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A secondary antibody format chemiluminescence immunoassay for the determination of estradiol in human serum

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ABSTRACT

A competitive immunoassay for estradiol (E2) based on secondary antibody format was established. The donkey anti-rabbit IgG was used as the secondary antibody to coat micro-plates, and the horseradish peroxidase (HRP)–luminol–H_2O_2 chemiluminescent system with high sensitivity was chosen as the detection system. The addition of sodium trichloroacetate (CCl_3COONa) in the enzyme buffer as a replaceable packing material can realize directly analysis of E2 in human serum without extraction, which improved reproducibility and resolution of the assay. Additionally, the method showed specific recognition of estrogen, without cross-reaction for the major steroids (estrone (E1), estriol (E3), dihydrotestosterone (DHT), androstenedione, testosterone (T)) commonly found in human serum. The chemiluminescence immunoassay with secondary antibody can be applied to detect E2 with good precision at concentrations as low as 1.48 pg mL^{-1}. The proposed method has been successfully applied to the determination of E2 in 97 human sera and showed a good correlation compared with the commercially radioimmunoassay (RIA) kit with a correlative coefficient of 0.9881. This method has exhibited great potential in the fabrication of diagnostic kit and can be used in the clinical analysis of E2 in human serum.

1. Introduction

Estrogens are hormones that are responsible for the development and maintenance of the female reproductive organs and female secondary gender characteristics. Estrogens also participate in the regulation of the menstrual cycle and in the maintenance of pregnancy [1,2]. Estradiol (E2) is the most potent natural estrogen and is derived from the ovaries. Its concentration in blood is of great value for clinical endocrinological investigation in women, such as identifying and monitoring ovarian function, evaluating the condition of early pregnancy, and investigating estrogens-secreting tumors or menstrual disorder [3–5]. The measurement of E2 in human serum is also of great value to monitor replacement therapy.

However, the major challenge of serum estradiol measurement is the low concentrations of estradiol normally found in serum. Serum E2 levels vary over a range from 41 to 272 pg mL^{-1} during the menstrual cycle in premenopausal women, and the E2 levels decrease to about 4–14 pg mL^{-1} after menopause [5–7]. The current chromatography methodologies for quantification of E2 were usually gas chromatography–mass spectrometry [8,9], and liquid chromatography–mass spectrometry [1,10]. But these chromatography methodologies have lower sensitivity, need well-trained personnel and are costly. So, these methods cannot meet the requirements of clinical diagnosis. Radioimmunoassay (RIA) [11,12] has been established for several years as a classical immunoassay method, although this method is reliable and sensitive. The use of ^{125}I as the radioisotopes restricts the application of practical detection in human serum. Enzyme-link immunoassay (ELISA) [13,14] cannot meet the requirements for accurate assessment of steroid hormone in low concentrations because of low sensitivity. Fortunately, chemiluminescence immunoassay (CLIA) based upon secondary antibodies format overcomes these defaults [15,16]. The application of secondary antibody has these advantages as follows: (a) promoting the sensitivity of the assay; (b) eliminating the nonspecificity for many analytes; (c) decreasing the relative standard deviation of the assay; (d) reducing dosage of the antibody. Meanwhile, the secondary antibody can be multivalently bound to a primary antibody to form a constant immunological complex [5]. Thus, CLIA based upon secondary antibodies format is very effective for determining low steroid hormone concentrations.

In this paper, CLIA based upon secondary antibodies format with low detection limit was designed for determination of E2 in human serum samples. The proposed method used the second antibody coating microplate as solid phase, and a horseradish peroxidase (HRP)–luminol–H_2O_2 system with high sensitivity was introduced in the assay. Additionally, the employment of sodium trichloroac-

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etate in the enzymatic buffer as a block agent enhanced displacing E2 from the bind proteins separation. The methodological potentially shown here might be available for the determination of E2 in clinical diagnosis as a commercial kit.

2. Experimental

2.1. Apparatus and reagents

BHP9504 microplate chemiluminescence immunoassay reader was from Beijing Hamamatsu Technology Co., Ltd., (Beijing, China). DEM-III automatic plate washer was got from Tuopu Analytical Instruments Co., Ltd. (Beijing, China). XW-80A blending shaker (Shanghai Jingke Industry Co., Ltd., Shanghai, China) was employed to mix the solutions. HH.W21-Cr000 Electric homoiothermic water-bath tank was from Changan Scientific Equipment Co., Ltd. (Beijing, China). Data acquisition and treatment were performed with an integrated 16-bit microprocessor system. In using the commercial immunoradiometric assay kit for sample analysis, a Gamma counter (GC-400, USTC Chuangxin Co., Ltd., Zonkia Branch, Hefei, China) was used.

