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DIRECT TIME-RESOLVED FLUOROIMMUNOASSAY OF ESTRIOL IN SERUM

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Summary—A rapid, direct, solid-phase, time-resolved fluoroimmunoassay for free estriol in serum, using Europium-chelate–protein A as a label, is described. The coefficient of correlation with the results of RIA was 0.983.

INTRODUCTION

Assay of serum estriol levels is of great clinical value for following the progress of a pregnancy. The levels of free estriol are especially informative because they are not affected by maternal renal function, as total estriol levels are [1]. Many immunoassays for estriol, with different markers and detector systems, have been described [2–5].

Among the procedures that do not use radioactive isotopes, time-resolved fluoroimmunoassay (TR-FIA) is interesting. This technique employs as labels some Europium chelates which, because of their excellent emission characteristics, eliminate the interference from background fluorescence of biological fluids [6]. TR-FIAs for steroids require Europium-labeled specific antibodies [7, 8]. Since it would be useful to have a general marker that can be bound to antibodies *in situ*, protein A from *Staphylococcus aureus*, which binds rapidly to IgG of various species without influencing its antigen binding capacity [9], has been bound covalently to isothiocyanate-EDTA-Eu [10].

In this article we will describe a TR-FIA for free serum estriol that uses protein A labeled in this way. This assay is better than the similar TR-FIA for progesterone [11] because the incubation of antigen, antibody and Europium-labeled protein A is a one-step operation, not a two step, which is not only easier, but also saves time.

EXPERIMENTAL

Materials

Purified protein A from *Staphylococcus aureus*, estriol standard, agarose-anti-rabbit IgG raised in goats, 6-ketoestriol 6-(*O*-carboxymethyl)oxime, ovalbumin and bovine serum albumin (BSA) were obtained from Sigma (St Louis, MO, U.S.A.); [2,4,6,9-^{3}H]estriol (sp. act. 2.89 TBq/mmol) was purchased from Amersham (Bucks., U.K.). Rabbit anti-6-ketoestriol 6-(*O*-carboxymethyl)oxime–BSA antiserum was obtained through the courtesy of Dr G. Bolelli (Fisiologia della Riproduzione, Università di Bologna, Italy). The antiserum was used at a 1/5000 working dilution. Lyphochek immunoassay control sera were purchased from Bio-Rad (Hercules, CA, U.S.A.). The serum samples assayed were those used for the Italian interlaboratory quality control. The scintillation solution Picofluor 40 was purchased from Packard (Groningen, Holland). The enhancement solution was kindly given by Pharmacia (Uppsala, Sweden). The other chemicals were obtained from Carlo Erba (Milan, Italy).

Apparatus

An Arcus model 1230 fluorometer with time-resolution (Wallac) was used for the Europium measurements. Radioactivity was counted with a Packard Tricarb 1500 β-counter.

Label synthesis

The label was prepared as follows: 250 mg of 1-(*p*-aminophenyl)ethylene-diaminotetraacetic acid (sodium salt), synthesized by the Sundberg method [12], were dissolved in 10 ml 2 M HCl and 1 ml thiophosgene dissolved in 2 ml CCl_4 was added; the reaction mixture was stirred at room temperature in a fume hood for 5 h [13]. After extraction with ethyl ether, the aqueous layer was evaporated. The residue was dissolved in 10 ml citrate buffer (0.1 mol/l, pH 6.0); 500 mg $EuCl_3.6H_2O$ were added and the

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solution was evaporated; the chelate–protein A coupling was made as already described [10]. The molar ratio Eu^{3+}–protein A (11:1) of the label was determined by comparison with the fluorescence of $EuCl_3$ standard solutions.

