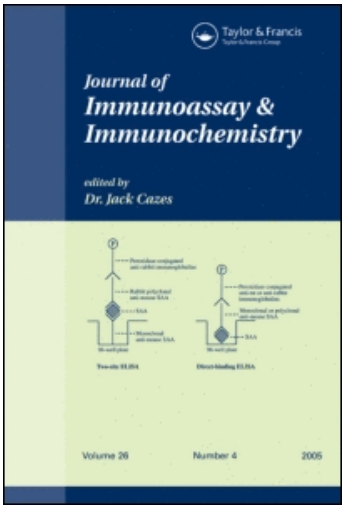


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# NONCOMPETITIVE IMMUNOASSAY FOR FOLLICLE-STIMULATING HORMONE IN HUMAN SERUM USING CAPILLARY ELECTROPHORESIS WITH CHEMILUMINESCENCE DETECTION

Yingxue Zhang[a]; Hong Zheng[a]
[a] College of Chemistry, Chongqing Normal University, Chongqing, P. R. China

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# NONCOMPETITIVE IMMUNOASSAY FOR FOLLICLE-STIMULATING HORMONE IN HUMAN SERUM USING CAPILLARY ELECTROPHORESIS WITH CHEMILUMINESCENCE DETECTION

**Yingxue Zhang and Hong Zheng**
*College of Chemistry, Chongqing Normal University, Chongqing, P. R. China*

□ *A noncompetitive immunoassay based on capillary electrophoresis (CE) with chemiluminescence (CL) detection has been developed for the determination of follicle-stimulating hormone (FSH) in human serum. The work involved the development of separation and CL conditions allowing for routine analysis of serum samples. In this study, horseradish peroxidase (HRP)-labeled monoclonal anti-FSH can catalyze the luminol–hydrogen peroxide reaction. The determined FSH can react with an excessive amount of HRP-labeled anti-FSH. Within 15 min, free enzyme conjugate and immune complex could be separated in alkaline borate buffer by a high voltage of 15 kV. To improve the sensitivity, a series of measures was adopted, including the choice of sodium tetraphenylboron as a new CL enhancer, with a unique design in the detect window. Under optimal conditions, the calibration curve for FSH was established in the concentration range of 1–150 IU/L and the detection limit was 0.08 IU/L. Compared with enzyme-linked immunosorbent assay (ELISA), this method decreased the detection limit by about 25-fold, and it has been successfully employed on determination of the FSH in human serum.*

**Keywords** capillary electrophoresis, chemiluminescence, FSH, human serum, noncompetitive immunoassay



## INTRODUCTION

Follicle-stimulating hormone (FSH) is a glycoprotein gonadotropin secreted by the anterior pituitary in response to gonadotropin-releasing hormone (GnRH), which is released by the hypothalamus. The approximate molecular mass of FSH is 32 kD. The same pituitary cell type also secretes luteinizing hormone (LH), another gonadotropin. FSH and LH are composed of alpha and beta subunits. The specific beta subunit confers the unique biologic activity. FSH and LH bind to receptors in the testis and ovary and regulate gonadal function by promoting sex steroid production and

Address correspondence to Yingxue Zhang, College of Chemistry, Chongqing Normal University, Chongqing 400047, P. R. China. E-mail: zhyxsnow@yahoo.com

gametogenesis. In women, FSH helps control the menstrual cycle and the production of eggs by the ovaries. The amount of FSH varies throughout a woman's menstrual cycle and is highest just before she releases an egg (ovulates). In men, FSH helps control the production of sperm. The amount of FSH in men normally remains constant. The amounts of FSH and other hormones (luteinizing hormone, estrogen, and progesterone) are measured in both a man and a woman to determine why a couple cannot become pregnant (infertility). The FSH level can help determine whether male or female sex organs (testicles or ovaries) are functioning properly. Therefore, it is very important to determine FSH in human serum for evaluation and diagnosis of a wide range of physiological conditions in both women and men.

