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Simultaneous determination of alpha-fetoprotein, human chorionic gonadotropin and estriol in serum of pregnant women by time-resolved fluoroimmunoassay

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Abstract

We have developed a simple and rapid time-resolved fluoroimmunoassay (TR-FIA) for simultaneous determination of alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG) and estriol (E3) using europium and samarium ion chelate. In the proposed method, we used a combination of a 96-well microtiter plate for the AFP and hCG assay and transferable solid phase plate for the E3 assay. Therefore, these analytes could be measured simultaneously. The measurable ranges for AFP, hCG and E3 by the proposed method were 3.91–1000 ng ml^{-1}, 877–250 000 IU l^{-1} and 0.39–100 ng ml^{-1}, respectively. The proposed method which utilized characteristics of a rare earth ion chelate, was convenient (unnecessary diluting samples), quick (96 assays for 2 h), and required only a small quantity sample (50 μl). The principle of this proposed method is applicable to other antigens. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Alpha-fetoprotein; Human chorionic gonadotropin; Estriol; Europium ion chelate; Samarium ion chelate; Simultaneous determination

1. Introduction

A rare earth ion chelate has large a Stokes shift, narrow emission bands and long fluorescence decay time (over 10 000 times longer than general fluorescence). Therefore, time-resolved fluoroimmunoassay (TR-FIA) [1–3] which uses a rare earth ion chelate for labeling and detection is a highly sensitive measurement. We also have reported TR-FIA for 17 alpha-hydroxyprogesterone [4], cholecystokinin-8 [5] and pituitary adenylate cyclase activating peptide-27 [6]. Furthermore, TR-FIA can measure multiple substances with simultaneous use of various rare earth ions e.g. Eu(III), Sm(III), Tb(III) [7–9].

Recently, Xu et al. [10] reported simultaneous quadruple TR-FIA which was labeled with four

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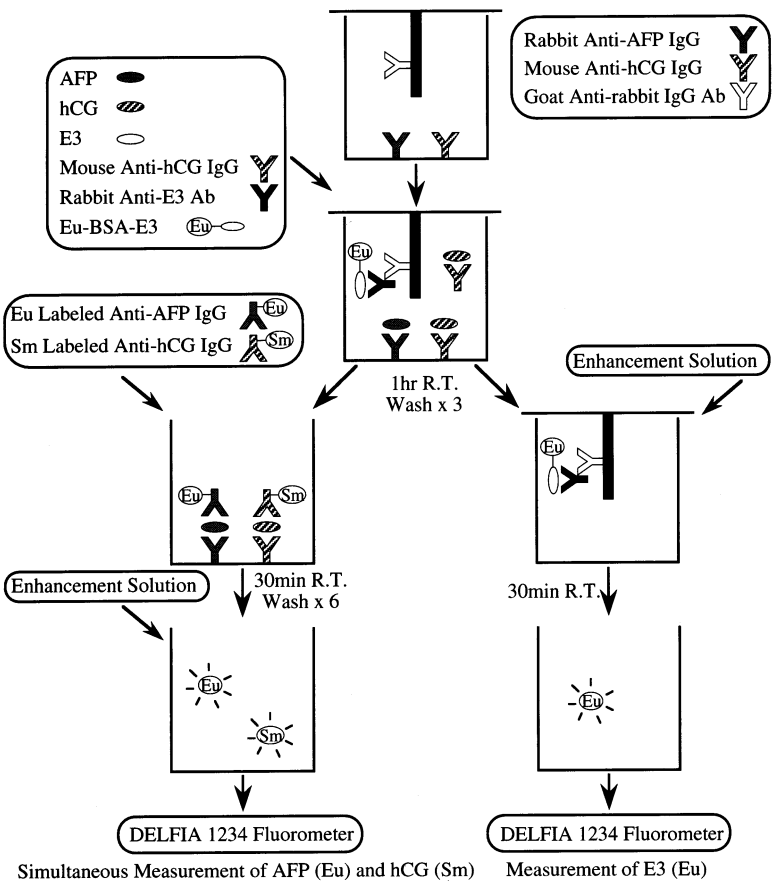
kinds of rare earth ion chelate (Eu(III), Sm(III), Tb(III) and Dy(III)). Their method was very significant, but particular enhancement solutions for Tb(III) and Dy(III), which are less sensitive than the other ions, is required. The TR-FIA developed in this study measures three analytes simultaneously, despite using only two ion chelates of Eu(III) and Sm(III). This method is combined and uses both microtiter and transferable solid phase (TSP) plates (Scheme 1). The TSP plate has 96 pins which correspond to a 96-well microtiter plate. An immunoreaction is performed on both plates. As TSP plates can be quickly and easily transferred to another microtiter plate, multiple analytes can be measured simultaneously.