Preparation of estriol–ovalbumin conjugate

The conjugate was prepared as follows: 25 mg of 6-ketoestriol 6-(*O*-carboxymethyl)oxime were dissolved in 3 ml anhydrous dioxane, cooled to 4°C and 47 μl of tri-*n*-butylamine, were added. After 5 min 8 μl of *iso*butylchloroformate were added and the solution was left for 30 min at 4°C. This mixture was added to 80 mg of BSA dissolved in 8 ml 40% dioxane in water, to which 200 μl 1 N NaOH was added. The reaction mixture was stirred at 4°C for 1 h while the pH was kept at 8.5–9.5 by repeatedly adding 20 μl 1 N NaOH then it was left at 4°C overnight. The conjugate was purified by gel filtration on Sephadex G-25 (2.5 × 35 cm column by using 0.05 M phosphate buffer, pH 7.3, as eluting solution) and lyophilized.

Coating of polystyrene microtiter strips

The estriol–ovalbumine conjugate was adsorbed onto the wells of polystyrene microtiter strips. The wells were coated overnight at 27°C with 0.25 ml of 5 μg/ml conjugate solution in 0.1 M sodium carbonate buffer, pH 9.0. After washing with carbonate buffer, a second coating was made with 0.3 ml of 4% BSA solution in carbonate buffer. After 18 h at 27°C, the wells were washed 5 times with assay buffer (0.05 M Tris–HCl, pH 8.2, containing 0.9% NaCl, 0.25% BSA and 0.05% NaN_3) by an immunowash NUNC.

Europium measurement

Europium fluorescence was measured at emission wavelength (em) = 615 nm with excitation wavelength (ex) = 345 nm as β-diketone chelate obtained with an enhancement solution (phthalate–HCl buffer 0.1 M, pH 3.2; 2-naphthoyltrifluoroacetone 15 μM; tri-*n*-octyl-phosphine oxide 50 μM and Triton X-100 0.1%) [14]. The measuring time was 1 s, the delay time 400 μs, the counting time 500 μs. The reading was made 15 min after the enhancement solution was added to the sample.

Immunoassay

A mixture of diluted antiserum and Eu-labeled protein A (50 pmol/ml) was prepared 15 min before use. A similar mixture was prepared with normal rabbit serum for evaluation of non-specific binding (NSB). A standard curve was run with 50 μl of serial dilutions of the estriol, over the range 0–50 ng/ml in assay buffer: the standard solutions were pipetted in triplicate into microwells containing 10 μl of normal male serum; 10 μl of undiluted samples were also pipetted in triplicate into 50 μl of buffer; 140 μl of antiserum/Eu-labeled protein A were added to each well, except those for NSB, to which 140 μl of non-immune mixture were added. The wells were agitated for 15 min and then incubated for 30 min at room temperature. After washing 6 times with 0.9% NaCl solution, 250 μl of enhancement solution were added, the wells agitated for 10 min and the fluorescence determined.

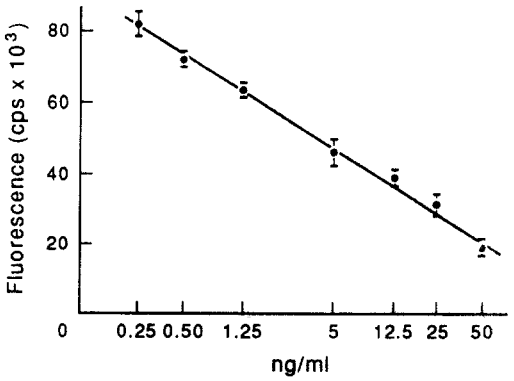

Fig. 1. Standard curve for estriol using TR-FIA. Bars correspond to ±SD. Average of 10 determinations.