Analysis of FSH in cattle has been carried out by sensitive radioimmunoassay (RIA) procedures that were established many years ago using $^{125}I$ as the label.[1–3] Although all these methods are reliable and accurate, they suffer from the problems associated with the use of radioisotopes, which restrict their use to specialized laboratories, and with a short half-life. Enzyme-linked immunosorbent assay (ELISA) methods,[4–8] which often depend on some commercial kits including labeled reagents and lacunaris reaction board, correspondingly provide a better alternative due to their nonradioactive nature, but they have additionally suffered from manual operation with washing and rising steps that take hours to complete, and cross-polluting, which resulted in inaccurate results. Currently, available random-access, fully automated, commercial systems have been developed[9,10] for the determination of FSH, while the main conventional methods are still RIA and ELISA in clinical diagnosis. High-performance liquid chromatography (HPLC) is the regular means in comparison with ELISA due to its broad application in pharmaceutical applications and biologic samples analysis as well as clinical diagnosis,[11–14] and one report was found for the determination of FSH.[15] It can be seen in triennial reviews[16–18] that capillary electrophoresis (CE) has proven to be a powerful separation tool, and therefore it is used as a useful separation method for rapid and efficient immunoassays (IA). Laser-induced fluorescence (LIF) detection is still the usual choice in CEIA because of its high sensitivity, commercially available apparatus, and easy preparation of fluorescent-labeled tracer, and CEIA-LIF has also been applied to some drugs and biological hormones.[19–23] In addition, chemiluminescence (CL) detection has been already used in CEIA[24,25] due to its high sensitivity, low cost, wide linear range, and lack of requirement of exciting sources. Nevertheless, there was no report for analysis FSH in CE methods.

In this article, a noncompetitive IA based on CE-CL detection has been developed for the determination of FSH in human serum. The conditions for the CL reaction and electrophoresis were investigated in detail using horseradish peroxidase (HRP)-labeled monoclonal anti-FSH as the

performance of the system because of its catalytic effects on the luminol–hydrogen peroxide reaction with sodium tetraphenylboron (NaTPB) as a CL enhancer. In CEIA, some problems occurred in terms of a diluent effect post column, with dead volume in the detection window so as to reduce the sensitivity or broaden the peaks. To overcome these disadvantages, a unique CL detector without any dead volume or diluent effect was designed so wonderfully in the CEIA system that it could obtain the desirable sensitivity. In addition, several enhancers were also tested and at last, *para*-iodophenol (PIP),[24–26] which was broadly used in all CEIA based on CL methods, was replaced by NaTPB, which can enhance the CL intensity more strongly than PIP does. Linearity, detection limit, and experimental results were also discussed in detail. This method has been used to determine the FSH in human serum, and the feasibility of this new assay was evaluated in clinical diagnosis.

## EXPERIMENTAL

### Apparatus

An MPI-A CE-CL detection system including a 0–20 kV high-voltage (HV) power supply and analytical system of multicenter data collection (Ruimai electronic technological corporation, Xi'an, China) was used throughout the experiment (Figure 1). A fused-silica capillary (50 cm × 75 μm ID) coated with polyimide (Polymicro Technologies, Phoenix, AZ) was used for separation, and then was inserted into a reaction capillary (22 cm × 600 μm ID; Chongqing Optical Fiber, Chongqing, China). The detection window was formed by burning 8 mm of the polyimide of the reaction capillary and setting it in front of the photomultiplier tube (PMT; Hamamatsu, Japan), and synchronously putting a reflector on the other side. A section of the end of the separation capillary was inserted in the middle of the detection window. The CL reaction proceeds immediately in the detection window and there is no dead volume or diluent effect in the reaction capillary in such an elaborate device. The PMT of the detector was operated at 700–800 V. CL reagents were delivered by a double microsyringe pump (Shanghai Instrument Plant, Shanghai, China) and flowed through a reagent capillary (22 cm × 250 μm ID; Lanzhou Institute of Chemical Physics, China) to the reaction capillary. CL reagent solutions were fed at a rate of 15–20 μL/min. The end section of the reaction capillary exited the detector and entered a buffer reservoir to complete the circuit. Data acquisition and collection were processed using commercially available software (IFFM–D data analysis system, Xi'an, China). The pH of buffer solutions was measured by a precise pH meter (pHs-2, Shanghai Second Analytical Instrument Factory, Shanghai, China).

