA-3-carboxymethyloxime/BSA (CPA-3-CMO/BSA), and C-3-carboxymethyloxime/BSA (C-3-CMO/BSA) (immunogens)

The two haptens, CPA-3-CMO and C-3-CMO were coupled to a carrier, the bovine serum albumin (BSA), by the mixed anhydrid method of Erlanger [8]. After coupling, the immunogens were dialyzed overnight, then lyophilized and kept at $+4$°C.

2.5. Tritiated Cyproterone acetate

(TRQ9098) (SA 4 Ci/mmol) and Cyproterone (TRQ9097) (SA 56 Ci/mmol) were obtained from Amersham France.

2.6. Obtention of CPA and C antisera

Four New Zealand white female rabbits were immunized with CPA-3-CMO/BSA immunogen and four others were immunized with C-3-CMO/BSA, according to the Vaitukaitis method [9]. 0.5 mgr of each immunogen was first injected into each animal with complete Freund adjuvant, then, with incomplete Freund adjuvant every month. Blood was drawn from the ear marginal vein. Antisera were separated, and the titers, i.e. dilutions of the antisera which bind 50% of the tritiated tracers, were studied at each bleeding.

2.7. Special reagents devices, and apparatus for TR-FIA

Microtitration plates 12×8 NUNC (Ref 1244-550 Wallac).

Goat anti-rabbit antibody from Valbiotech (75010 France), (4 mg/2 ml) for coating.

Washing solution: physiological saline solution with Tween 20.

Eu-labelled streptavidin: 0.1 mg/ml, 2.5 ml (Ref 1244-360, Wallac), diluted 1/1000 in the following buffer: BSA 5 g/l + tween 40, in physiologic saline solution + Na azide (0.5%), diethylene triamine penta-acetic acid 15 mg/l, adjusted to pH 7.8 with Tris–HCl.

Coating buffer 0.05 M, pH 9.6. Anhydrous Na carbonate ($Na2CO_3$) 1.55 g Na hydrogen carbonate (NaHCO3) 2.97 g qsp 1000 distilled water. Adjusted to pH 9.6 with diluted HCl.

Saturation solution and assay buffer: pH 7.4 0.05 M,. Disodium hydrogen phosphate (Na2HPO4, $2H_2O$) 7.2 g, sodium di-hydrogen phosphate (NaH2PO4, $2H_2O$) 1.3 g, distilled water qsp 1000 ml. Dissolve bovine serum albumin 15 g (Sigma A-9647) and Na azide 2 g (Merck, ref 106-688), kept at + 4°C.

Fluorometer: Time-resolved fluorescence was measured with a 1234 Delfia Fluorimeter (Perkin Elmer, Wallac 91945 Courtaboeuf, France). A model 1296-024 platewash device (Perkin Elmer, Wallac) was also used.

2.8. Coating and saturation of microtiter plates

Microtiter plate wells were coated by adding 250 μl of goat *anti*-rabbit antibody, 1000 diluted fold in coating buffer. After washing three times with a washing solution, free well sites were saturated by adding 300 μl of the saturation solution. Microtitration plates were covered with sealing tape (Corning 430454), and kept at + 4°C.

2.9. Biotinylated solutions

Stock solutions of biotinylated CPA (B-ACP) and biotinylated C (B-C) were obtained by disolving, respectively 10.4 mg) of CPA and 12 mg of C in 100 ml of ethanol. Two biotinylated tracer intermediary solutions (SI) were prepared by diluting the two stock solutions 1/1000 in ethanol. Stock and intermediary ethanolic solutions were kept at + 4°C.

2.10. CPA and C standard solutions

Mother solutions of CPA and mother solutions of C containing 10 mg of steroids per 100 ml of ethanol were used to prepare daughter solutions containing 100 times less. These two alcoholic standard solutions were kept at + 4°C, and used for the preparation of aqueous standards necessary to establish the two immunocompetition standard curves.