In this paper, we selected alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG) and estriol (E3) for analytes as an example in the proposed TR-FIA. These are important substances that were measured in maternal screening for Down's syndrome [11]. In addition, we attempted to measure maternal serum samples by the proposed method.

2. Material and methods

2.1. Reagents

Estriol (E3) and estron were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 6-Ketoestriol 6-(*O*-carboxymethyl) oxime (E3-6CMO), estriol 3-carboxymethyl ether (E3-3CME) and other E3 relative derivatives were purchased from



Scheme 1. Schematic illustration of simultaneous determination for AFP, hCG and E3 by the proposed TR-FIA.

Sigma Chemical Co. (St. Louis, MO). An alpha-fetoprotein (AFP) and a human chorionic gonadotropin (hCG) were purchased from Scripps Laboratories (San Diego, CA). Rabbit anti-AFP IgG for immobilization was purchased from DAKO Japan Co. (Kyoto, Japan). Mouse monoclonal anti-AFP IgG for labeling, mouse monoclonal anti-hCG IgG for labeling or immobilizing and AFP free human pooled serum were obtained from Sankyo Co. (Tokyo Japan). Four types of rabbit anti-E3 antisera were obtained from Hycor Biochemical Inc. (Irvine, CA), Cosmo Co. (Tokyo, Japan), UCB Bioproducts (Poole, UK) and Teikokuzoki Pharmaceutical Co. (Kawasaki, Japan), respectively. Goat anti-rabbit IgG antibody was obtained from Tosoh Co. (Tokyo, Japan). Eu(III) and Sm(III) chelate labeling reagents were purchased from Wallac Oy (Turku, Finland). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (WSC) and *N*-hydroxysuccinimide (NHS) were purchased from Dojindo Laboratories (Kumamoto, Japan). The 96-well microtiter plate and TSP plate were purchased from Nunc Co. (Roskilde, Denmark). Other reagents used were of analytical grade.

2.2. Solutions

Assay buffer: 0.1 M Tris–HCl buffer (pH 7.75) containing 0.15 M NaCl, 0.05% NaN_3, 20 μM DTPA, 0.5% bovine serum albumin (BSA), 0.05% bovine gamma globulin and 0.01% Tween 40. Enhancement solution: 0.1 M acetate–phthalate buffer (pH 3.2) containing 0.1% Triton X-100, 15 μM 2-naphthoyltrifluoroacetone and 50 μM tri-*n*-octylphosphine oxide. Washing buffer: 50 mM Tris–HCl buffer (pH 7.0) containing 0.9% NaCl and 0.05% Tween 20. Post coating solution: 50 mM phosphate buffered saline (pH 7.0) containing 5 mM DTPA and 1% water soluble gelatin. Assay buffer and enhancement solution were purchased from Wallac Oy (Turku, Finland).