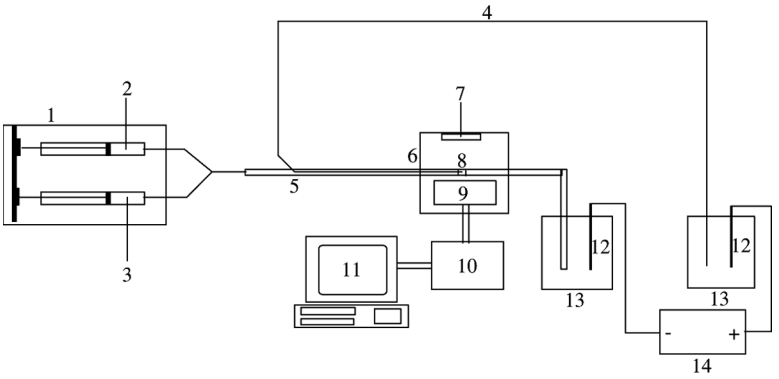


**FIGURE 1** Schematic diagram of the capillary electrophoresis instrument with chemiluminescence detection. (1) double syringe pumps; (2) luminol solution; (3) $H_2O_2$ solution; (4) electrophoretic separation capillary; (5) reaction capillary; (6) black box; (7) reflector; (8) PMT window; (9) PMT; (10) signal amplifier; (11) personal computer; (12) Pt electrodes; (13) electrolyte reservoirs; (14) high-voltage power.



## Chemicals (Reagents and Solutions)

Luminol was from Merck (Darmstadt, Germany). Hydrogen peroxide (30% $H_2O_2$), acetic acid, sodium hydrogen carbonate ($NaHCO_3$), sodium hydroxide (NaOH), ethylenediamine tetraacetic acid (EDTA), and sodium tetraborate ($Na_2B_4O_7$) were purchased from Shanghai Chemical Plant (Shanghai, China). Horseradish peroxidase (HRP) was obtained from Boaosen Biochemcals (Beijing, China). PIP, NaTPB, *p*-cresol, and *p-tert*-butylcatechol were products from J&K (Shanghai, China). The FSH ELISA kit (catalog number 2110Q-48), which contained enzyme conjugate, standard set of FSH serum, and control serum, was purchased from Syntron Bioresearch, Inc. (Abbott Diagnostics, Sydney, Australia). The electrophoresis buffer was 4 mmol/L $Na_2B_4O_7$ (pH 10.20), and the CL reaction buffer was 0.4 mmol/L $H_3BO_3$–$Na_2B_4O_7$ (pH 9.1) containing 5.0 mmol/L $H_2O_2$, 0.8 mmol/L luminol, 0.1 mmol/L EDTA, and 0.3 mmol/L NaTPB. Sodium phosphate-buffered saline (PBS; 10 mmol/L sodium phosphate, 138 mmol/L NaCl, 27 mmol/L KCl, pH 7.4) was used as diluent. All solutions were prepared with deionized water obtained from a Milli-Q water purification system (Millipore, Bedford, MA) and filtered through 0.22-μm membrane filters prior to use. Human serum samples were provided by Southwest University Hospital and kept at −20°C.

