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Contributed Paper

Magnetic Particles-based Chemiluminescence Immunoassay for Progesterone Determination

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ABSTRACT

A magnetic particles-based chemiluminescence immunoassay was investigated for progesterone detection by using luminometer. In this work, progesterone was determined based on the competitive binding between progesterone in the sample and progesterone-horseradish peroxidase (HRP) conjugate for a constant amount of rabbit anti-progesterone. Initially, anti-rabbit IgG coated magnetic particles conjugated with primary progesterone antibody were bound to progesterone in the samples. Then, the amount of progesterone was quantified by reacting with the residual unoccupied antibody sites with HRP-progesterone, followed by HRP substrate (luminol, H_2O_2, and p-iodophenol (PIP)) and finally detection of the generated chemiluminescence by a luminometer. The intensity of the emitting light was proportional to the amount of enzyme present (HRP-progesterone) and was inversely related to the amount of unlabeled progesterone in the sample. The optimum conditions for determination of progesterone were obtained at 0.15 μg L^{-1} magnetic particles, $5.0x10^{-4}$ mol L^{-1} luminol, $5.0x10^{-3}$ mol L^{-1} H_2O_2, $1.0x10^{-3}$ mol L^{-1} PIP, and phosphate buffer saline buffer pH 9. The optimal dilutions of both anti-progesterone antibody and HRP-progesterone conjugate were 1:1000. The linear relationship between chemiluminescence intensity (RLU) and various concentrations of progesterone was over the concentration range of 0.5-50.0 μg L^{-1}. This proposed method had been successfully applied to the evaluation of progesterone in human sera.

Keywords: progesterone, chemiluminescence immunoassay, magnetic particles

1. INTRODUCTION

Progesterone is a natural steroid hormone involved in the maintenance of the female reproductive system. Progesterone remains relative constant throughout the follicular phase of the menstrual cycle [1-3]. The concentration then increases rapidly following ovulation and

remains elevated for 4-6 days and decreases to the initial level 24 hours before the onset of menstruation. In pregnancy, placental progesterone production rises steadily to level of 10 to 20 times those of the luteal phase peak. Progesterone measurements are thus performed to determine ovulation as well as to characterize luteal phase defects. Monitoring of progesterone therapy and early stage pregnancy evaluations comprise the remaining uses of progesterone assays.

Current methods for the quantification of progesterone can be mainly divided into two categories: chromatographic methods and immunoassay methods. Chromatographic methods [4,5], as the reference methods for the measurement of progesterone in human serum, have numerous predominant advantages of high accuracy, selective, reproducibility and sensitivity. However, chromatographic methods have some disadvantages such as the requirement of expensive instrument, intensive labour and complicated pretreatment of sample. Therefore, the chromatographic methods could not meet the requirements of clinical measurement such as rapidity, convenience, low cost and high throughput. In contrast, immunoassay methods are specific, easy to use and cost-effective and have been widely used for the clinical determination of progesterone. Radioimmunoassay (RIA) [6,7], enzyme linked immunoabsorbent assay (ELISA) [8,9], time resolved fluoroimmunoassay (TRFIA) [10] and fluoroimmunoassay (FIA) [11] have also been reported for the evaluation of progesterone in human serum. RIA has the drawbacks of environmental contamination, short useful-life of reagent and tardiness of determination with the use of radioisotopes, which restrict its further application. The sensitivities of ELISA and FIA are limited and they cannot be used for the measurement of low concentration of progesterone in serum. The chemiluminescence enzyme immunoassay (CLEIA) overcomes the disadvantages of the forementioned immunoassay methods and is automated completely abroad. CLEIA, which integrates the advantages of immunoassay and chemiluminescence determination such as high specificity and throughput, rapidity (does not require long incubations), convenience in operation and relatively simple and inexpensive instrumentation, is a type of clinical diagnostic technique, which is currently applied worldwide.

