Comparison of Two Time-Resolved Fluoroimmunoassays (TR-FIA), as Applied to Oestriol in Human Serum and Progesterone in Bovine Milk

By A. Ins, C. Iacobello, G. Meroni and Maria Angela Bacigalupo

1 Istituto Chimica Ormoni C. N. R., Milano, Italy
2 Terzo Laboratorio Spedali Civili, Brescia, Italy

(Received January 18/April 26, 1993)

Summary:
We compared two time-resolved fluoroimmunoassay systems for measuring free oestriol in human serum and progesterone in bovine milk. By reading the fluorescence of europium complex of 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid in solution, the measuring range is increased for both oestriol (10—50 000 ng/l instead of 25—50 000 ng/l) and for progesterone (10—50 000 ng/l instead of 25—10 000 ng/l). In addition, the interassay coefficients of variation were lowered from 9.5 to 5.7% for oestriol and from 7.5 to 5.4% for progesterone, at the smaller hormone concentrations detectable by each method.

Introduction
Commercial kits currently used for time-resolved fluoroimmunological assay (TR-FIA) employ two different europium chelators, p-isothiocyanatophenyl-EDTA europium complex in the Delfia method (1) and 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid in the Cyber Fluor method (2). In the first method the fluorescence is read in solution after addition of an enhancement solution that extracts the europium ion and forms a new complex. In the second method, the fluorescence is read directly on the solid phase. Kropp et al. (3) have introduced a modification of the Cyber Fluor method in which the fluorescence of 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid europium complex is read in solution after dissociation of the complex from the solid phase. Unlike the Delfia method, this system is not affected by contamination with europium and uses a dissociation solution that is less expensive and more stable than the enhancement solution. It has the advantage over the Cyber Fluor method of using common microwells, instead of those of opaque material required for reading in the solid phase. In addition, quantification of 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid in solution may make the assay more sensitive, because in the solid phase measurement only the fluorescence emitted from a small spot on the surface is recorded, while in solution a larger number of tracer molecules are available for measurement. We have compared the two detection systems of the Delfia and the Kropp methods for assaying free oestriol in human serum and progesterone in cow's milk. The assays were direct solid phase assays, using protein A labelled with either p-isothiocyanatophenyl-EDTA europium complex (4, 5) or 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (6) as universal markers for immunoglobulins G.

Materials and Methods

Materials
Purified protein A from Staphylococcus aureus, oestriol standard, 6-keto-oestriol 6-(O-carboxymethyl)oxime, progesterone standard, 11α-hydroxyprogesterone hemisuccinate, ovalbumin and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The enhancement solution (1.0 g/l Triton X-100, 6.8 mmol/l potassium hydrogen phthalate, 0.1 mmol/l acetic acid, 0.05 mmol/l tri-octyl phosphine oxide, 0.015 mmol/l 2-naphthoyltrifluoroacetone) was kindly given to us by Pharmacia (Uppsala, Sweden). The other chemicals were obtained from Carlo Erba (Milan, Italy). Rabbit anti-6-keto-oestriol 6-(O-carboxymethyl)oxime-bovine serum albumin conjugate and rabbit anti 11α-hydroxyprogesterone hemisuccinate-bovine serum albumin conjugate antisera were obtained through the courtesy of Dr. G. Bolelli, Centro Fisiologia della Riproduzione (Bologna, Italy).

Apparatus
An Arcus model 1230 fluorometer with time-resolution (Wallac, Finland) was used for the europium measurements.

Synthesis
Oestriol-ovalbumin conjugate and p-isothiocyanatophenyl-EDTA europium complex progesterone-ovalbumin conjugate (5), 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (2), protein A labelled with p-isothiocyanato-
phenyl-EDTA europium complex (7), and protein A labelled with 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (6) were synthesized as described in earlier publications.

Preparation of the solid phase
Hormone ovalbumin conjugates were adsorbed onto polystyrene microwells. Carbonate buffer (0.1 mol/l, pH 9) (250 μl) containing 5 mg/l oestriol-ovalbumin conjugate or 2 mg/l progesterone ovalbumin conjugate was placed in each well and left to stand overnight at room temperature. After washing with carbonate buffer, the wells were saturated with Tris buffer (0.05 mol/l, pH 7.5) containing 40 g/l bovine serum albumin.