The amounts of CPA and C (expressed in pg) put into the wells to establish the standard curves were: 1200, 600, 300, 150, 75, 37.5, 18.75, 9.37, and 4.68, which correspond to 2.87, 1.44, 0.72, 0.36, 0.18, 0.09,- 0.045, 0.0225, and 0.0112 pmol of CPA, and to 3.16, 1.58, 0.79, 0.39, 0.19, 0.099, 0.049, 0.024, and 0.0-12 pmol of C.

2.11. TR-FIA of CPA and C

The immunoassay procedures for these two steroids were very similar: 50 μl of aqueous solutions of ACP or C (standards and aqueous solutions obtained by extraction or extraction + chromatography from sera, see below), were put into coated wells in duplicate, followed by addition of appropriate dilutions of biotinylated CPA or biotinylated C aqueous solutions (50 μl) followed by the addition of optimal dilutions of the anti-CPA antibody or anti C antibody (100 μl). Plates were incubated under 350 rpm agitation at room temperature for 180 min. The immunoreaction was stopped by washing the wells three times with washing solution. Two hundred microlitres of Eu-labelled streptavidin solution were added, and the plates agitated for 20 min before washing three times. After the last washing, the plates were removed from the automatic washer, pour off by turning the plates upside down, then washed one more time. The Eu was dissociated by adding 200 μl of enhancement solution to each well, agitated 350 rpm for 20 min, and the fluorescence was measured.

2.12. Determination of the optimal dilutions of anti-CPA and anti-C antisera and biotinylated CPA and biotinylated C for TR-FIA

Anti-CPA antiserum and anti-C antiserum were diluted from 10^2 to 10^7 times with phosphate assay buffer, 0.05 M, pH 7.4, in presence of various dilutions of the corresponding biotinylated tracers. Standard binding curves for CPA and C were established for each antiserum-tracer combination.

2.13. Specificity of anti-CPA and anti-C antisera

The specificity of CPA and C antisera were measured as cross-reactivities for a displacement of 50% of the biotinylated tracers by analog steroids.

2.14. Reagents for extraction, and chromatography

Merck (Nogent-sur-Marne, France), Carlo-Erba (Rueil Malmaison, France), and Prolabo (Paris, France) supplied isooctane, cyclohexane, ethylacetate, dichoromethane, and ethylene glycol. The tributylamine, isobutyl choroformate, and albumin bovine came from Aldrich-Sigma (Saint Quentin Fallavier 38297 France), and isobutyl chloroformate from Fluka (Saint Quentin Fallavier 38297 France). Johns Manville (Touzart et Matignon, Vitry-sur-Seine, France) supplied the Celite analytical filter aid, which was pretreated with cyclohexane, as described [10].

2.15. Extraction of CAP, and C from plasma samples

After adding minute doses of tritiated CAP or 4-androstenedione or/and C to plasma samples and incubation 15 min, steroids were extracted with cyclohexane/ethylacetate, 50/50, 1–10 ml, by mixing for 2 min with a multivortex. After allowing the phases to separate, the aqueous lower phase was frozen and the upper organic phase transferred into a glass tube for evaporation.

2.16. Chromatographic separation of steroids on celite columns (Fig. 3)

The dried residues of steroids were redissolved in 1.5 ml isooctane, and introduced into a 5 ml siliconized pipette filled with 0.75 gr of a homogeneous mixture of Celite + ethylene glycol (2:1). CPA, Δ4-androstenedione, and C were separated by sequential addition of set volumes of solvent mixtures of increasing polarity, i.e. firstly isooctane/ethyl acetate 94/6 (v/v) 5 ml, then isooctane/ethyl acetate 85/15 (v/v) 6 ml. By collecting ml-by-ml solvents eluted from the chromatographic columns, measurements of radioactivity of the tritiated steroids allowed us to determine the chromatographic elution diagram of each steroid, as well as the recovery rates following extraction and chromatography.

2.17. Biological material

Blood samples were drawn from daily CPA 50 mg treated women; between 8 and 10 h.

3. Results and discussion

3.1. Preparation of haptens

The formation of haptens was achieved by reacting cyproterone, or cyproterone acetate with oximinoacetic acid in the presence of sodium acetate. The NMR spectra indicated, in both cases, the formation of a mixture of two stereoisomers (E/Z). They were not separated and were used to prepare the antigens and the biotinylated tracers.