Magnetic particles (MPs) are typically made of a magnetic iron oxide core surrounded by a non-magnetic polymer that can be functionalized with chemically functional groups, such as, -$CONH_2$ and -COOH, or biomolecules of interest [12]. Inconsequence, MPs are usually used as solid supports for immunoassay reactions. Their features have many advantages because MPs have potentially large binding surface area per volume and hence, a large number of analyte molecules are bound within a small volume, allowing for sensitive detection [13]. Moreover, MPs-based technique eliminates the need for sample pretreatment by centrifugation or filtration, therefore, shortening the time needed. Combining MPs with immunoassay provide a much faster and easier than conventional immunoassay because, in such a homogenous system, the mass-transfer distance of analytes (Antigen; Ag) to the immobilized antibody (Ab) on the MPs is significantly decreased. In addition, the molecules, which are immobilized on MPs, can be easily and quickly separated or dispersed in solution by an external magnetic field.

In this paper, the anti-rabbit IgG is immobilized on carboxylic acid magnetic particles. After immobilization, the competitive binding between progesterone in the sample and progesterone-HRP conjugate for a constant amount of rabbit anti-progesterone has been taken. Next, a solution of chemiluminescence substrate (luminol, H_2O_2, PIP) is then added and the intensity of the emitted light is proportional to the amount of enzyme present and is inversely

related to the amount of progesterone in the sample, was detected.

2. MATERIALS AND METHODS

2.1 Apparatus

A chemiluminescence microplate reader (The POLARstar OPTIMA multi-mode micro-plate reader; BMG LABTECH, Germany) with white opaque 96-well micro-plate (PerkinElmer, USA) that could be placed into the luminometer was used for the magnetic particles-based chemiluminescence detection. The NdFeB magnet of 5 mm length and 2 mm diameter (Swindon, UK) was used for the magnetic separation. A vortex mixer was used to shake the solution after adding the washing solution.

2.2 Chemicals

Progesterone and progesterone-horseradish peroxidase (HRP) conjugate (labeled-progesterone), were provided by the ELISA kit (Progesterone ELISA kit, cat # 9025-16, Calabasas, California). Anti-progesterone IgG (immunoglobulin G) from rabbit (Ab) was purchased from Abcam, England. The Ab was immobilized on the surface of super-paramgnetic particles (2.8 μm diameter, carboxylic acid active groups on the surface, Dynabeads®, Norway), using the following reagent 1-ethyl-3-(3-dimethylminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), all from Fluka, Switzerland. The coupling buffer for surface activation of –COOH groups was a 2-(*N*-Morpholino) ethanesulfonic acid, pH 5 (MES, Sigma-Aldrich). Tris HCl (Sigma-Aldrich) pH 7.4 was used for eliminate the risk of unspecific binding.

The HRP substrate mixture (luminol, p-iodophenol (PIP) and hydrogen peroxide (H_2O_2), all from Fluka, Switzerland) was prepared by dissolution in 10 mM phosphate buffer saline, pH 7.4 (PBS, Sigma-Aldrich).

The human serum samples were collected from local Hospitals and stored at -20 °C until required without any pretreatment.

2.3 Immobilization of Antibody (Ab) on Magnetic Particles

The anti-rabbit IgG (Ab) was immobilized on carboxylic magnetic particles (Figure 1), according to the specific protocol provide by invitrogen®[M 270]. In the first step, 100 mL

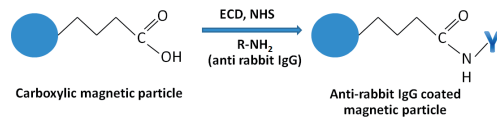

Figure 1. Immobilization of antibody (Ab) on carboxylic magnetic particles.