Immunoassays
Oestriol in human serum was assayed immunologically in parallel by the two methods, in 0.05 mol/l Tris/HCl, pH 7.5, using the same concentrations of standard solutions (0—50 μg/l) and the same working dilution of antibody (1:5000), which was mixed 15 min before use with protein A labelled with p-isothiocyanatophenyl-EDTA europium complex or with 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (50 nmol/l). Various concentrations of standard solution (50 μl) and normal male serum (10 μl) were placed in wells with 160 μl of a solution containing antibody and labelled protein A. All assays were performed in duplicate. The wells were shaken for 15 min, incubated for 30 min at room temperature, then washed 6 times with 9 g/l NaCl before addition of the enhancement solution or of the dissociation solution. Non-specific binding for both methods was evaluated by adding to the wells a mixture of rabbit non-immune IgG and protein A labelled with β-isothiocyanatophenyl-EDTA europium complex (non-specific binding < 5% of the value of the zero concentration of added oestriol) or with 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (non-specific binding < 3.5%).

Progesterone was also assayed by both methods, but with a double incubation. The first was for 90 min at room temperature with 100 μl of antisem (working dilution 1:8000), in duplicate, in coated microwells containing 10 μl of whole milk from cows in oestrus to which different concentrations of standard progesterone, from 0 to 50 μg/l had been added. After aspiration of the liquid phase and washing with 9 g/l saline, a second incubation was performed for 1 hour at room temperature with 200 μl of a 5 nmol/l solution of protein A labelled with one of the two chelating agents. The microwells were then washed again with 9 g/l saline before the fluorescence was read. Non-specific binding, evaluated as described above, was ≤ 3% for p-isothiocyanatophenyl-EDTA europium complex and ≤ 2% for 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid.

Measurement of fluorescence
After incubation and washing, 250 μl of enhancement solution for the p-isothiocyanatophenyl-EDTA europium complex method or of dissociation solution (4 mol/l urea, 10 g/l sodium dodecylsulphate and 1 μmol/l europium trichloride) (3) for the 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid method were added to each well. For both methods fluorescence was measured after 20 min, using the emission wavelength of 615 nm and the excitation wavelength of 345 nm. The delay time was 400 μs after excitation.

Results and Discussion
Under optimal conditions, with minimal quenching and matrix effects, the greater quantum efficiency of the method with β-naphthoyl-trifluoroacetone europium complex (the complex obtained with the enhancement solution in the Delfia method) makes it possible to read as little as 10⁻¹³ mol/l of Eu³⁺, while the lower limit for reading 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid europium complex in solution is 10⁻¹¹ mol/l (1, 8).

Labeling with p-isothiocyanatophenyl-EDTA europium complex or with 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid affects the binding capacity of protein A.
for immunoglobulins G. The ratio of incorporation, 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid/protein A, must be relatively low. With a ratio of 8, the binding capacity of the protein A is decreased by 50% (6).

Because of these two facts, the method using the enhancement solution gives optimal results when the labelling ratio and intensity of fluorescence are both high.

However, the non-specific binding to the solid phase of protein A labelled with p-isothiocyanatophenyl-EDTA europium complex or with 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid is a determining factor for the range of applicability of the assay, and this depends on the chemical and physical interactions of the label and the molar ratio of label to protein A. Non-specific binding is lower for both oestradiol in serum and progesterone in milk when measured with 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid-labelled protein A in solution.

Measurement of fluorescence with the enhancement solution involves transfer of the europium ion from its complex with p-isothiocyanatophenyl-EDTA on the solid phase to the β-diketone complex in the aqueous phase, and is therefore affected by time and by concentration. With the other method, the entire 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid chelate complex is read in the presence of excess europium, so the signal is more stable with time and more reproducible (see the % coefficients of variation in figs. 1 and 2).

In conclusion, as the standard curve in figure 3 shows, even though the fluorescence signal in the Kropf method is weaker in absolute terms, the sensitivity of the method for the assay of serum oestradiol is greater, the lower limit being 10 ng/l instead of 25 ng/l.

The measuring range of the progesterone assay in cow's milk is increased even more (fig. 4), from 25 ng/l to 10 μg/l to 10 ng/l to 50 μg/l. This is particularly useful when progesterone concentrations are high, because it lessens the error that would be contributed by dilution of the samples.

Acknowledgements

This work was supported by the Consiglio Nazionale delle Ricerche, Progetto Finalizzato Biotecnologie e Biostrumentazioni.

References


Dr. Adriano Ius
Istituto Chimica Ormoni, C.N.R.
via Mario Bianco 9
I-20131 Milano
Italy