3.2. Establishement of CPA and C TR-FIA standard curves (Fig. 1 and Fig. 2)

Optimal dilutions of anti-CPA n°4001 and anti-C n°8001 antisera were, respectively $1/10^5$ and $1/6.10^4$ using, respectively 156 and 150 pg of biotinylated tracers per assay well.

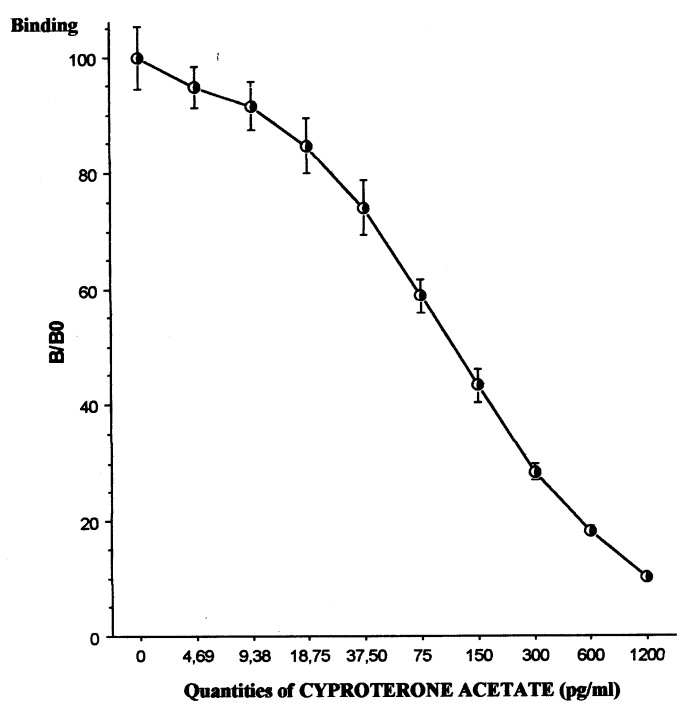

Fig. 1. Dose-response curve of the CPA TR-FIA. Means ± 3 SD ($n = 12$) are plotted.

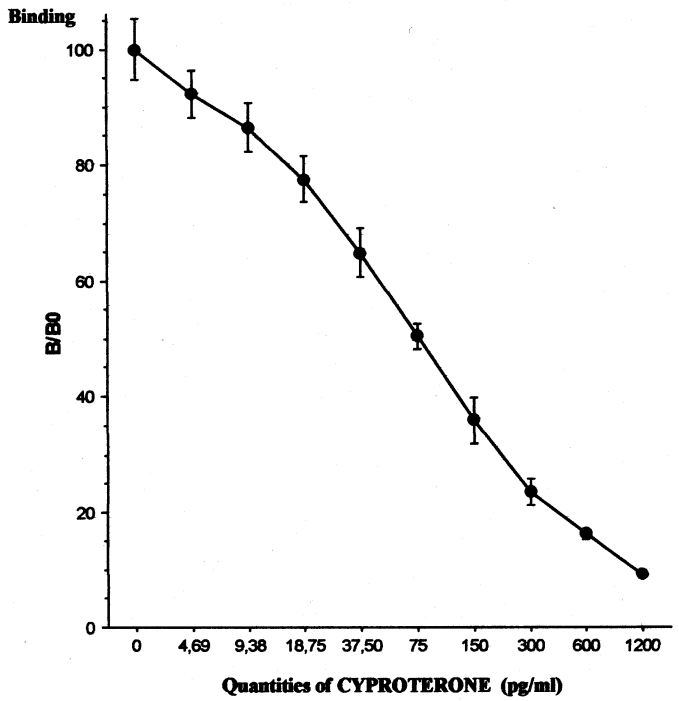

Fig. 2. Dose response curve of the C TR-FIA. Means ± 3 SD ($n = 12$) are plotted.