Donkey anti-rabbit antibody was obtained from Beijing ChemClin Biotechnology Co., Ltd. (Beijing, China). Rabbit anti-E2 polyclonal antibody, the solution of horseradish peroxidase-labeled estradiol derivative (E2-6-HRP), and estradiol were obtained from Fitzgerald, (USA). Bovine serum albumin (BSA), sodium trichloroacetate (Na-TCA), estrone (E1), estriol (E3), dihydrotestosterone (DHT) and testosterone were from Sigma–Aldrich (St. Louis, MO, USA). Normal human serum and chemiluminescent (CL) substrate were all obtained from Beijing ChemClin Biotechnology Co., Ltd. (Beijing, China).

2.2. Buffers

Coating buffer was 0.05 mol L^{-1} carbonate solution (pH 9.6) and 0.06 mol L^{-1} citrate solution (pH 4.8). Blocking buffer was 0.05 mol L^{-1} phosphate solution (PBS, pH 7.4) containing 1% BSA and 0.1% proclin-300. The PBS buffer was 0.05 mol L^{-1} phosphate buffer, 0.5% saline solution, pH 7.38. The assay buffer was 0.05 mol L^{-1} PB buffer solution containing 1.5% (w/v) BSA and 0.5% (w/v) hydrolyzed gelatin, pH 7.38. The estradiol antibody dilution buffer was 0.01 mol L^{-1} assay buffer solution containing 0.9% (w/v) NaCl, pH 7.38. Washing solution was PBS with 0.01% Tween-20 (PBST). CL substrate solution is luminal, H_2O_2 and *p*-iodophenol solution.

2.3. Preparation of calibrators

The E2 stock solution of 150 ng mL^{-1} was prepared in 25% ethanol solution and stored at −20 °C. For calibration, a serial dilution of the stored solution was prepared in fresh hormone free human serum for 0, 15, 50, 150, 500, 1000 pg mL^{-1}, designated as S_0, S_1, S_2, S_3, S_4, and S_5, correspondingly. The prepared calibrators were stored at 4 °C.

2.4. Purification of donkey anti-rabbit antibody

The saturated ammonium sulfate (SAS) precipitation method [13] was used for the purification of donkey anti-rabbit antibody. Certain volume of donkey anti-rabbit antiserum was diluted with physiological saline and was placed on the blender of magnetic force. The same volume of SAS solution was slowly added to make the saturation of 50% and the last admixture of antiserum and SAS solution was placed at room temperature for 2 h. A centrifuge of low-temperature was used at 4 °C for 30 min and the supernate was discarded. The obtained deposition was dissolved in physiological saline and SAS solution was slowly added to make the saturation of 35%. The centrifugation and dissolution steps were repeated, and then the SAS solution was slowly added to make the saturation of 33%. The centrifugation step was performed further. The deposition obtained at this time was dissolved in 0.02 mol L^{-1} PBS (pH 7.4), dialyzed and then was centrifuged to remove the slight deposition. The final product was the solution of purified donkey anti-rabbit antibody. Its absorbency was determined using ultraviolet (UV) spectrophotometer at 280 nm with 1 cm of photic distance, and the concentration of IgG was calculated according to the molar adsorption coefficient method. An in-split charging product was obtained after the addition of 0.05% (v/v) biological preservative of proclin-300 and stored at −20 °C.

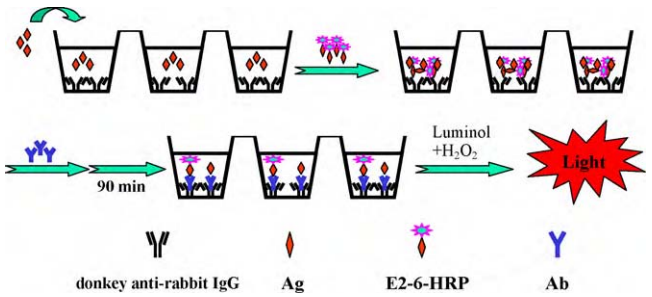

Fig. 1. Schematic illustration of the proposed CLIA.