## Preparation of Capillaries

The new capillary was initially rinsed with 1 mol/L NaOH for half an hour, followed by 0.1 mol/L NaOH for 20 min, then with Milli-Q water

for 20 min, and finally with the buffer solution for 20 min. To maintain reproducible migration times, the capillary was flushed with 0.1 mol/L NaOH for 2 min, water for 2 min, and running buffer for 3 min between runs. The sample was introduced by electrokinetic injection with a voltage of 10 kV for 9 s, and 15 kV was applied to it for 850 s with current reading of about 20 μA. The CL reaction and running buffer were refreshed every 2 h. In order to keep the capillary wall in good condition, 0.1 mol/L NaOH filled it when it was not being used.

### CL Conditions

Combined with the preceding procedure, the double microsyringe pumps were switched on to provide a mixed constant flow of CL reagent (luminol–$H_2O_2$ solution) to the reaction capillary during analysis. The rate of the double microsyringe pumps was 10 μL/min with 5 mL microsyringe volume. The reaction capillary was also washed by 0.5 mol/L NaOH after use for a day, and rinsed with water and CL reagent before use.



### IA Procedure

HRP-labeled anti-FSH and FSH solutions were diluted to the appropriate concentrations with 10 mmol/L PBS, pH 7.4. To perform noncompetitive assay, a 25-μL volume each of fixed HRP-labeled anti-FSH and a series of 25-μL different FSH solutions were mixed in a 500-μL microcentrifuge tube and incubated at 37°C for 30 min, then diluted 1:10 before injection for CE-CL assay of FSH.

## RESULTS AND DISCUSSION

### Optimization of CL Conditions

Some phenol derivatives including PIP[24,26,27] have been used in the HRP–luminol–hydrogen peroxide CL system as broad enhancers, while other enhancers were scarcely mentioned. In this study, we have tested four enhancers (*p*-cresol, *p-tert*-butylcatechol, PIP, and NaTPB) and compared their enhancement ability on the luminol–$H_2O_2$ reaction catalyzed by HRP-labeled FSH in $H_3BO_3$–$Na_2B_4O_7$ buffer (concentration 0.1 to 5 mmol/L). With 4 mmol/L $Na_2B_4O_7$ (pH 10.20) electrophoresis buffer, with CL detection, the intensities of four enhancers were showed on the performance of HRP-labeled anti-FSH with a dilution of 1:20 in Figure 2. The ratio of signal to noise (S/N) can express the CL intensities. As seen in Figure 2, the S/N ratio values for NaTPB were higher than those for

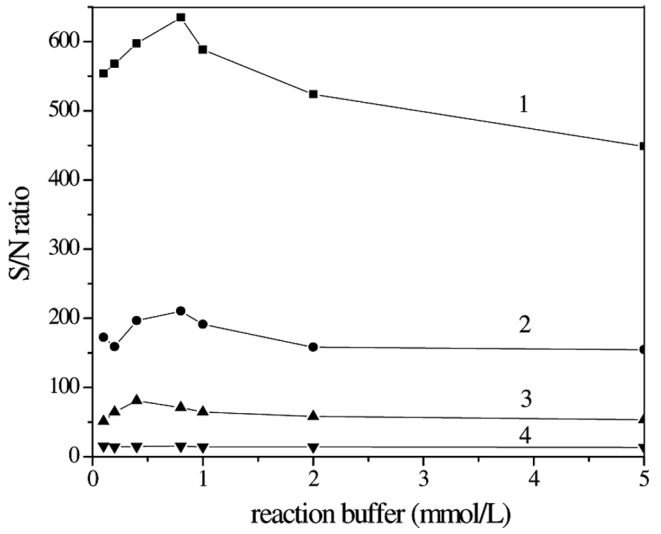


**FIGURE 2** Effects of the concentration of reaction buffer in the presence of HRP-labeled anti-FSH with a dilution of 1:20. $H_3BO_3$-$Na_2B_4O_7$ solutions (concentration from 0.1 to 5 mmol/L) containing 5.0 mmol/L $H_2O_2$, 0.8 mmol/L luminol, 0.1 mmol/L EDTA and 0.3 mmol/L enhancer were used as a reaction buffer. 1. NaTPB; 2. PIP; 3. *p*-cresol; 4. *p*-tert-butylcatechol.