suspension of magnetic particles, placed in an Eppendrof vial, were separated from the solution using a permanent magnet (Swindon, UK). The supernatant solution was removed, and magnetic particles were washed by re-suspension in 100 mL solution of 25 mmol L^{-1} MES buffer (pH 5), following magnetic separation and removal of the solution. Then, the –COOH groups grafted on the particles surface were activated by adding 50 mL of 50 mg mL^{-1} 1-ethyl-3-(3-dimethylminopropyl)-carbodiimide hydrochloride (EDC) and 50 mL of 50 mg mL^{-1} *N*-hydroxysuccinimide (NHS) in cold MES buffer. Further, the Eppendrof content was vortexed for 30 second, and then incubated for 30 minutes at room temperature under gentle mixing. The excess of reagents was eliminated and the magnetic particles were washed by magnetic separation in/from 100 mL solution of 25 mmol L^{-1} MES buffer (pH 5). The activated magnetic particles were then suspension in 60 mL of Ab solution (1 mg mL^{-1} protein dissolved in 10 mmol L^{-1} PBS (pH 7.4)), and the coupling reaction was performed at room temperature (25 °C), under gentle stirring for 60 minutes. Finally, the resulting magnetic particles was re-suspended in 100 mL 0.05 mol L^{-1} Tris HCl pH 7.4 for 15 minutes to eliminate the risk of unspecific binding, and then washed five times with 100 mL of 10 mmol L^{-1} PBS

solution. The anti-rabbit IgG-coated magnetic particles were further stored as suspension in 10 mmol L^{-1} PBS at 4 ^{o}C.

2.4 The Method for Progesterone Determination Using Luminometer

The progesterone CLEIA is based on the principle of competitive of progesterone present in the sample and progesterone-HRP conjugate (labeled-progesterone) binding to a limited number (constant amount) of anti-progesterone immobilized on anti-rabbit IgG magnetic particles (Figure 2). In the incubation, anti-rabbit IgG-coated magnetic particles were incubated with 50 mL anti-progesterone, 25 mL progesterone standards/samples and 100 mL progesterone-HRP conjugate (labeled-progesterone) at room temperature (18-25 ^{O}C) for at least 30 minutes with slow tilt rotation. After competition has reached equilibrium, unbound labeled-progesterone was eliminated by washing, and the chemiluminescence substrate (luminol solution containing H_2O_2 and p-iodophenol) was then added to give 3-aminophthalate and chemiluminescence emission was produced and detected by using Luminometer. The intensity of the emitting light (Relative Light Units; RLU) was proportional to the amount of enzyme present and was inversely related to the amount of progesterone in the sample (Figure 3).

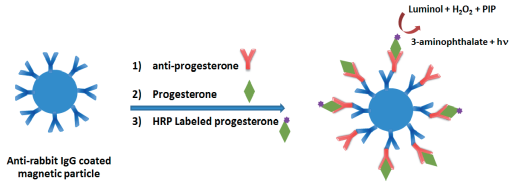

Figure 2. Competitive assay.

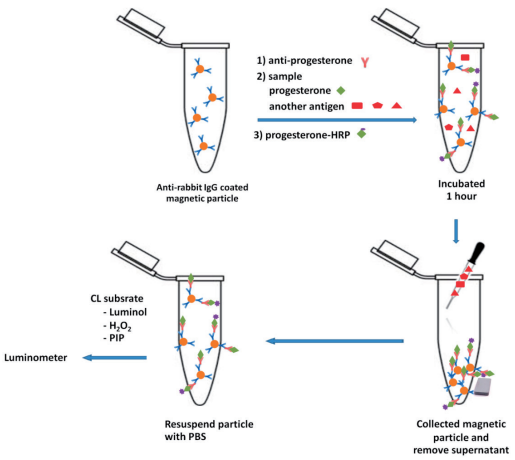

Figure 3. Magnetic particles-based procedures for progesterone determination using a luminometer.