2.3. Preparation of antibody coated plates

The microtiter plate was coated with 200 μl of 50 mM sodium bicarbonate buffer (pH 9.5) containing rabbit anti-AFP IgG for immobilization and mouse monoclonal anti-hCG IgG for immobilization (10 μg ml^{-1}, respectively). The microtiter plates were allowed to stand at room temperature overnight. The IgG solution was removed and then 200 μl of the post coating solution was added to the wells. The TSP plates were coated with goat anti-rabbit IgG antibody by covering a microtiter plate to which 200 μl of goat anti-rabbit IgG antibody solution (10 μg ml^{-1}) was added and incubated at room temperature overnight. The TSP plates were then post coated using the post coated buffer in the anti-AFP IgG and anti-hCG IgG coated microtiter plates. These plates were stored at 4°C prior to use and remained stable for over 6 months.

2.4. Preparation of Eu(III) chelate or Sm(III) chelate labeled IgG

We labeled mouse monoclonal anti-AFP IgG with Eu(III) chelate and mouse monoclonal anti-hCG with Sm (III) chelate, respectively. The procedures are described below. One milligram per ml of IgG in 50 mM sodium bicarbonate buffer (pH 9.5) reacted with 100 μl of Eu(III) or Sm(III) chelate labeling reagent (2 mg ml^{-1}). After incubation at 4°C overnight, the reaction was stopped by adding 100 μl of 1 M Tris–HCl buffer (pH 7.0). The Eu(III) or Sm(III) chelate labeled IgG was purified on a Sephadex G-25 column (2 cm i.d. × 47 cm) equilibrated and eluted with 50 mM Tris–HCl buffer (pH 7.0) containing 0.9% NaCl and 0.05% NaN_3. We collected 1 ml fractions of Eu(III) or Sm(III) chelate labeled IgG. The fractions with high immunoreactivity and time-resolved fluorescence intensity were stored at −20°C prior to use after addition of 10 μl of 7.5% BSA solution.

2.5. Preparation of Eu(III) chelate labeled E3-BSA conjugate (Eu-BSA-E3)

In this study, we used Eu(III) chelate labeled E3-BSA conjugate as a tracer for E3 assay. Initially, we prepared E3-BSA conjugate, and then E3-BSA conjugate which was labeled with Eu(III). Preparation of E3-BSA conjugate was as

following: The carboxyl group of E3-6CMO or E3-3CME was activated to an ester derivative by using NHS and WSC. Briefly, 10 mg of E3-6CMO was dissolved in 500 μl of anhydrous dimethylsulfoxide (DMSO) and 7.7 mg of WSC and 4.6 mg of NHS was added. After incubation at room temperature for 3 h, the reaction mixture was added to 150 ml of ethyl acetate, and then washed three times with 50 ml of saturated NaCl solution. The organic layer was dried by sodium sulfate anhydrous. The activated ester derivative of E3-6CMO was obtained by evaporating the organic layer. Ten moles excess amount of the derivative was dissolved in 50 μl of DMSO and added to 1 mg ml^{-1} of BSA in 50 mM sodium bicarbonate buffer (pH 9.5). After standing at room temperature overnight, the mixture was purified by a Sephadex G-25 column (2 cm i.d. × 47 cm) equilibrated and eluted with 50 mM sodium bicarbonate buffer (pH 9.5). The fractions of E3-BSA conjugate which have higher absorbance at 280 nm were collected and concentrated by ultrafiltration. Further, the E3-BSA conjugate was reacted with 30 moles excess of Eu(III) chelate labeling reagent. An excess Eu(III) chelate labeling reagent was removed by using a Sephadex G-25 column (2 cm i.d. × 47 cm) which was equilibrated and eluted with 50 mM Tris–HCl buffer (pH 7.0) containing 0.9% NaCl and 0.05% NaN_3. The fractions containing Eu-BSA-E3 were stored at −80°C after addition of 10 μl of a 7.5% BSA solution. Furthermore, we prepared activated ester derivative of E3-3CME with 8.3 mg of WSC and 4.98 mg of NHS, conjugated to BSA, and then labeled with Eu(III) chelate by using the method described above.