Mean bindings ($n = 12$) *of the tracers as reported in the CPA and C standard curves, for each standard* (4.69, 9.38, 18.75, 37.50, 75, 150, 300, 600, and 1200 pg) were, respectively 95/92, 91/87, 85/78, 74/65, 59/50, 43/36, 28/23, 18/16, and 10/9% of the binding of the zero standards.

Fluorescence unit ranges were between 721499 and 73273 in the CPA standard curve and between 682542 and 62237 in the C standard curve.

CV of the binding of each standard were between 1.1 and 2.1% in the CPA standard curve, and between 1.4 and 3.7% in the C standard curve.

The lowest amounts of CAP and C in standard curves that were significantly different from the zero values, in the wells of the microtitration plates, calculated from 12 consecutive assays (B_0-3SD) were 5.13 pg and 3.38 pg, respectively.

The sensitivities of the CPA and C standard curves expressed as the quantities of steroid per well, which displaced 20, 50 and 80% of the tracers were 27, 111 and 510 pg of CPA, and 13, 72, and 465 pg of C.

3.3. Specificities of anti-CPA and anti-C antisera

The following steroids: CPA, C, cortisol, 17-OH-progesterone, and Δ4-androstenedione,-ethinylestradiol, exhibited the following cross-reactivities (%), 100, 0.16, < 0.0001, < 0.0001, < 0.0001, < 0.0001 with anti-CPA antiserum (rabbit 4001), and 0.05, 100, $<$ 0.0001, 0.31, < 0.0001, < 0.0001 with anti-C antiserum (rabbit 8001).

3.4. CPA and C TR-FIA specificities in plasma of CPA treated patients

3.4.1. CPA TR-FIA specificities in plasma

We decided to measure CPA and C in blood after organic solvent extraction. However, was it necessary to pursue the purification of extracted plasma samples by chromatography or not before TR-FIA? To resolve this question, after extraction, we first separated CPA and C by celite chromatography, in two different elution fractions, and we applied adequate TR-FIA on each elution fraction.

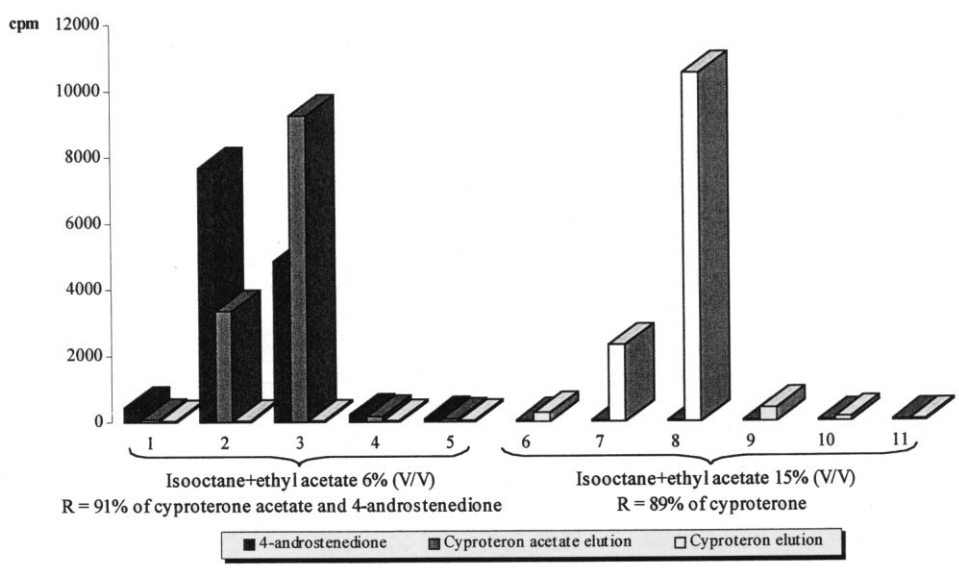

Fig. 3. Chromatographic elution diagram of 4-androstenedione, cyproterone acetate, cyproterone with % of recoveries (R).

