Competitive solid-phase immunoassay of testosterone using time-resolved fluorescence

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A competitive solid-phase immunoassay for the determination of testosterone in serum samples using time-resolved fluorescence is described. The solid phase is a testosterone–3-(*O*-carboxymethyl)–oxime–ovalbumin conjugate coated to polystyrene microtiter strips. Europium-labelled polyclonal and monoclonal antibodies against testosterone–3-(*O*-carboxymethyl)–oxime–bovine serum albumin were compared. Their behavior was quite similar although the polyclonal antibody was more sensitive, giving a detection limit of 15 fmol testosterone per assay. Correlation with RIA was very good ($r = 0.982$ and $y = -0.150 + 0.969x$).

Testosterone *Time-resolved fluoroimmunoassay* *Fluoroimmunoassay* *Immunoassay* *Solid-phase*

1. INTRODUCTION

In recent years immunoassays based on time-resolved fluorimetry [6] have proved to be an alternative method to RIA [3–5]. Because of the long decay fluorescence of the europium chelate used as marker, disturbing fluorescence from other components of the sample is avoided [6]. Other features of the method are the short counting time, only 1 s per sample, and the stability of the label as compared to radioactive isotopes.

We describe here a simple time-resolved fluoroimmunoassay (TR-FIA) for testosterone, in which monoclonal or polyclonal antibodies are labelled with a europium chelate. The antigen, in the form of a testosterone–3-CMO–OVA conjugate, is fixed by adsorption to the surface of polystyrene microtiter strips. This is the first assay for hapten molecules using the principle of time-resolved fluorescence.

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Abbreviations: CMO, carboxymethyl oxime; BSA, bovine serum albumin; OVA, ovalbumin

2. EXPERIMENTAL

2.1. *Purification and labelling of antibodies*

Monoclonal rat antibodies to testosterone–3-CMO–BSA (clone H_6) were purified from ascites fluid by Na_2SO_4 precipitation and dialyzed overnight at 4°C against 0.05 M K_2HPO_4. Polyclonal rabbit antitestosterone–3-CMO–BSA antibodies were purified from serum in the same way. The antibodies were labelled with an isothiocyanatophenyl–EDTA–Eu chelate as in [1], and purified from excess label on a 1.5 × 45 cm Sepharose 6B column using 0.05 M Tris–HCl buffer (pH 7.4) containing 0.9% NaCl and 0.05% NaN_3 as eluent. The europium-to-protein ratio in the labelled monoclonal and polyclonal antibody preparations was 7 and 6, respectively. The preparations were stored as such at 4°C.

2.2. *Preparation of testosterone–3-CMO–ovalbumin conjugate*

The testosterone–3-CMO–OVA conjugate was prepared essentially as in [2]. A cold solution of 0.5 mg testosterone–3-CMO–*N*-hydroxysuccini-

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mide in 0.1 ml dioxane was carefully added to 3.1 mg ovalbumin in 1.6 ml of 0.05 M phosphate buffer (pH 7.3) containing 0.1% gelatin, 0.9% NaCl and 0.1% NaN_3 (molar testosterone-to-ovalbumin ratio ~15). The reaction mixture was held at 4°C for 2 h after which it was centrifuged at 2000 × *g* for 15 min. The conjugate was purified from excess testosterone derivative by gel filtration on Sephadex G-25 M (column PD 10, Pharmacia, Sweden). Elution was performed with 0.05 M phosphate buffer containing 0.1% NaN_3. The protein concentration in the conjugate preparation was 470 μg/ml, and the total protein yield was about 50%. Analysis of the ultraviolet absorption spectra of the conjugate indicated a testosterone-to-ovalbumin ratio of 0.8.

2.3. *Coating of polystyrene microtiter strips*

The testosterone–3-CMO–OVA conjugate was immobilized by adsorption to the well walls of polystyrene microtiter strips (Eflab, Helsinki). One strip comprises 12 wells and the dimensions of each well are 6.5 × 11 mm. The wells were coated overnight at room temperature with 0.25 ml of a 0.05 μg/ml conjugate solution diluted in 0.1 M sodium carbonate buffer (pH 9.3). After coating the wells were washed with 0.9% saline containing 0.05% NaN_3 using the Nunc Immuno-Wash system. Finally, the wells were dried and stored at room temperature in plastic bags over silica gel until use.

2.4. *Extraction of serum samples*

One hundred μl of serum-based standards or unknown samples were extracted for 15 min in 1 ml freshly prepared diethyl ether–ethyl acetate (9:1): 400 μl of the ether phase was evaporated under a stream of air, 120 μl of assay buffer was added and the sample was left for at least 30 min before 50-μl samples in duplicate were taken for testosterone measurement. The assay buffer contained 0.9% NaCl, 0.05% NaN_3, 0.5% BSA, 0.05% bovine globulin, 0.01% Tween-40 and 20 μM diethylenetriaminepentaacetic acid in 0.05 M Tris–HCl buffer (pH 7.7).

2.5. *Time-resolved fluoroimmunoassay*

Fifty μl of standards in assay buffer or 50 μl extracted serum-based standards or samples were added to the coated wells: 200 μl antibody solution (containing 25 ng labelled antibody diluted in assay buffer) was then added. The immunoreaction took place at room temperature for 1.5 h and was stopped by washing 3 times with 0.9% NaCl containing 0.05% NaN_3.

2.6. *Measurement of fluorescence*

The Eu bound to the solid phase was dissociated into the solution and measured as a 2-naphthoyltrifluoroacetone chelate [1] using the single photon counting time-resolved fluorimeter in [7].