2.6. Time-resolved fluoroimmunoassay

An aliquot of 50 μl of standard solution which contained AFP, hCG and E3 in AFP free human pooled serum or sample serum, 25 μl of Eu-BSA-E3-6CMO solution (0.39 μg ml^{-1}), 25 μl of rabbit anti-E3 antiserum solution (1: 40 000, obtained from Teikokuzoki Pharmaceutical Co.) and 100 μl of mouse anti-hCG IgG (same antibody of immobilization) solution (40 μg ml^{-1}) were added to anti-AFP IgG and anti-hCG IgG coated microtiter plate. The goat anti-rabbit IgG antibody coated TSP plate was put on the microtiter plate, and incubated for 1 h at room temperature. Both plates were then washed using the washing buffer. We added 100 μl of Eu(III) labeled anti-AFP IgG solution (0.26 μg ml^{-1}) and 100 μl of Sm(III) labeled anti-hCG IgG solution (1.32 μg ml^{-1}) to the microtiter plate. After incubation for 30 min at room temperature, the microtiter plate was rewashed and 200 μl of enhancement solution added. The both time-resolved fluorescent intensity of Eu(III) and Sm(III) as AFP and hCG was measured by model 1234 DELFIA fluorometer (Wallac Oy, Turku, Finland). On the other hand, an intact microtiter plate which was filled with 200 μl of enhancement solution was covered with the TSP plate and allowed to stand for 30 min. The TSP plate was then removed and Eu(III) fluorescent intensity in the microtiter plate was measured as described above.

3. Results and Discussion

3.1. Standard curves

In this study, we attempted to measure three analytes which are AFP, hCG and E3 in maternal serum using two ion chelates labeled. We used a 96-well microtiter plate for AFP and hCG assay and a TSP plate for E3 assay. Therefore, these analytes were available to measure simultaneously. Initially, we resolved optimum assay conditions for each analyte, individually.

In the E3 assay, we prepared two kinds of labeled antigen (Eu(III)-BSA-E3-3CME, Eu(III)-BSA-E3-6CMO) and used with four kinds of anti-E3 antiserum (Hycor, Cosmo, UCB, Teikokuzoki). Each antisera was produced by rabbit using E3-6CMO-carrier protein conjugate as an immunogen. Optimum assay conditions were determined for every eight combination of four antibodies and two Eu(III) labeled antigens, sensitivity and cross-reactivity, were also tested. The use of Eu-BSA-E3-3CME as a conjugated antigen resulted in higher sensitivities at the concentration yielding 50% inhibition (IC_{50}) than use of in each antisera (Table 1). However, cross-reac-

Table 1
Cross-reactivity of anti-estriol antibody by the proposed TR-FIA[a]

Anti-E3 Ab	Hycor		Cosmo		UCB		Teikokuzouki	
Conjugated antigen	E3-3-CME	E3-6-CMO	E3-3-CME	E3-6-CMO	E3-3-CME	E3-6-CMO	E3-3-CME	E3-6-CMO
IC_{50} (ng ml^{-1})	2.80	7.00	2.50	11.00	2.70	30.00	2.55	7.14
Compounds								
Estriol	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Estriol 3-Sulfate	58.82	24.14	40.00	9.17	53.33	0.80	29.33	5.46
Estriol 3-Glucronide	100.00	1.39	100.00	52.00	–	–	100.00	53.33
Estriol 3,16,17-Trisulfate	0.15	0.06	0.02	0.09	0.04	0.05	0.02	0.03
Estriol 16-Glucuronide	0.97	63.64	0.67	0.85	0.86	3.64	0.44	0.81
Estriol 17-Glucuronide	0.02	0.08	0.02	0.09	0.07	6.15	0.02	0.03
16-Epiestriol	5.00	21.88	0.91	0.92	80.00	72.73	44.00	12.00
Estrone	<0.01	0.18	<0.01	<0.01	<0.01	0.07	<0.01	<0.01
Estrone 3-Sulfate	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Estrone 3-Glucuronide	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Estradiol	0.12	1.80	0.03	0.15	0.08	2.00	0.12	0.42
Estradiol 3,17-Disulfate	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Estradiol 17-Glucuronide	0.03	0.06	0.05	0.05	0.07	0.25	0.07	0.07