2.5. Preparation of solid phase antibody

Coating involves the interaction between the solid phase surfaces and reagents in immunoassay. First, each well of the microplates was coated with 300 μL of the purified donkey anti-rabbit antibody (1:1000) in citrate solution (pH 4.8). The plates were allowed to stand sealed at 4 °C overnight. Then, the plate was washed by PBS solution twice and was gently tapped against tissue paper to remove all fluid. After that, 300 μL of the blocking buffer was added into each well and the plate was put at room temperature for 4 h in order to block the active sites on the plate. Subsequently, the solution in the well was aspirated and the plate was made dry. Finally, the plate was vacuumed and stored at 4 °C for further use [13,14].

2.6. Immunoassay procedure

Immunoassay procedures were present in Fig. 1. Firstly, 50 μL E2 calibrators or serum samples were added into the coated microplate. Then 100 μL HRP-labeled E2 and 100 μL rabbit anti-E2 polyclonal antibody were added stepwise. After incubation with gently shaking at 37 °C for 90 min (immunoreaction time), the microplate was washed five times by washing solution to separate free compounds from the bound immunocomplex. The remnants solution was discarded by tapping the plates against tissue paper. Finally, 100 μL chemiluminescent substrate solution was pipetted into each well, the mixture was incubated for 5 min at room temperature in the dark, and the emitted photons were measured.

2.7. Data analysis

Standards and samples were measured in double tubes, and CL intensity values were integrated. Standard curves were obtained by plotting CL intensity against the logarithm of analyte concentration and fitted to the equation of logit *Y*–log *X*, in which the value of log *Y* was calculated according to the formula as follows:

$$\text{logit}\, Y = \ln\left[\frac{y}{1-y}\right] \quad \left(y = \frac{B}{B0}\right) \quad (\text{B} : \text{RLU}_{S1-S5},\ \text{B}_0 : \text{RLU}_{S0})$$

Table 1
Comparison between indirect coating and direct coating ($n = 5$).

	Sensitivity (pg mL^{-1})	CV (%)	Volume (μL)	Dilution ratio	Ratio
Indirect coating	1.48	1.35–12.2	100	1:15,000	<1:10
Direct coating	1.81	5.4–17.1	150	1:2000	

2.8. Samples

The ultimate goal of this paper is widely applied for clinical diagnosis. Thus, human sera obtained from some hospitals in Beijing, China, were tested as the assay samples. All the samples were analyzed using the proposed CLIA without any pretreatment.

3. Results and discussion

3.1. Comparison between second antibody and direct coating of anti-E2 antibody

For the small molecule competitive immunoassay, weak reproducibility and precision are two of the main problems. In order to solve this defect, the second antibody coating method (indirect coating) was carried out and compared with the traditional direct coating. The comparison results are shown in Table 1. As could be seen, the sensitivity of the second antibody coating was higher than direct coating. Moreover the coefficient of variation (CV) of different concentrations serum for the second antibody coating method was 1.35–12.2%, better than direct coating. In addition, using the same anti-E2 polyclonal antibody dilution ratio of 1:15,000, RLUmax of indirect immobilization was 200,811, whereas that of direct coating was only 8890. When RLUmax was arrived at 197,538 for the direct coating, the dilution ratio of anti-E2 polyclonal antibody was 1:2000. Obviously, more than 10 times saving ability for anti-E2 polyclonal antibody was achieved by the second antibody coating. This may be because the second antibody can capture more anti-E2 polyclonal antibody than micro-plates, so that the proposed method uses less antibody and RLU is higher. Therefore, we chose the second antibody format in this experiment.

3.2. Optimization of amount of second antibody

According to the above comparison, the second antibody coating method was selected for the development of E2 CLIA. Generally, an appropriate dosage of the second antibody is one of the important factors for the sensitivity and homogeneity of the assay. It is easy to see from Fig. 2, the RLU of S_0 was increased sharply with the increasing dosage of the second antibody. When the dilution ratio of the second antibody was up to 1:1000, the RLU was maximal, then the RLU was declining. The possible reason for the phenomenon was the molecular superposition owing to superfluous second antibody, and the superposition of molecular was washed after the competitive reaction, so as to decrease the RLU. In addition, the ratio between the RLU of S_1 and S_0 reached an appropriate radio for 83.8%, when the dilution ratio of the donkey anti-rabbit IgG as the second antibody reached 1:1000, which meant that the sensitivity was higher at a dilution ratio of 1:1000. Finally, considering the sensitivity and the dosage of the second antibody, a dilution ratio of 1:1000 was chosen in the whole experiment.