others in the described conditions despite its undulation. The maximum point of S/N ratio can be seen in the NaTPB curve and it corresponded with the *X* axis at a concentration of 0.4 mmol/L $H_3BO_3$–$Na_2B_4O_7$ buffer. Several other CL reaction buffer solutions were also investigated and it was found that the CL emission in the presence of NaTPB as the enhancer was more sensitive in $H_3BO_3$–$Na_2B_4O_7$ solution than in other buffers such as PBS, $NaHCO_3$, $NaHCO_3$–NaOH, $Na_2CO_3$–$NaHCO_3$, Tris-HCl, and acetate buffer. Therefore, NaTPB was chosen as the new-style CL enhancer and 0.4 mmol/L $H_3BO_3$-$Na_2B_4O_7$ solution (pH 9.1) was selected as the optimum reaction buffer in the following work.

As the chemiluminescence reagent, luminol, and $H_2O_2$ concentrations affect the CL intensity, the effects of the concentrations of luminol and $H_2O_2$, CL reaction buffer solution and flow rate of double microsyringe pump on CL intensity were examined in the presence of HRP-labeled anti-FSH with a dilution of 1:20. The CL intensity reached a maximum value at the $H_2O_2$ and luminol concentrations of 5.0 mmol/L and 0.8 mmol/L, respectively.

To mask the heavy metal ions from the analytical-grade reagents or human serum, 0.1 mmol/L EDTA was added to the luminol CL buffer in the presence of $H_2O_2$. Considering the stability and sensitivity of CL emission, the flow rates of the luminol–NaTPB mixture and $H_2O_2$ were selected at 10 μL/min, respectively. The total flow rate of substrates through the

capillary to the flow cell was 20 μL/min; 4.0 mmol/L $Na_2B_4O_7$ (pH 10.2) was used as a run buffer. The sample was introduced by electrokinetic injection with a voltage of 10 kV for 9 s, and 15 kV was applied to it for 850 s with current reading of about 20 μA. The temperature was about 25°C.

## Optimization of Immunoassay Procedure

In an immunoassay based on the CE-CL system, analysis time is important because the complex is not stable within a long separation time. During the course of the slower run, the complex dissociates, giving no peak or poorly formed peaks for the complex. The separation time is shorter when using a narrower capillary with higher separated voltage, although this can cause the adsorption of the capillary of proteins and higher current. Integrating the two factors, the high pH run buffer was used for suppressing the adsorption of proteins and gaining an appropriate electroosmotic flow (EOF). Therefore, a voltage of 15 kV was selected for the separation.



The pH of the run buffer influences the surface characteristics of the fused-silica capillary. It also plays significant roles in the conformation and charge of the HRP-labeled antibody, antigen, and immunocomplex. The results showed that the conformation and charge of HRP-labeled anti-FSH and immunocomplex changed greatly between pH 8.5 and pH 10.8. When the pH of the run buffer exceeded 11.2, the HRP-labeled antibody would denature and become immunologically unresponsive and only one peak could be observed in the electropherogram. Therefore, the optimum pH of the buffer was 10.2.

Under a given set of conditions, the buffer concentration can offer different ionic intensity and may affect resolution and migration time. When the buffer concentration was increased, the migration time and resolution of HRP labeled anti-FSH and immunocomplex increased too. Therefore, the borate solution (4 mmol/L $Na_2B_4O_7$, pH 10.2) gave the optimization in which the HRP labeled anti-FSH and immunocomplex were efficiently separated within 14 min and sensitively detected.

As shown in Figure 3, incubation time of the HRP-labeled anti-FSH and FSH binding reaction (from 10 min to 60 min) was optimized: 25 μL of HRP-labeled anti-LH ($Ab^*$) mixed with a 25 μL of 100 IU/L LH (Ag) in a 500-μL centrifugal tube, then diluted with 1:10. At last, the suitable incubation time was 30 min at 37°C.