[a] Cross-Reactivity (%)

tivities against estriol 3-sulfate and estriol-3-glucuronide with Eu-BSA-E3-3CME was higher than those with Eu-BSA-E3-6CMO. Generally, a site-heterologous system that uses differently positional derivatives for immunogen and labeled antigen is more sensitive than site-homologous systems that use similarly positional derivatives [12]. This phenomenon was also observed in our proposed TR-FIA, but use of a site-heterologous system resulted in higher cross-reactivities. In a site-homologous system, combination to antiserum of Hycor and Eu-BSA-E3-6CMO was more sensitive (7.00 ng m^{-1}, IC_{50}) than other site-homologous system, but it showed higher cross-reactivities of estriol-16-glucuronide and 16-epiestriol. Each combination of Eu-BSA-E3-6CMO and antiserum from Teikokuzoki or Cosmo had almost similar cross-reactivities (Table 1). In their optimum assay conditions for the proposed TR-FIA, the antisera were used with 3125-times and 40 000-times diluted solutions for Cosmo and Teikokuzoki, respectively. Therefore, we used a combination of antiserum of Teikokuzoki and Eu-BSA-E3-6CMO for the proposed assay as a optimum condition.

For the AFP assay, we selected Eu(III) chelate labeled IgG, due to its higher sensitivity than Sm(III) chelate labeled IgG. One molar of anti-AFP IgG was labeled with 24.5 molar of Eu(III) chelate. We used 0.26 μg ml^{-1} of Eu(III) chelate labeled IgG solution under optimum conditions.

In the hCG assay, the concentration of Sm(III) labeled anti-hCG IgG, which was 9.4:1 of a molar ratio of Sm(III) chelate to the IgG, was 1.32 μg ml^{-1} at optimum conditions. However, under the conditions of this study, the measurable range of hCG was from 7.81 to 500 IU l^{-1}. Furthermore, in order to measure a high concentration hCG in

serum without dilution, we tried the modified method of Stenman et al. [13] (Scheme 1). In the first immunoreaction, we added anti-hCG IgG, which is the same antibody of immobilization, in order to have a competitive reaction of high concentration hCG between immobilized and liquid phase antibody. Therefore, unnecessary hCG was captured by liquid phase antibody and then removed by washing. When 40 μg ml^{-1} of anti-hCG IgG solution was added into the first immunoreaction, the measurable range of hCG was expanded to 250 000 IU l^{-1}.

Fig. 1 shows typical standard curves for AFP (a), hCG (b) and E3 (c) by using our proposed simultaneous TR-FIA. These measurable ranges of AFP, hCG and E3 were 3.91–1000 ng ml^{-1}, 877–250 000 IU l^{-1} and 0.39–100 ng ml^{-1}, respectively. The accuracy of intra-assay with each point of standard ($n=8$) for AFP, hCG and E3 were 1.8–9.8, 3.0–9.5 and 3.1–8.9%, and inter-assay with various samples ($n=5$) were 4.9–15.8, 5.3–21.0 and 6.3–21.7%, respectively.

3.2. Recovery test of AFP, hCG and E3 in human pregnancy serum samples

We carried out the recovery tests of AFP, hCG and E3 by using the human pregnancy sera by the proposed simultaneous TR-FIA. As shown in Table 2, different known quantities of AFP (a), hCG (b) and E3 (c) were spiked to various concentration of samples. The mean recovery of AFP was 97.5% ($n=18$), and that of E3 was 100.3% ($n=15$). The range of recovery test for hCG was restricted, because we were not able to obtain extremely high concentration hCG standards. However, we were also able to obtain mean recovery of hCG, that is 109.8% ($n=9$) below 40 000 IU l^{-1}. The each of all mean recoveries was acceptable.