RESULTS AND DISCUSSION

The TR-FIA standard curve is shown in Fig. 1. The values reported were means of 10 determinations in triplicate and corrected for NSB (4528 ± 880 cps). The sensitivity of the method was 0.25 ng/ml at twice the SD. Within-assay and between-assay coefficients of variation assayed for 3 serum samples at high (22.4 ng/ml), medium (5.4 ng/ml) and low (2.8 ng/ml) concentration of estriol were 11.5, 9.2 and 9.7% for 12 replicates and 9.6, 10.8 and 8.8% for 10 assays carried out over 4 weeks. Recovery was evaluated by assay of known quantities of standard estriol added to male serum and incubated overnight at 4°C. The

Table 1. Recovery evaluation of estriol in male serum

Estriol added (ng)	Found (ng ± SD, n = 10)	Recovery (%)
5	5.3 ± 0.5	106
10	11.0 ± 1.2	110
20	20.6 ± 2.4	103

Table 2. Parallelism test of 3 different dilutions of serum sample

Estriol concentration (ng/ml)		Recovery (%)
Expected	Measured	
22.5	24.3	108.0
11.2	10.7	95.5
5.6	6.1	108.9

results are given in Table 1. RIA was carried out for comparison in duplicate, with 10,000 cpm of tritiated estriol as the tracer on the same dilution of standard and antiserum as in the TR-FIA, in a total volume of 200 μl. A suspension of agarose antirabbit IgG (50 μl) was used to separate the bound hapten; part of the supernatant (150 μl) was transferred into scintillation vials for measurement of radioactivity. The regression line for 18 samples was: $Y(FIA) = 0.75X(RIA) + 1.07$. The slope is 0.75, probably because of the greater affinity of the antibody for the immobilized hapten (which is bound to ovalbumin through a bridge homologous with that to the immunogen) than for the tritiated tracer used in the RIA. For accuracy evaluation the parallelism test was carried out on a serum with known estriol concentration after dilution with different volumes of a normal male serum. The measured and expected estriol concentration values are shown in Table 2. We have also assayed by RIA and TR-FIA the Lyphochek immunoassay control serum at different concentrations of estriol; the values agreed with those reported for interlaboratory quality control as shown in Table 3. Cross-reactions of antiserum with various steroids were measured in the presence of 10 μl of normal male serum as reported in Table 4. The good coefficient of correlation ($r = 0.983$) means that this method can be used for direct determination of free estriol in the serum. In addition the overestimate that usually is obtained in direct methods is limited in our method to the low cross-reactivity of the antiserum with interfering metabolites. The major interfering substance, because of its concentration in serum, is estriol 3-sulfate, and this would not contribute more than 10% to an overestimate. The specificity of the antiserum is, of course, fundamental and the very high

Table 3. Accuracy evaluation by 3 control sera

Control[a] (ng/ml)	RIA (ng/ml)	TR-FIA (ng/ml)
3.4	3.6	3.8
14.5	18.7	15.0
36.6	40.8	35.2

[a]Bio-Rad quantimune

Table 4. Cross-reactions expressed as % concentrations giving 50% inhibition of estriol binding

	Percent cross-reactivity	
	RIA	TR-FIA
Estriol	100	100
16-Epiestriol	15.2	14.5
15α-Hydroxyestriol	8.0	6.5
Estriol 3-sulfate	4.5	5.0
16α-Dihydroxyestrone	2.5	3.2
Estradiol	0.5	0.1
16-Ketoestradiol	0.1	0.2
Estriol 3-(β-D-glucuronide)	<0.1	<0.01
Estriol 16a-(β-D-glucuronide)	<0.01	<0.01
17-Epiestriol	<0.01	<0.01
Estrone	<0.01	<0.01
Estrone 3-sulfate	<0.01	<0.01

overestimates in the literature [15] can be attributed to partial recognition by the antibody of fractions of the conjugated estriol. No overestimates have been reported when more specific monoclonal antibodies were used [16]. Our method covers the concentration range covered by the commonly-used commercial kits and, as for clinical analyses using the kits [17], each laboratory must provide its own range of normal values. The single-step procedure is simpler than the double-step procedure for progesterone [11] and the time needed for distribution of the reagents and the incubation time are both shorter. The entire procedure requires 90 min for 100 samples, including the fluorimeter reading. We feel therefore, that this method is a valid alternative for RIA.

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