## Linearity, Detection Limit, and Reproducibility

The IA protocol was a noncompetitive format. HRP-labeled anti-FSH was examined carefully in terms of linearity, detection limits, and

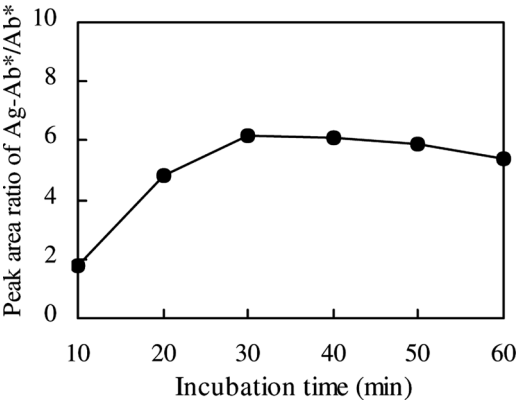


**FIGURE 3** Effects of incubation time. Reaction buffer: 0.4 mmol/L $H_3BO_3$-$Na_2B_4O_7$ solution (pH 9.1) containing 5.0 mmol/L $H_2O_2$, 0.8 mmol/L luminol, 0.1 mmol/L EDTA, and 0.5 mmol/L NaTPB. Sodium borate (4.0 mmol/L, pH 10.2) was used as a run buffer. Applied voltage 15 kV, sample injection time 9 s with electrokinetically introduced at 10 kV. The temperature was about 25°C.



reproducibility of our CE-CL system. Under the optimal conditions, the peaks areas of the immunocomplex were directly proportional to the concentrations of FSH in a linear range of 1 to 150 IU/L ($R = .9992$), and the detection limit was 0.08 IU/L for FSH. The relative standard deviation (RSD) of peak area was 1.8% for 100 IU/L FSH ($n = 7$). The good reproducibility was likely due to the utilization of a high-pH separation buffer. Under this condition, the proteins, such as FSH and HRP-labeled anti-FSH, were changed into negatively charged species and their adsorptions were efficiently suppressed. The high sensitivity of our CE-CL system was mainly attributed to the use of an on-line CL reactor, and the end of the separation capillary was directly inserted in the reaction capillary without any dead volume and exactly in the middle of the detection window with a reflector. In addition, employment of an enhancer was also important. Figure 4 shows the electropherogram of 25 μL FSH at concentration of 0, 50, and 100 IU/L mixed with 25 μL HRP-labeled anti-FSH. The mixture was incubated at 37°C for 30 min, then diluted 1:10 before injection for CE-CL assay of FSH.

## Determination of FSH in Human Serum

Serum samples were obtained from Qufu Normal University Hospital. Each serum sample was filtrated through the 0.45 μm PTFE; then 25 μL of each serum sample was mixed with 25 μL HRP-labeled anti-FSH and incubated at 37°C for 30 min after shaking. The mixture was diluted 10-fold with 10 mmol/L PBS. The contents of FSH in human serums were analyzed by the CE-CL system as described earlier. The electropherograms of a