3.3. Optimization of immunoreaction reagents

Theoretically, the amounts of immunoreaction reagents are the key parameters affecting the sensitivity of an assay. Therefore, the chessboard dilution ratios of E2 polyclonal antibody and E2-6-HRP were studied and optimized in this experiment. The results are shown in Table 2. In general, the use of the less amount of antibody often results in high sensitivity in a competitive immunoassay. To achieve higher sensitivity in CLIA, the dilution ratio of the antibody was examined in the range 1:10,000–1:20,000. The results showed that when the dilution ratio was higher than 1:15,000 for the E2 polyclonal antibody, the sensitivity was low. While the dilution ratio was lower than 1:15,000, the sensitivity was not increased obviously instead of reducing the RLU. Therefore, the dilution ratio of 1:15,000 for E2 polyclonal antibody was applied in the experiment.

The dilution ratio of E2-6-HRP conjugation was also evaluated using the proposed method. When the dilution ratio of E2-6-HRP conjugation was lower than 1:2000, it would affect the sensitivity for the determination of E2, and the RLU would be decreased apparently. The value of S_5/S_0 was also lower than 10%, which suggested it gave us a narrow linear range. It is because that less amount of E2-6-HRP conjugation cannot meet the equilibrium between the limited antibody and antigen. Thus, in this experiment, the concentration of 1:2000 for E2-6-HRP conjugation was chosen.

3.4. Influence and optimization of protein blocking agent

During the detection of E2, most E2 exists in the form of protein-binding compounds in human serum and these combinations could severely affect the immunoreaction between antigen and the antibody. To make determination of E2 content more accurate and to keep E2 exhibited in a free station, some protein blocking agents were applied in the system to eliminate the E2 effect on the other protein. Nowadays, 8-aniline-1-naphthol sodium (ANS) and sodium trichloroacetate (CCl_3COONa) are used as protein blocking agents generally. As for ANS, it could make these binding proteins denaturized and release steroid hormone into the serum, but at the same circumstance, its RLU dropped more than 20% comparison with CCl_3COONa, and the use of ANS become one of the main limiting factors on increasing the sensitivity of the assay [15]. Therefore, CCl_3COONa was selected as the protein blocking agent in this study. Meanwhile, the amount of CCl_3COONa was also studied and optimized, and the results are shown in Table 3. It could be seen that

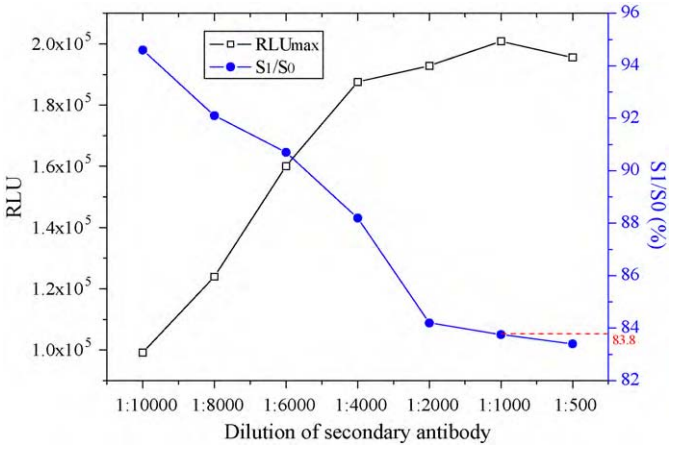

Fig. 2. Effect of the secondary antibody dilution on RLUmax and sensitivity (ratio between RLU of S_0 and S_1) for E2 CLIA. S_0 is the zero calibrator and S_1 is the calibrator with the lowest concentration. S_1:S_0 is the RLU ratio between S_1 and S_0.

Table 2
Optimization of dilution of anti-E2 and E2-HRP ($n = 5$).