3. Results and Discussion

3.1 Effect of Concentration of Magnetic Particles

The amount of MPs was relative to the amount of anti-rabbit IgG; more antibodies reacted with more antigens, thereby leading to enhanced sensitivity. However, a large amount of the MPs caused particle aggregation. Therefore, effect of the amount of MPs on the chemiluminescence intensity was studied the greatest to obtain sensitivity. The effect of concentration of magnetic particles on chemiluminescence intensity was investigated in the range of 0.015-0.15 mg mL^{-1}, the results obtained were shown in Figure 4A. It was found that, an increase in magnetic particles concentrations leads to improvement in assay sensitivity. Thus, 0.15 mg mL^{-1}, corresponding to $1x10^7$ particles, was chosen as the optimum concentration of magnetic particles with reasonable cost-effective.

3.2 Effect of Dilution of Anti-Progesterone

The effect of anti-progesterone antibody dilution was investigated in the range of 1:5000 to 1:20, the results obtained were shown in Figure 4B. The chemiluminescence intensity (Relative Light Units; RLU) decreased with increasing the dilution of anti-progesterone antibody. Considering the anti-progesterone cost in the use, the dilution ration 1:1000 was chosen in all subsequent with reasonable of a compromise between high sensitivity and the

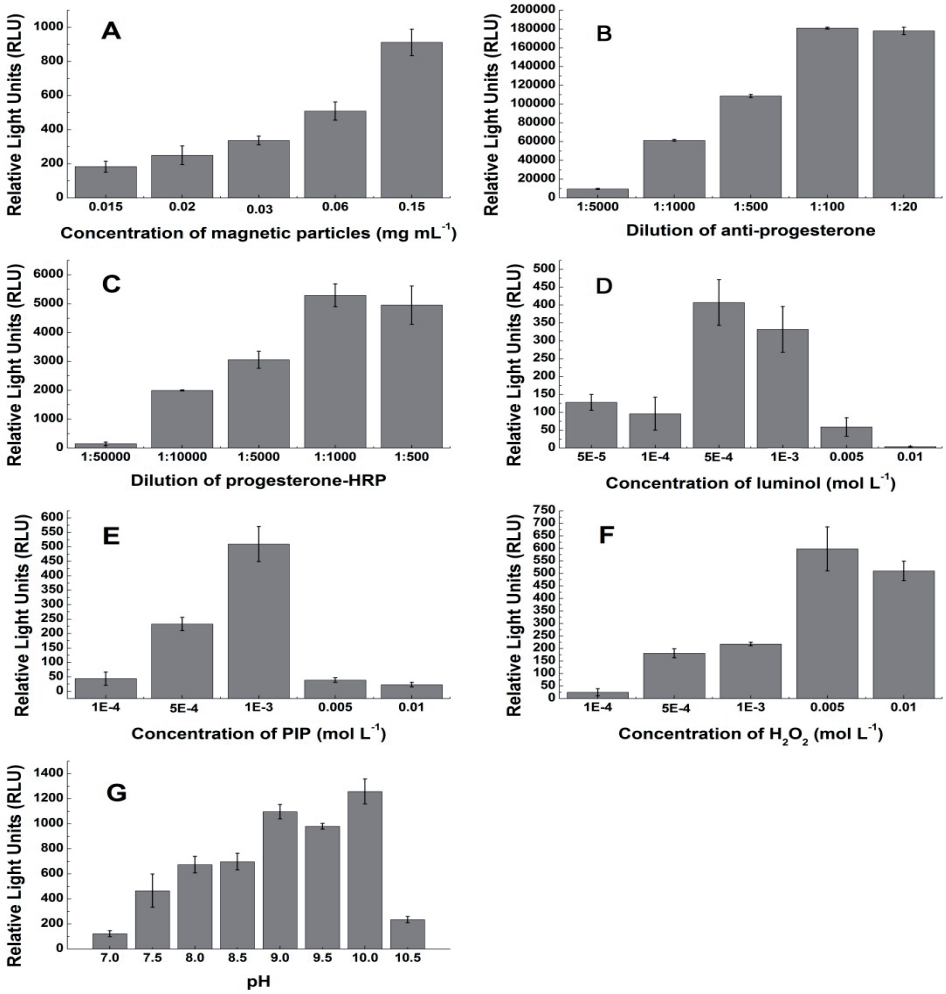

Figure 4. Optimization of reagent concentrations for progesterone determination by using MPs-based CLEIA method: CL signal (RLU) as function of: (A) magnetic particles concentration; (B) anti-progesterone dilution; (C) progesterone-HRP concentration; (D) luminol concentration; (E) PIP concentration; (F) H_2O_2 concentration; and (G) pH.

cost assay. To increase sensitivity, 1:100 diluted of anti-progesterone was being used instead.

3.3 Effect of Progesterone-HRP Dilution

This study was carried out at various progesterone-HRP dilutions between 50000 to 500 fold dilutions, the results obtained were shown in Figure 4C. It was found that, the RLU increased with increasing dilution of progesterone-HRP from 50000 diluted to 1000 folds and then, slightly decreased at 500 folds due to limited number (constant amount) of anti-progesterone immobilized on magnetic particles. Hence, 1000 fold dilutions progesterone-HRP was chosen for further progesterone determination.