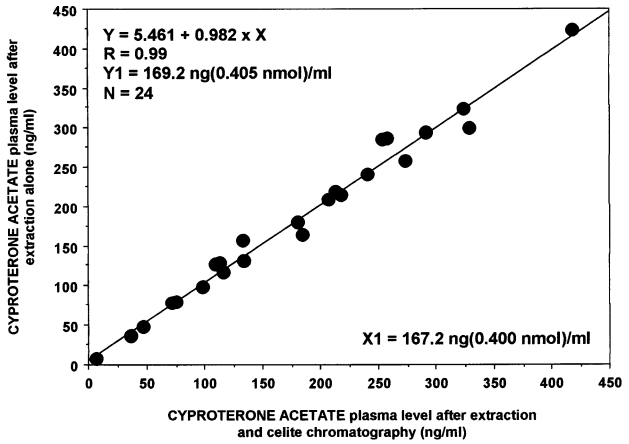

Fig. 4. Comparison of cyproterone acetate (CPA) plasma levels measured by TR-FIA, after extraction plus chromatography, and after extraction alone in 24 CPA-treated women. Regression curve (Y = TR-FIA after extraction alone: X = TR-FIA after extraction + chromatography), correlation coefficient (R), means of the values obtained with each procedure ($X1$ = TR-FIA after extraction plus chromatography: $Y1$ = TR-FIA after extraction alone).

We achieved complete separation between tritiated CPA and C, without overlap, as reported in Fig. 3. With a recovery of 91% of CPA in 5 ml of isooctane/ethylacetate, 94/6, v/v, and a recovery of 89% of C in 6 ml of isooctane/ethylacetate, 85/15, v/v.

However, the low specific activity of tritiated CPA (4 Ci/mmol) did not allow the use of this tracer to monitor recovery after extraction + chromatographiy prior to TR-FIA of CPA in the plasma of treated patients. This is why we determined the chromatography elution diagram and recovery of a steroid of similar polarity as CPA, such as Δ4-androstenedione, which specific activity was sufficient (58 Ci/mmol).

We observed (Fig. 3) that Δ4-androstenedione was eluted with the same recovery as CPA, (91%), in the same elution fraction (isooctane + ethyl acetate, 94/6, v/v).

To ascertain the specificity of CPA and C measurements in treated women plasma, we assayed these two steroids according a method including a purification phase, extraction + celite chromatography, before TR-FIA. CPA was measured in the elution fraction isooctane/ethyl acetate 94/6, and C was measured in the more polar elution fraction isooctane/ethyl acetate 85/15. We measured comparatively CPA and C by TR-FIA, after extraction of plasma not followed by a chromatography step.

The CPA plasma levels obtained after extraction alone on one hand, and after extraction + celite chromatography on the other were not significantly different. The levels found were between 6.5 ng/ml and 418 ng/ml of plasma. The regression diagram (Fig. 4) generated the regression curve: $Y = 0.982\ X + 5.46$ (Y = extraction alone; X = extraction + chromatography) with $r = 0.99$, with mean values using each purification method $X1 = 167.2$ ng/ml (extraction + chromatography), and $Y1 = 169.2$ ng/ml (extraction).

Thus we concluded that CPA measurements in the plasma of CPA-treated women can be carried out directly after extraction, without a chromatographic step, prior to TR-FIA, with a recovery rate not significantly different from 100%.

This result is a consequence of the rather low plasma levels of C (see below), and of the high specificity of the anti-CPA antiserum, which cross-reacts only 0.16% with C.

In comparison, the only other anti-CPA antiserum that has been published (to our knowledge) [2] cross-reacts 25% with C. This difference in the specificity of the two anti-CPA antisera, our anti-CPA-3-CMO/BSA antiserum, and the anti-CPA-**11**α-succinoyloxy/BSA, is very likely due to the different positions (3 or 11) of the coupling of the link arm on the steroid skeleton. The **3** position is better than the 11-position allowing to distinguish steroids whose structural variations are situated on carbons 17, 16, or 15 of the steroid skeleton [11].