Dilution of anti-E2	Dilution of E2-HRP	Relative light units (RLU)			Binding percent (%)	
		S_0	S_1	S_5	S_1/S_0	S_5/S_0
1:10,000	1:2000	258,712	239,835	44,524	92.0	17.2
	1:4000	167,342	151,110	22,089	90.3	13.2
1:15,000	1:2000	200,811	170,829	20,400	85.1	10.2
	1:4000	132,257	119,957	7142	90.7	5.4
1:20,000	1:2000	145,350	120,811	9457	83.1	6.5
	1:4000	108,215	91,117	5086	84.2	4.7

Table 3
Influence of content of CCl_3COONa ($n = 5$).

CCl_3COONa (mol L^{-1})	Relative light units (RLU)	Binding percent (%)		Recovery (%)	
	S_0	S_1	S_1/S_0	Spiked 20 pg mL^{-1}	100 pg mL^{-1}
0.25	267,114	249,217	93.3	81.5	90.2
0.5	199,817	172,842	86.5	96.1	110.8
0.75	105,731	83,633	79.1	110.3	126.7

the RLUs were decreased along with the increase of the amount of CCl_3COONa. Moreover, the recovery nearly 100% and the inhibition were lowest when 0.5 mol L^{-1} CCl_3COONa was applied. Thus, 0.5 mol L^{-1} CCl_3COONa was used as an optimal concentration in the experiment.

3.5. Optimization of immunoreaction condition

The three immunoreaction conditions, including incubation in water-bath at 37 °C, oscillation at room temperature (25 ± 2) °C and standing at room temperature, were compared and their effects on CL intensity were studied. For this experiment, we varied the competitive reaction time from 30 to 120 min to evaluate the effect of incubation time. The result is shown in Fig. 3. RLU of the incubation performed in water-bath at 37 °C, was higher compared with other incubation conditions such as oscillation at room temperature and standing at room temperature. This is attributed to the fact that immunoreaction can be significantly accelerated by temperature and can be brought into equilibrium between antibody and antigen more easily. Also, it was presented that the immunoreaction time for water-bath at 37 °C and oscillation at room temperature were quite close to each other, this may be because higher temperature and gentle shaking both could accelerate the diffusion process and increase the interaction probability between antibody and antigen. By considering the RLU and immunoreaction time of the assay, 90 min incubation in water-bath at 37 °C was selected as the optimal immunoreaction condition in the present work.

3.6. Optimization of CL substrate volume

The CL substrate volume was directly related to the light intensity as well as the sensitivity of an assay. Therefore, the effects of volumes of CL substrate on RLUmax ranging from 50 to 200 μL were also studied. The results are shown in Fig. 4. It was easy to see the RLUs were promoting sharply with the increasing CL substrate volume. However, the ratio between the RLU of S_1 and S_0 was higher than 90% while the volume of CL substrate went beyond 100 μL. In other word, the ratio between the RLU of S_1 and S_0 was directly influenced the sensitivity of the assay, the higher the ratio between the RLU of S_1 and S_0 was, the lower sensitivity was. Considering the sensitivity and practicability of the proposed assay, 100 μL CL substrate volume was selected in the whole experiment.

3.7. Kinetics of CL reaction

In this system, a mixed normal female serum was used for analyzing kinetics of CL reaction after adding CL substrate. A platform of CL lighted by HRP–luminol–H_2O_2 system appears with the stabilizing agent and intensifier. The intensity of CL was the highest

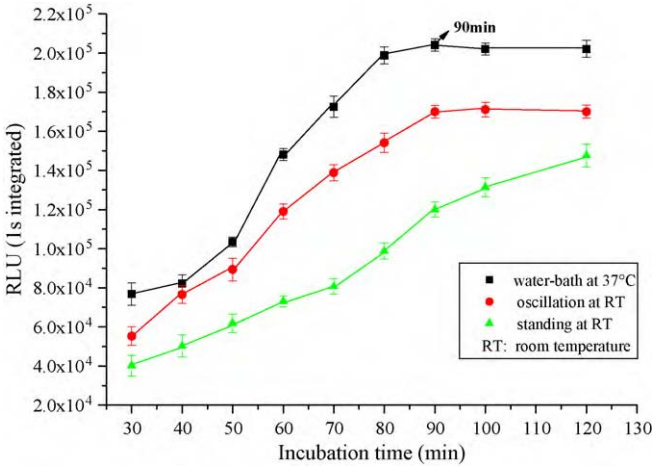

Fig. 3. Results for optimization of immunoreaction condition.