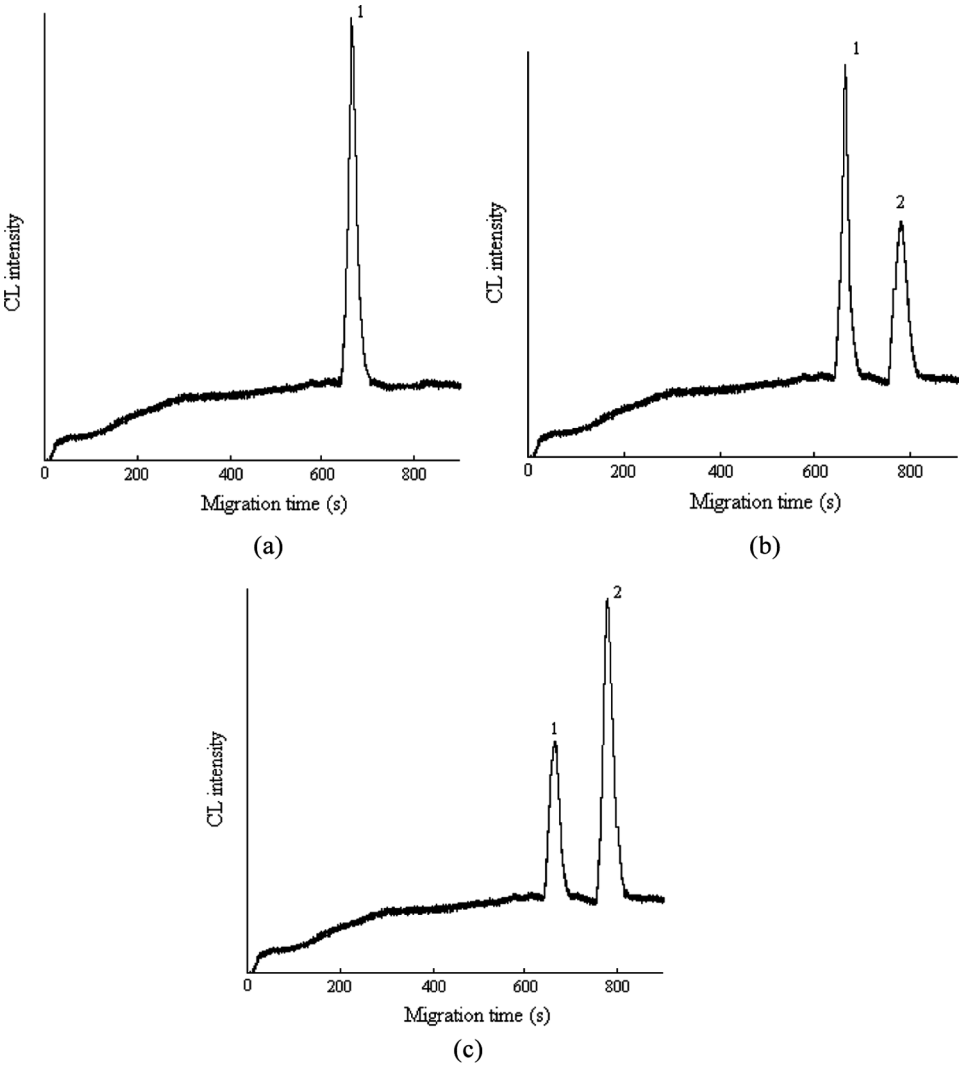


**FIGURE 4** Electropherograms of noncompetitive immunoassay based on CE-CL of standard FSH. (a) FSH 0 IU/L; (b) FSH 50 IU/L; (c) FSH 100 IU/L. CE and CL conditions as in Fig. 3. Peaks: 1 HRP labeled anti-FSH; 2 immunocomplex.

typical female serum sample and a male serum sample are shown in Figure 5.

Table 1 shows the results of determination of FSH in 14 human serum samples by noncompetitive immunoassay based on the CE-CL system. In the table, samples 1 to 5 are male serum samples and samples 6 to 10 are female serum samples. It was found that the average FSH concentration was 10.8 IU/L for the male serum samples, and the individual concentrations varied from 4.4 to 19.1 IU/L. The average of FSH concentration was 61.0