3.3. Correlation between the proposed TR-FIA and other methods

The AFP, hCG and E3 concentrations of plasma samples were explored by the proposed TR-FIA and conventional methods. The methods compared were: enzyme immunoassay (EIA, Boeringer mannheim, Tokyo, Japan) for AFP, and dissociation enhanced lanthanide fluorescent immunoassay (DELFIA, Wallac Oy, Turku, Finland) for hCG. The regression equation determined for the results was y (TR-FIA) $= 0.972x$ (EIA) $= 6.568$ ($n=86$, $r=0.987$) for AFP (Fig. 2a). We were able to measure the high concentration samples of hCG directly using our TR-FIA method, but DELFIA were required to dilute samples. As shown in Fig. 2b, the high degree of correlation was observed in various concentration samples between the values by the proposed TR-FIA and those by DELFIA (y (TR-FIA) $= 0.850x$ (DELFIA) $= 8251.9$ ($n=47$, $r=0.924$).

For E3, we compared two type of radioimmunoassays (RIA) measurements of total E3 (tE3) (Ortho-Clinical Diagnostics Inc., Tokyo, Japan) and unconjugated E3 (uE3) (Laboratory of Clinical Pathology, Showa University, Tokyo, Japan). The regression equation between tE3 levels by RIA and E3 levels by the proposed TR-FIA was y (TR-FIA) $= 0.298x$ (tE3, RIA) $= 24.80$ ($n=24$, $r=0.887$). On the other hand, the correlation coefficient between uE3 levels by RIA and E3 levels by the proposed TR-FIA was 0.938 (Fig. 2c). This result was better than that between tE3 levels and E3 levels by the proposed TR-FIA. However, the regression equation between uE3 levels by RIA and E3 levels by the proposed TR-FIA was y (TR-FIA) $= 3.367x$ (uE3, RIA) $=$ 16.50 ($n=41$), and our observed E3 levels were about three-times higher than uE3 levels. Furthermore, the regression equation between uE3 levels by RIA and tE3 levels by RIA was y (uE3) $=$ $0.094x$ (tE3) $= 1.59$ ($n=24$, $r=0.784$). This result was due to our anti-E3 antiserum having cross-reactivities of E3 3-sulfate and E3 3-glucuronide (Table 1) which were major conjugates of E3. However, the E3 levels by the proposed TR-FIA was reflected uE3 levels better than tE3 levels.

4. Conclusion

We developed a simultaneous TR-FIA for determination of AFP, hCG and E3 using labeled Eu(III) and Sm(III) ion chelates. In the proposed method, the measurable range of simultaneous