3.4 Effect of Luminol Concentration

The effect of luminol concentration on the RLU was studied by varying the concentration of luminol from $5x10^{-5}$ - $1x10^{-2}$ mol L^{-1}, the results obtained were shown in Figure 4D. It

was found that, the RLU increased rapidly up to $5.0x10^{-4}$ mol L^{-1} luminol, and then, further increment of luminol concentration, the RLU decreased rather rapidly and reaching its minimum value as soon as the luminol concentration increased to $1x10^{-2}$ mol L^{-1}. Hence, the $5x10^{-4}$ mol L^{-1} luminol was chosen as optimum luminol concentration for the proposed method, which was used throughout the experiments.

3.5 Effect of PIP Concentration

Light emission from luminol-H_2O_2-peroxidase system can be greatly enhanced by the addition of PIP. The underlying mechanism of this enhanced chemiluminescence is not completely understood. The most probable explanation is that these chemiluminescence enhancers can accelerate one or more of the oxidation step to generate luminol radicals during the complex reaction pathway of the enzymatic oxidation of luminol. This study was carried out at various PIP concentrations between $1x10^{-4}$ - $1x10^{-2}$ mol L^{-1}. As can be seen in Figure 4E, the maximum RLU was obtained with $1x10^{-3}$ mol L^{-1} PIP, above this concentration the RLU decrease. Therefore, the $1x10^{-3}$ mol L^{-1} PIP was selected as optimum condition for further work.

3.6 Effect of H_2O_2 Concentration

The effect of hydrogen peroxide concentration was investigated over the range $1x10^{-4}$ - $1x10^{-2}$ mol L^{-1}, the results obtained were shown in Figure 4F. It can be seen that RLU increased with increasing H_2O_2 concentration from $1x10^{-4}$ -$5x10^{-3}$ mol L^{-1}, and then decreased as soon as the H_2O_2 concentration was beyond $5x10^{-3}$ mol L^{-1}. Therefore, the $5x10^{-3}$ mol L^{-1} H_2O_2 was regarded as the optimum concentration for the following studies.