As a consequence of the 3 coupling position of the link arm of immunogen choosen to elicite our anti-CPA anti serum, it is likely that our anti-serum exhibites low cross-reactivities with polar metabolites of CPA and C, i.e. 15β-hydroxycyproterone acetate, and 15β-hydroxycyproterone. It has been published [2], that 15β-hydroxycyproterone acetate, and 15β-hydroxycyproterone cross-react 12.14 and 2.07%, respectively with the anti-CPA-**11**α-succinoyloxy-/BSA.

3.4.2. C TR-FIA in plasma

C plasma levels measured and compared after a single extraction were significantly higher than the levels measured after extraction plus a chromatographic step. The regression curve was $Y = 1.231\ X + 0.897$ (Y = extraction alone; X = extraction + chromatography) with $r = 0.64$. After extraction plus chromatography, C plasma levels of the CPA treated women were between 0.3 and 1.1 ng/ml, and after extraction alone, C plasma levels were 1 and 2.9 ng/ml. This lack of specificity of C TR-FIA after extraction alone is likely due to the much higher plasma CPA levels and the insufficient specificities of anti-C antiserum related to CPA and to endogenous steroid plasma concentrations.

The exact measurement of C plasma levels in CPA treated women therefore requires a purification step with extraction plus chromatography prior to TR-FIA.

3.5. Precision

Interassay CV of plasma CPA and C were, respectively between 4 and 6%, and between 5 and 9% ($n = 13$).

Table 1
TR-FIA of CPA and C in different dilutions of the same plasmas

Dilutions	CPA levels in 3 different sera (ng/ml)			C levels in 3 different sera (ng/ml)		
Dilution 1/1	55	158	232	0.50	0.88	1.05
Dilution 1/2	53	150	228	0.45	0.85	1.00
Dilution 1/4	56	159	236	0.51	0.90	0.97
Dilution 1/8	51	152	227	0.50	0.87	0.96

3.6. Dilution assays

Dilutions of 3 sera of CPA treated women were assayed after extraction alone for CPA, and after extraction plus chromatography for C. The results are reported in the Table 1.

3.7. The lower limits of quantification of CPA and C in plasma or serum

The lower limits of quantification of these steroids in biological samples depend on the least detectable doses on the standard curves, 5.13 pg of CPA, and 3.38 pg of C (as reported before), on volume of the samples extracted, on volume of aqueous buffer used to redissolve the steroids following extraction or extraction plus chromatography, and on the recovery (monitored by tritiated steroids) before TR-FIA.

Thus, the lower limits of quantification of these two steroids are below 50 pg/ml of plasma or serum, if sample volumes of 1 ml are extracted. When the plasma ACP level is in the range of usual post-therapeutic levels (100–200 ng/ml during ACP treatment), 0.1 ml of sample is sufficient. To measure normal post-therapeutic plasma concentrations of C (0.2–1.5 ng/ml) a sample volume of 0.5 ml is sufficient.

3.8. CPA and C plasma levels in CPA-treated women

The CPA and C determinations were carried out in the plasma of patients for whom androgen radioimmunoassays (RIA) had been ordered by endocrinologists. The blood samples were obtained between 8 and 10 h. No control was possible to verify the hour of the last CPA intake. It was not the aim of this work to determine the CPA and C plasma levels at a precise interval after CPA intake.

It was the reason why the range of plasma CPA levels, we measured were rather large, i.e. between 6.5 and 418 ng/ml, whereas in the literature it has been reported that the CPA plasma levels after administration of a daily dose of 50 mg of CPA were between 199 and 228 ng/ml [12].

4. Conclusion

We report for the first time a Time Resolved-Fluoroimmunoassay of CPA and C in plasma. These assays exhibit a high sensitivity and specificity and are well-suited for pharmacokinetic and bioequivalence future studies. Equally these assays will be useful for the follow-up of patients treated with CPA (contraception, hirsutism, prostate cancer, sexuality excess).

Moreover, these non-isotopic assays exhibited a good praticability. The biotinylated tracers alcoholic solutions have showed until to-day a good stability (>24 months). At last, only small doses of radioactive tritiated tracers were used for recovery, contrary to the precedent published CPA RIA [1].

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