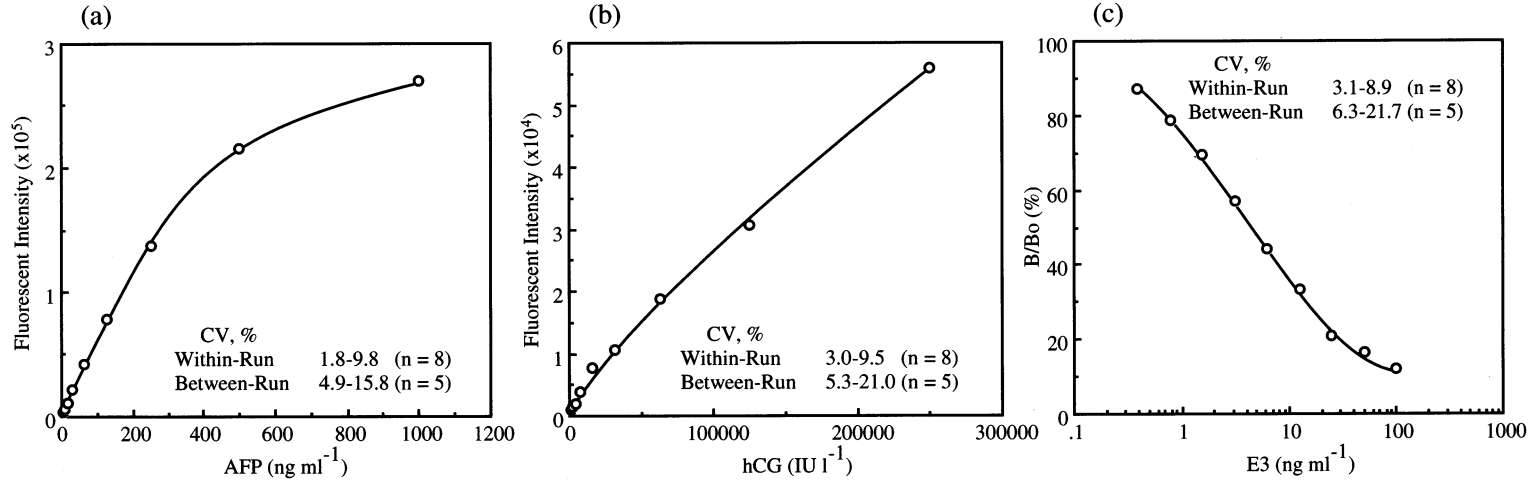


Fig. 1. Standard curves and corresponding within- ($n = 8$) and between-run ($n = 5$) precision of simultaneous determination for AFP (a), hCG (b) and E3 (c) by the proposed TR-FIA.

Table 2
Recoveries for AFP (a), hCG (b) and E3 (c) by the proposed TR-FIA

(a) AFP					(b) hCG					(c) E3				
Sample	Added ng ml^{-1}	Determined ng ml^{-1}	Recovered ng ml^{-1}	Recovery %	Sample	Added IU l^{-1}	Determined IU l^{-1}	Recovered IU l^{-1}	Recovery %	Sample	Added ng ml^{-1}	Determined ng ml^{-1}	Recovered ng ml^{-1}	Recovery %
1	0.00	34.52			1	0.0	7826.4			1	0.00	2.01		
	31.25	69.32	34.80	111.37		7812.5	15106.0	7279.6	93.18		3.13	5.29	3.28	104.96
	125.00	161.38	126.86	101.48		15625.0	24467.6	16641.2	106.50		12.50	14.19	12.18	97.44
	500.00	533.46	498.94	99.79		31250.0	46601.4	38775.0	124.08		50.00	50.51	48.50	97.00
2	0.00	23.85			2	0.0	13743.5			2	0.00	3.46		
	31.25	54.86	29.01	92.84		7812.5	22866.4	9122.9	116.77		3.13	6.58	3.12	99.84
	125.00	145.72	121.87	97.49		15625.0	32340.1	18596.6	119.02		12.50	17.16	13.70	109.60
	500.00	499.01	475.16	95.03		31250.0	43460.0	29716.5	95.09		50.00	52.51	49.05	98.10
3	0.00	21.13			3	0.0	1328.0			3	0.00	6.10		
	31.25	47.90	26.77	85.65		7812.5	9665.9	8337.0	106.71		3.13	9.46	3.36	107.52
	125.00	147.27	126.14	100.91		15625.0	18235.2	16906.3	108.20		12.50	18.69	12.59	100.72
	500.00	501.56	480.43	96.09		31250.0	38410.7	37081.8	118.66		50.00	54.18	48.08	96.16
4	0.00	33.69						Mean ± SD,	109.8 ± 10.8		0.00	6.76		
	31.25	59.55	25.86	82.75							3.13	10.53	3.77	120.64
	125.00	156.63	122.94	98.35							12.50	20.52	13.76	110.08
	500.00	513.76	480.07	96.01							50.00	55.05	48.29	96.58
5	0.00	22.22								5	0.00	10.76		
	31.25	57.85	35.63	114.01							3.13	13.36	2.60	83.20
	125.00	153.20	130.98	104.78							12.50	22.43	11.67	93.36
	500.00	591.37	569.15	113.83							50.00	55.49	44.73	89.46
6	0.00	16.56											Mean ± SD,	100.3 ± 9.2
	31.25	41.36	24.80	79.35										
	125.00	137.96	121.40	97.12										
	500.00	461.22	444.66	88.93										
			Mean ± SD,	97.5 ± 9.7										