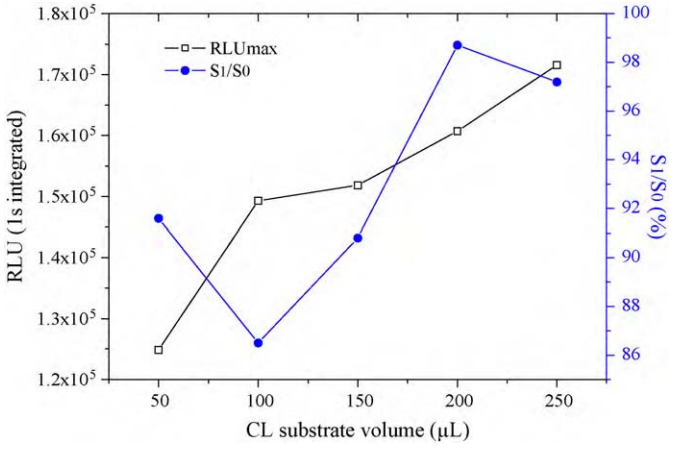

Fig. 4. Optimization of CL substrate volume by estimating the ratio between the RLU of S_1 and S_0. S_0 is the zero calibrator and S_1 is the calibrator with the lowest concentration. S_1:S_0 is the RLU ratio between S_1 and S_0.

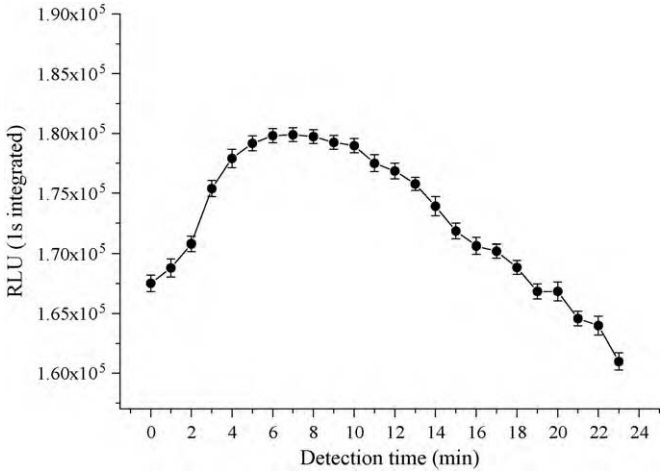

Fig. 5. Kinetics of chemiluminescence reaction.

and can keep constant during the range of the platform. As can be seen from Fig. 5, the intensity of CL sharply increased with the CL reaction time in the range of 0–5 min. From 5 to 10 min, a platform of CL intensity appeared and during this period of reaction time, CL intensity was higher and quite stable. After 10 min, the CL intensity rapidly decreased. This phenomenon could account for the sufficient effect between luminol–H_2O_2 and HRP-labeled E2. Hence, 5–10 min of the detection time was chosen as the optimal reaction time for the purpose of rapid analysis. Comparing with the former magnetic particles CLIA [7], the detection time reduced largely. The possible reason for the phenomena is that the active groups of magnetic particles can keep a long time between luminol–H_2O_2 and HRP-labeled E2, resulting in delaying the platform of CL lighted by HRP–luminol–H_2O_2 system.

3.8. Methodology evaluation

3.8.1. Analytical parameters of the optimized immunoassays

Under the optimal conditions, dose–response curve for E2 was developed and presented in Fig. 6. The linear ranges determined as the concentration causing 15–85% inhibition of the maximal CL intensity was 15–1000 pg mL^{-1}. The detection limit, defined as the minimal dose that can be distinguished from zero, the minimum detected concentration (mean – 2S.D. of zero standard, 10 replicates) of E2 was 1.48 pg mL^{-1}.