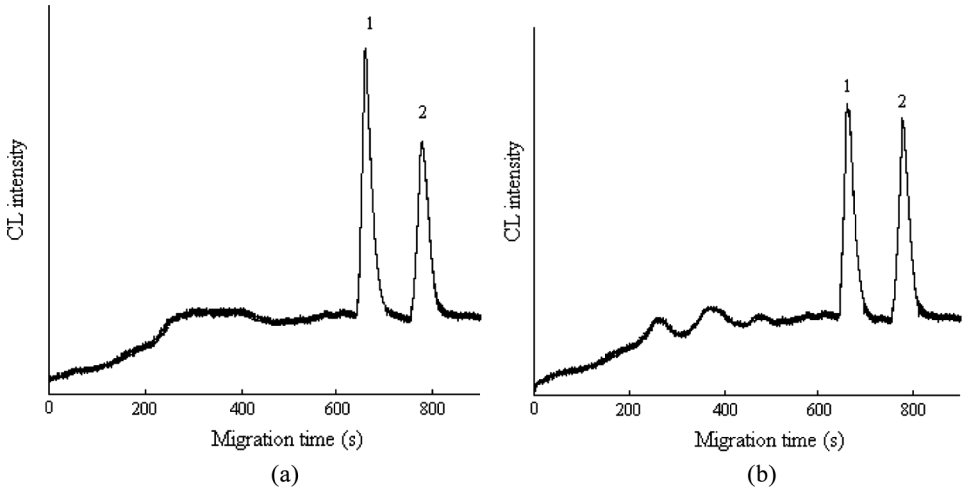


**FIGURE 5** Electropherograms of noncompetitive immunoassay based on CE-CL of FSH in human serum. (a) female serum sample; (b) male serum sample. CL and CE conditions as in Fig. 3. Peaks: 1 HRP labeled anti-FSH; 2 immunocomplex.



**TABLE 1** Determination Results of FSH in Human Serum

| Serum Sample | ELISA $OD_{450\,nm}$ | Determined (IU/L) | CE-CL Determined (IU/L) | Added (IU/L) | Found (IU/L) | Recovery % | RSD % ($n=7$) |
|---|---|---|---|---|---|---|---|
| Blank | 0.008 | – | – | – | – | – | – |
| 1 | 0.206 | 4.4 | 4.2 | – | – | – | – |
| 2 | 0.585 | 19.1 | 19.3 | 25 | 43.4 | 96.4 | 1.9 |
| 3 | 0.313 | 8.3 | 8.4 | – | – | – | – |
| 4 | 0.341 | 9.3 | 9.5 | – | – | – | – |
| 5 | 0.427 | 12.8 | 12.9 | 10 | 23.2 | 103 | 2.3 |
| 6 | 0.868 | 29.6 | 29.8 | | | | |
| 7 | 0.956 | 32.8 | 33.1 | – | – | – | – |
| 8 | 1.174 | 50.1 | 50.3 | 50 | 99.1 | 97.6 | 1.6 |
| 9 | 0.311 | 8.9 | 9.0 | – | – | – | – |
| 10 | 5.016 | 183.5 | 185.8 | 100 | 286.2 | 100.4 | 2.1 |
| Control 1 | 0.320 | 9.7 | 9.5 | – | – | – | – |
| Control 2 | 0.802 | 26.3 | 26.5 | 25 | 51.3 | 99.2 | 2.6 |

IU/L for the female serum samples, and the individual concentrations varied from 8.9 to 183.5 IU/L. The recoveries of the method were from 96.4 to 103%, and the relative standard deviations were less than 3% ($n=7$).

## CONCLUSIONS

In the present work, a method for the determination of FSH was developed in human serum using a noncompetitive immunoassay based

on CE-CL system. The unique CL detector without any dead volume or diluents effect was designed so wonderfully that it can obtain the most luminous intensity. PIP and *p*-fluorophenol[27] as conventional enhancers were replaced by NaTPB, a new style enhancer, which can exert more powerful enhancing action than phenolic enhancers. The sensitivity was about 25 times greater than that of ELISA (detection limit was 2 IU/L). As compared with conventional immunoassay methods, CE-CL has some unexampled merits such as proper sensitivity, no contaminative, needless exciting sources, lower cost, and especially flexible design in the detection cell. In the prospective work, the analytical system of CE-IA based on CL detection could be integrated into an array capillary electrophoresis chip, and thus, a large number of samples could be synchronously determined and the analytical efficiency was greatly enhanced.

## ACKNOWLEDGMENTS

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