3.7 Effect of pH

It is well-known that the chemiluminescence intensity of luminol is pH dependence. Oxidation of luminol catalyzed by a peroxidase system is affected by the pH value of the solution. Although HRP is stable in the pH range 4.2-12.0, its activity in the soluble state is maximal at pH 7.0 and greatly reduced under alkaline conditions [14]. However, luminol CL system is most efficient at pHs above 9.0 [15], and this large difference in pH optima significantly limits the application possibilities of peroxidase-catalyzed CL. Hence, the influence of pH values of the solution was carried out at various pH values between pH 7.0-10.5. As can be seen in Figure 4G, the optimum pH (9.0-10.0) for luminol CL catalyzed by a peroxidase-antibody conjugate in solution probably reflects a compromise where peroxidase retains some activity and luminol CL is possible. When the pH of luminol system exceeded 10, the HRP-labeled antibody would denature and become immunologically unresponsive. Although the highest RLU was obtained at pH 10, however at pH 9.0 a high response was also obtained with better repeatability. Hence, this pH was chosen for further progesterone determination.

3.8 Analytical Characteristics

3.8.1 Calibration Curve

A calibration curve was obtained by measurement the RLU of the immobilized magnetic particles using progesterone standard solutions in the range of 0 to 50 μg L^{-1} under the optimum conditions. The optimum conditions for determination of progesterone were obtained at 0.15 μg L^{-1} magnetic particles, $5.0x10^{-4}$ mol L^{-1} luminol, $5.0x10^{-3}$ mol L^{-1} H_2O_2, $1.0x10^{-3}$ mol L^{-1} PIP, and phosphate buffer saline buffer pH 9.0. The optimal dilutions of both anti-progesterone antibody and HRP-progesterone conjugate were 1:1000. The measurement was made in three replicates for each standard solution. Calibration curve were obtained by plotting CL intensity (RLU) against the logarithm of analyte concentration and fitted to the equation of logit Y – log X, in which

the value of logit Y was calculated according to the formula as follows:

$$\text{Logit } Y = \ln\left[\frac{y}{1-y}\right]$$

Where, $Y = C/C_0$, C_0 is the CL intensity of the zero calibrator, C is the CL intensity of other calibrators or samples, and X is concentration of progesterone. These results were shown in Figure 5. The calibration curve was linear over the range 0.5-50 μg L^{-1} and the linear regression equation of logit y = -2.24logx + 1.903. The linear correlative coefficient for this line was 0.9932.

3.8.2 Limit of Detection (LOD) and Repeatability

The detection limit was determined by calculating the minimum amount of progesterone that could be markedly distinguished from C_0 (mean binding at C_0-2SD). It was found to be 0.33 μg L^{-1} progesterone. The repeatability for determining progesterone (10 μg L^{-1}) were carried out by replicate injections (n = 11) within the same day was less than 17%.

3.9 Determination of Progesterone in Serum

Ten women sera were determined by both the proposed method and the progesterone kit (CLEIA). Three replicates of all measurements were made. It was shown that progesterone was found to be in the range of 0.72-16.97 μg L^{-1}. Comparative determination of progesterone in serum by using the proposed method and the progesterone kit (CLEIA) was also carried out. The results obtained from the proposed method compared favorably with those obtained using progesterone kit. Statistical analysis of