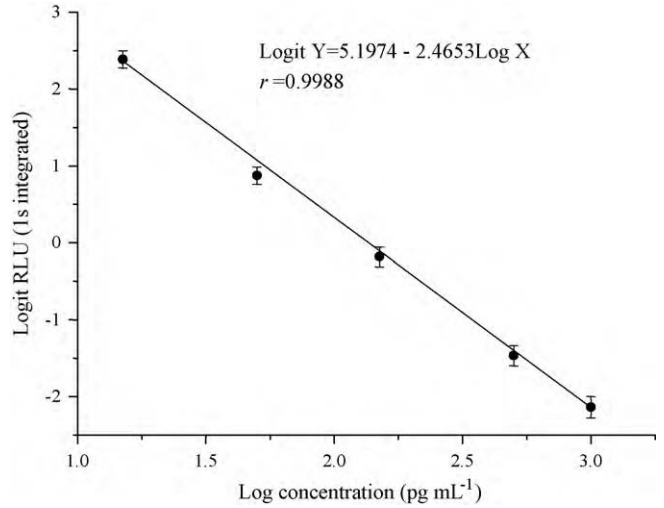

Fig. 6. Calibration graphs of the chemiluminescence immunoassay for E2.

Table 4
Cross-reactivity with some related steroids for E2.

Steroids	Cross-reactivity (%)
Estradiol (E2)	100
Estrone (E1)	2.75
Estriol (E3)	1.2
Androstenedione	<0.01
Dihydrotestosterone (DHT)	<0.01
Testosterone	<0.1

Assay precision was evaluated by spiking different amounts of E2 to normal human serum pools in the 15–85% inhibition range and determining the recovery on the same day (intra-assay) and on different days (inter-assay). Intra-assay and inter-assay coefficients of variation (CV) varied from 1.35 to 12.2%. When the concentration of samples was lowest, the highest CV values were showed. Moreover, obtained recoveries between 86.2 and 107% were also satisfied.

3.8.2. Specificity

Specificity of the immunoreaction should be considered as one of the most important factor in the immunological analysis. Of course, the specificity of the immunoassay was dependent on the antibody's specificity. The cross-reaction of E2 was evaluated using several endocrine disrupting compounds (Table 4) in this work. The results showed that E2 antibody did not cross-react with the structurally related steroids, including E1, E3, androstenedione, DHT, and testosterone, cross-reacted with the antibodies, containing CR lower than 3.0%, which was absolutely acceptable in the analysis.

3.8.3. Linear-dilution test

A linearity-dilution test was performed to investigate whether those samples beyond the detectable range could be accurately quantified after diluting with the free hormone human serum. A serum from a pregnant woman, containing E2 about 600 pg mL^{-1}, was diluted 2-, 4-, 8-, 16- and 32-fold with free hormone human serum and then the samples were analyzed by the proposed CLIA. As shown in Fig. 7, the concentrations of E2 in these samples were proportional to the degree of dilution and the linear correlation was good with correlation coefficient of $r = 0.9994$, indicating that the proposed method could provided accurate quantification for those samples which needed diluting before analysis.

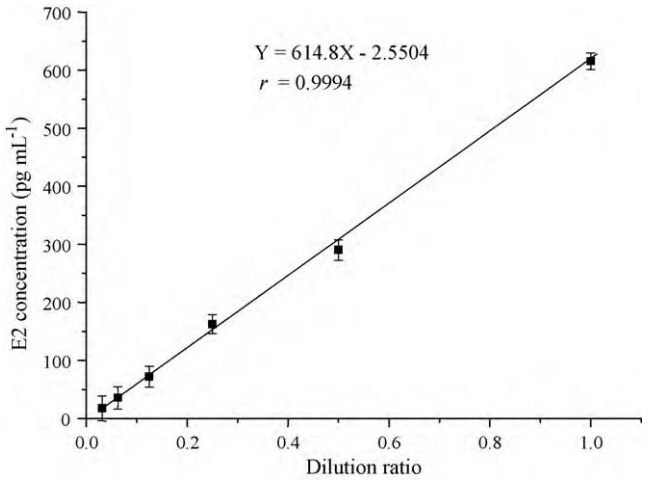

Fig. 7. Linearity-dilution test.