3. RESULTS AND DISCUSSION

The conjugate concentration used for coating of the microtiter strip wells was found to give optimal replacement for both the polyclonal and monoclonal antibodies. In 1.5 h incubation assays the obtained fluorescence was directly dependent on the concentration of the labelled monoclonal antibody up to 25 ng. In a prolonged incubation overnight, at which the reaction had reached equilibrium, the fluorescence was directly proportional to the label concentration up to 10 ng/well, whereafter the antigenic solid phase was saturated. An antibody concentration exceeding 50 ng gave a marked reduction on replacement. It was found that 25 ng of antibodies per assay gave enough high cps values as well as optimal replacement. This concentration was optimal for both polyclonal and monoclonal antibodies.

Dose-response curves obtained with the two labels at optimal conditions are shown in fig.1A. The polyclonal label was more sensitive in the assay. Taking a C.V. value of 13% as the highest acceptable limit, the lower detectable concentration is 0.3 nmol/l or 15 fmol/assay, which is comparable to most commercial RIA kits usually having a detection limit of 5–30 fmol. For the monoclonal antibody this concentration was 10-times higher. The measuring range with the polyclonal label was 0.3–10 nmol/l and for the monoclonal label 3.0–100 nmol/l. The precision profile of the assays is shown in fig.1B.

Cross-reactions in the competitive solid-phase immunoassay were compared to the data obtained with conventional RIA measurements reported by the manufacturers of the antibodies (table 1). The results obtained were essentially the same as in RIA. Thus, labelling of the antibodies with the

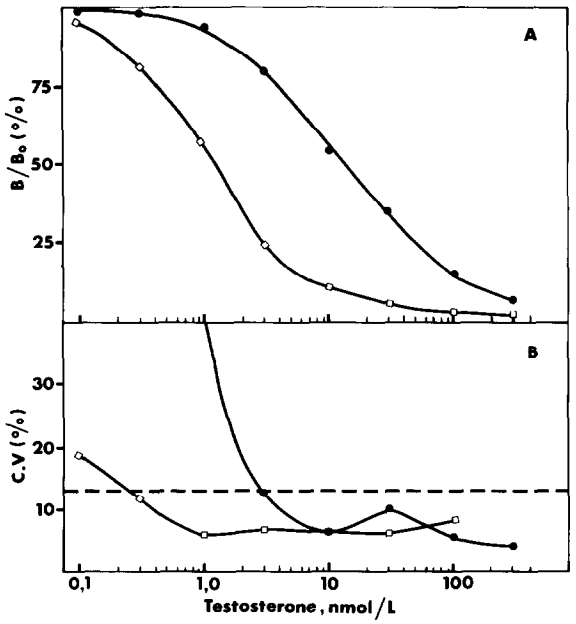

Fig.1. (A) Dose-response curves for TR-FIA of testosterone. Monoclonal antibody (●; B_0 = 226855 cps), polyclonal antibody (□; B_0 = 219709 cps). (B) Precision profile of the immunoassay. Nine duplicates were run for each concentration.

europium chelate does not affect their apparent properties.

Because of the cross-reaction properties and the higher sensitivity obtained, the polyclonal antibody was chosen for testosterone determination

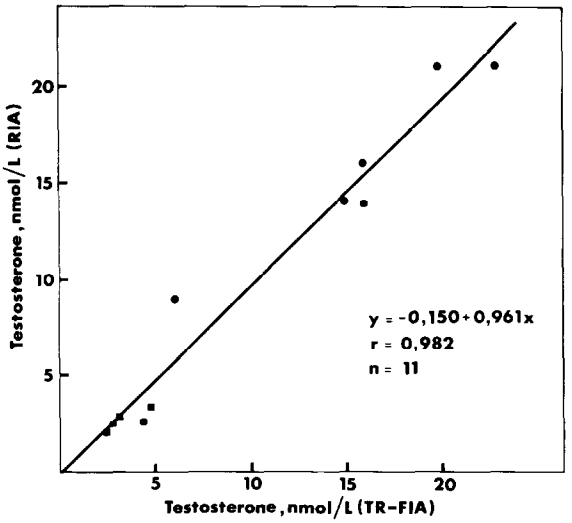

Fig.2. Correlation between the TR-FIA of testosterone and a commercial RIA. Male samples (●), female samples (■).

in extracted serum samples. Fig.2 illustrates the correlation between the TR-FIA and a commercial RIA on 5 female and 6 male samples. The correlation was found to be very good (r = 0.982).

In conclusion, the TR-FIA of testosterone was proved to be a very sensitive and reproducible test. The main features of the assay are its simplicity (only two pipettings and no centrifugation step), short fluorescence counting time (1 s), stability of the fluorescent probe and the avoidance of hazardous radioactivity.

Table 1

Cross-reactions expressed as percentages at a level of 50% replacement for testosterone using polyclonal and monoclonal antibodies in TR-FIA and RIA measurements

Compound	Monoclonal label		Polyclonal label	
	FIA	RIA	FIA	RIA
Testosterone	100	100	100	100
5α-Dihydrotestosterone	100	100	28	30
5β-Dihydrotestosterone	1.0	0.5	31	25
17α-Epitestosterone	0.1	0.2	0.1	0.8
Androstenedione	0.4	<0.1	2.5	0.2
Estradiol	<0.1	–	<0.1	<0.05
Progesterone	1.9	<0.1	<0.1	–
Cortisol	<0.1	<0.1	<0.1	≪0.0005

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