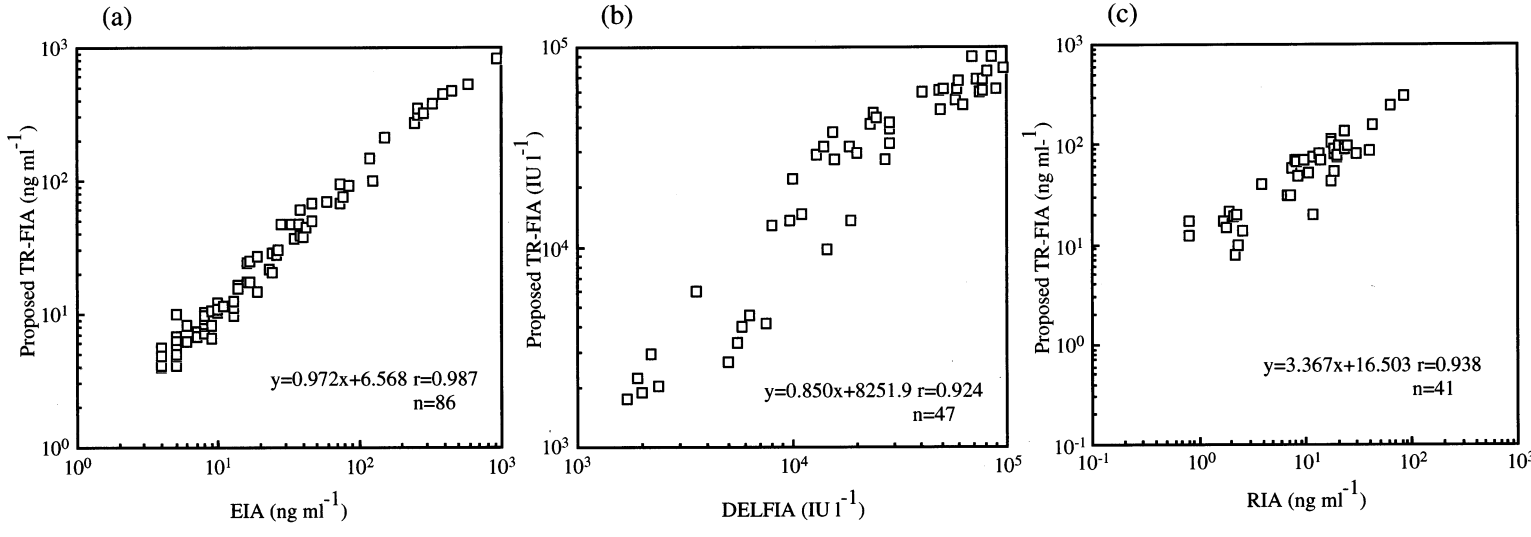


Fig. 2. Correlation of AFP (a), hCG (b) and E3 (c) levels in serum between conventional methods and the proposed TR-FIA.

determination for AFP, hCG and E3 were 3.91–1000 ng ml^{-1}, 877–250 000 IU l^{-1} and 0.39–100 ng ml^{-1}, respectively, and these ranges correlate to normal pregnancy samples. When the proposed TR-FIA was applied to determine maternal serum samples, the correlation of AFP, hCG and E3 levels in serum with the conventional methods were satisfactory.

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