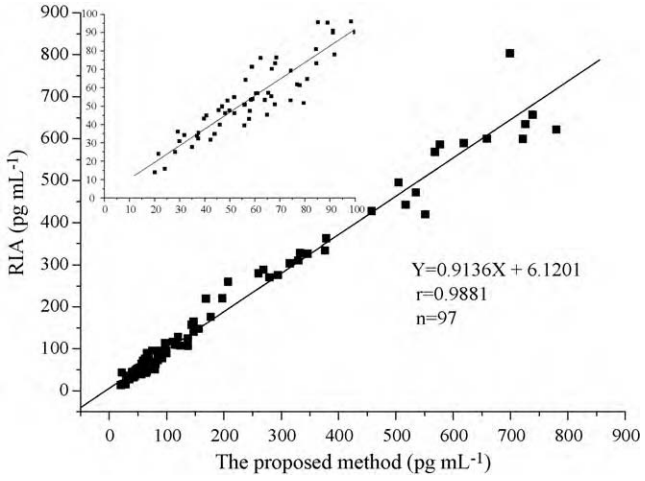

Fig. 8. Correlation between results measured by the proposed CLIA and RIA. The inset shows the correlation at E2 concentration <100 pg mL^{-1}. The equation for the regression line is: $Y = 0.9136X + 6.1201$ ($r = 0.9881$; $n = 97$). The equation of the line of regression in the inset is: $Y = 0.9065X + 1.2049$ ($r = 0.9074$; $n = 57$).

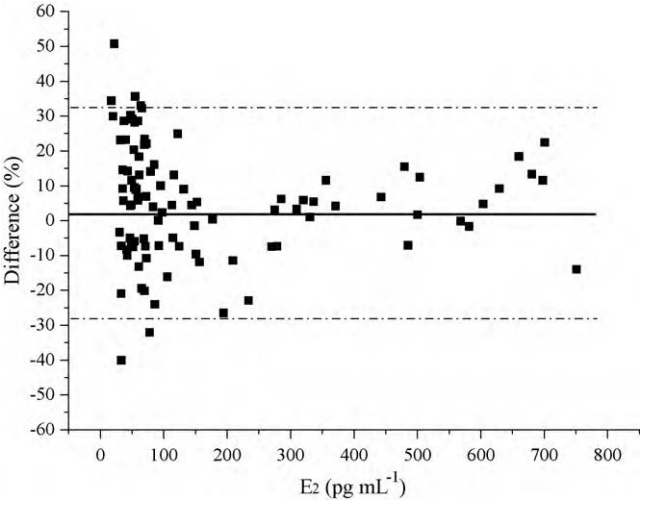

Fig. 9. Difference plot coming E2 values in 97 sera samples measured with the proposed CLIA and the RIA The *x*-axis represents (E2 CLIA assay + E2 RIA assay)/2, and the *y*-axis represents [100 × (E2 CLIA assay − E2 RIA assay)]/[(E2 CLIA assay + E2 RIA assay)/2]. The thick solid line indicates the mean difference between the two methods. The dashed lines indicate ± 2SD.

3.9. Analysis of serum samples

The proposed CLIA was applied to evaluate E2 in human serum. The accuracy was examined by calibration method. The results obtained using the proposed CLIA in the determination of E2 in 97 clinical sera samples were compared with those obtained by a commercial RIA kit. The results are shown in Fig. 7. There was good correlation between the proposed method and RIA with a satisfied coefficient of 0.9881. The difference plot (Fig. 8) for the proposed CLIA method and RIA method [16–18] showed that the mean percentage difference between the two methods was about 3%, and 86 of the 97 samples differed by <30%, 69 of the 97 samples differed by <18%. The assay variation (CV) was relatively high but still within the range of acceptability. An analytical CV equal to or less than one-half of the intra individual variation (<18%) would be considered as desirable performance [16]. Those results were indicated that the proposed CLIA was comparable and acceptable, and could be satisfactorily employed in clinical diagnose screening Fig. 9.

4. Conclusion

A high-throughput, sensitive and rapid CLIA was proposed for the clinical determination of E2 in human serum, using HRP–luminol–H_2O_2 CL system. The indirect immobilization method was used for the preparation of solid phase anti-E2 antibody. The proposed assay consists of donkey anti-rabbit IgG coated micro-plates, anti-E2 polyclonal antibody, E2 calibrators or samples, HRP-labeled E2, and CL substrate. As for the proposed CLIA, the detection limit of 1.48 pg mL^{-1} for E2 could be achieved by the assay with a larger linear range of 0–1000 pg mL^{-1}. The intra-assay and inter-assay coefficients of variation were both below 15% and the accuracy examination gave satisfied recoveries from 86.2 to 107%. This proposed method has been successfully applied for the clinical evaluation of E2 in 97 human sera. The results showed a good correlation with the accredited RIA, demonstrating that the proposed assay could meet the demands in daily clinical diagnosis.

Acknowledgements

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