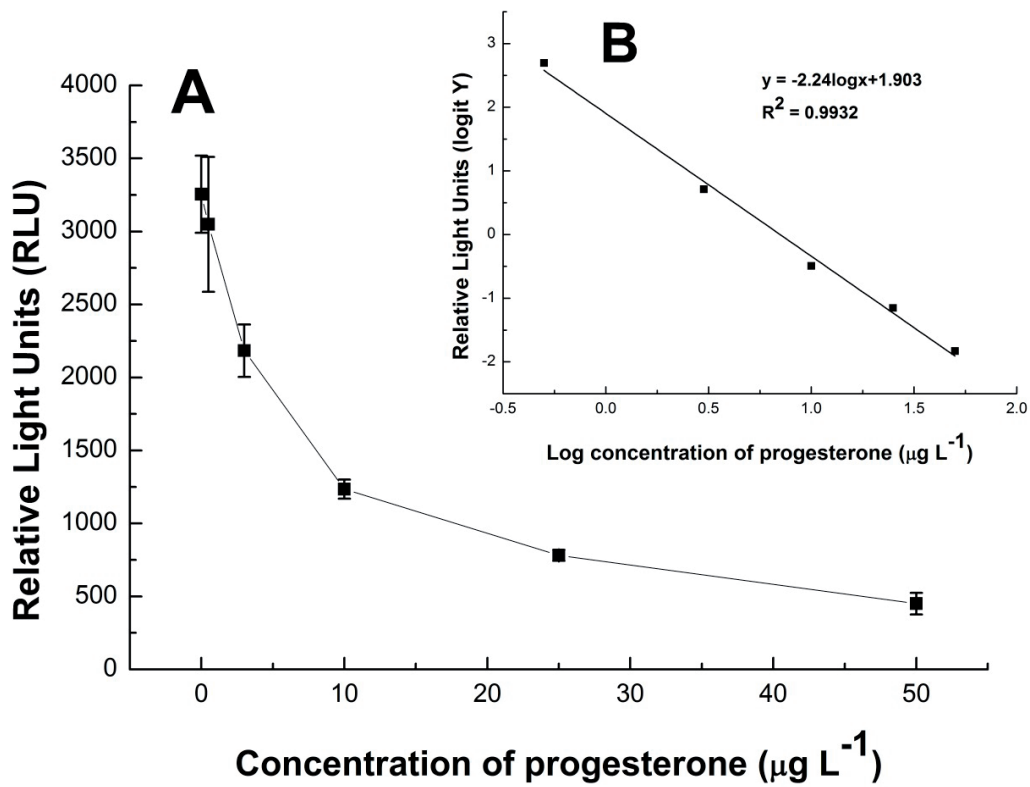

Figure 5. Relationship between progesterone concentrations and Relative Light Units (RLU) by plotting (A) various progesterone concentrations (μg L^{-1}) vs. RLU and (B) the log-log plot of (A).

the results by using t-test showed that there were no significant differences between the two methods at 95% confidence, demonstrating that the proposed method is as accurate and precise as the progesterone kit (CLEIA). The results were summarized in Table 1.

4. Conclusion

The magnetic particles-based chemiluminescence enzyme immunoassay (CLEIA) procedures have been developed for progesterone determination, using HRP-PIP-H_2O_2-luminol chemiluminescence system. The proposed method presented high accuracy, and sensitivity (LOD = 0.33 μg L^{-1}), simplicity and reliability. The proposed method has been successfully applied to the determination of progesterone in human sera and showed a good agreement between the proposed method and the commercial CLEIA kit (verified by Student's t-test). The proposed method seem to be promising as an alternative method for the routine assays and facilitated the development of high-throughput screening and automated operation systems in clinical diagnosis.

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Table 1. Comparison between results measured by the proposed method and the CLEIA kit.

Sample code	Concentration of progesterone (μg L^{-1})		t-values[a]	F-values[b]
	*The proposed method	*CLEIA kit		
S_1	0.75 ± 0.04	0.85±0.01	3.50	16.0
S_2	2.44 ± 0.06	2.68±0.05	4.99	1.28
S_3	1.19 ± 0.01	1.35±0.03	6.14	7.84
S_4	5.08 ± 0.01	5.04±0.02	2.02	4.84
S_5	3.35 ± 0.04	3.18±0.12	1.53	12.3
S_6	1.30±0.07	1.19±0.13	1.01	3.74
S_7	10.80 ± 0.33	12.03 ± 0.69	2.01	4.27
S_8	3.63 ± 0.42	3.43±0.31	0.83	1.84
S_9	16.97 ± 0.69	18.20±0.80	1.66	1.35
S_{10}	0.72 ± 0.06	0.83±0.11	1.19	4.16

*Mean ± RSD of three determinations (n= 3)
[a]Tabulated t-value for p = 0.05 and two degrees of freedom is 2.78.
[b]Tabulated F-value for p = 0.05 and two degrees of freedom is 19.

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