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Competitive immunoassay of progesterone by microchip electrophoresis with chemiluminescence detection

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ABSTRACT

A sensitive and rapid homogeneous immunoassay method based on microchip electrophoresis-chemiluminescence detection (MCE-CL) using luminol-hydrogen peroxide as chemiluminescence system catalyzed by horseradish peroxidase (HRP) was developed for the determination of progesterone (P). The assay was based on the competitive immunoreactions between HRP-labeled P antigen (HRP-P) and P with a limited amount of anti-P mouse monoclonal antibody (Ab), and MCE separation of free HRP-P and HRP-P-Ab immunocomplex followed by CL detection. The effect of various factors such as conditions for the CL reaction, MCE and incubation time for the immunoreactions were examined and optimized. Under optimal assay conditions, the MCE separation was accomplished within 80 s. The linear range of detection for P was 8–800 nM with a detection limit of 3.8 nM (signal/noise ratio = 3). This present method has been applied to determine P in human serum samples from normal and pregnant women. The result indicates that the proposed MCE-CL based homogeneous immunoassay method can serve as an alternative tool for clinical assay of P.

1. Introduction

Progesterone (P) is one of the hormones in human bodies that stimulates and regulates various functions, and plays an important role in maintaining pregnancy. It helps to prepare women body for conception and pregnancy, and regulates the monthly menstrual cycle [1]. The dynamic monitoring of P in human serum is important to reproductive system and investigation of the mechanism of various steroid prophylactics and anti-early-pregnancy drugs [2]. In addition, it has been indicated that P also have neuroprotective effect after traumatic brain injury [3].

Currently, the most common methods used to quantify P are chromatography and immunoassay methods. Chromatography methods such as LC–MS [4] and GC–MS [5] have been developed as reference methods for determination of P in human serum, which have advantages of high accuracy, selectivity, reproducibility and sensitivity. Immunoassays used in clinical practice including radioimmunoassay [6], enzyme linked immuno absorbent assay (ELISA) [7], fluoroimmunoassay [8], chemiluminescent enzyme immunoassay (CL-EIA) [9,10] and time-resolved fluorescence immunoassay [11] have also been reported for the evaluation of P in human serum. By far, the most immunoassays for P are heterogeneous, i.e. either antibody or antigen is immobilized at the solid phase. Heterogeneous immunoassays in general have low limits of detection (nM to pM), but they require a multi-step work flow (e.g. primary antibody incubation, washing, secondary antibody incubation etc.), and are therefore time-consuming [12].

Microchip electrophoresis (MCE) has become a very attractive separation technique for chemical and biological analyses [13]. It have various advantages such as reduced sample and reagent consumption, high separation speed and efficiency, short analysis time, simple operation, and easy integration and automation, which make it unequally suitable for biological and clinical analysis. Immunoassay is one application that makes use of these advantages. Many types of immunoassays on MCE have been applied in clinical diagnoses and biochemical studies. Over the past decade, MCE-enhanced immunoassays have been developed to determine cortisol [14], theophylline [15], matrix metalloproteinase-8 [16], goat anti-human IgG [17], B-type natriuretic peptide [18] and staphylococcal enterotoxin B [19]. Laser-induced fluorescence (LIF) detection is the most sensitive detection scheme available for MCE immunoassay. However, LIF detection requires relatively large and expensive apparatus systems. Chemiluminescence (CL) detection offers advantages including high sensitivity, simple optical

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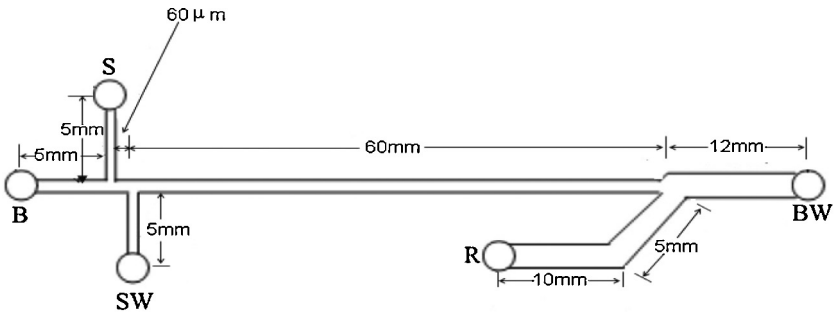

Fig. 1. Schematic diagram of the layout of the glass/PDMS microchip. S, sample reservoir; B, buffer reservoir; SW, sample waste reservoir; BW, buffer waste reservoir; R, the oxidizer reagent reservoir.

structure, low background noise and relatively simple and inexpensive instrumentation. CL is particularly suitable for integration on microfluidic devices. MCE-CL-enhanced immunoassays have been successfully used for analysis of rat IgG [20], human serum albumin [21], immunosuppressive acidic protein [22], atrazine [23], testosterone [24] and thyroxine [25].

Herein, we report on the development of a homogeneous immunoassay coupled with MCE-CL to quantify P in human serum. The assay was based on competitive immunoreactions between horseradish peroxidase (HRP)-labeled P antigen (HRP-P) and P with a limited amount of anti-P mouse monoclonal antibody (Ab), and MCE separation of free HRP-P and HRP-P-Ab immunocomplex followed by CL detection. It is well known that HRP catalyzes the luminol-H_2O_2 CL reaction and greatly enhances the CL emission [26]. Thus, two peaks for free HRP-P and HRP-P-Ab immunocomplex were observed in electropherogram. Both HRP-P and the HRP-P-Ab immunocomplex were sensitively detected. Because of the effective MCE separation, the CL analytical signal was less prone to sample matrix interference. The conditions for MCE separation and CL detection were investigated in detail, and the quantification of P in human serum from normal and pregnant women was demonstrated.

2. Experimental

2.1. Reagents and apparatus

HRP-P, P and Ab was purchased from Zhengzhou Biocell Biotechnology Co., Ltd. (Zhengzhou, China). Tween 20 was obtained from Shanghai Chemical Reagents Corporation (Shanghai, China). Luminol was purchased from Fluka (Buchs, Switzerland). Paraiodophenol (PIP), hydrogen peroxide (H_2O_2) were obtained from Taopu Chemicals (Shanghai, China). All other chemicals used in this work were of analytical grade. The P and Ab solutions were prepared by dissolving the reagents in 20 mM phosphate buffer (pH 7.4). The electrophoretic buffer and oxidizer solution were prepared according to method described previously [27]. Briefly, the electrophoretic buffer was 10 mM phosphate buffer (pH 10.2) containing 1.2 mM luminol and 0.08% (w/w) Tween 20. The oxidizer solution was 80 mM H_2O_2 and 1 mM PIP in 40 mM $NaHCO_3$ solution at pH 8.7. All solutions were filtered through 0.22-μm membrane filters prior to use. Water was purified by employing a Milli-Q plus equip from Millipore (Bedford, MA, USA), and used throughout the work.

A homemade MCE-CL system used in this work was similar to that described in our previous work [27]. The fabrication of the double "T" microchip was described previously [28] and illustrated in Fig. 1. The width of all microchannels except the oxidizer introduction channel (250 μm) was 65 μm, the depth of all microchannels was 25 μm, and the length of double T was 60 μm.

2.2. Human serum sample preparation

Human serum samples were kindly provided by the No. 5 People's Hospital (Guilin, China). A 250 μL of human serum sample was collected in a 1.5 mL vial, then 500 μL trichloroacetic acid was added and votexed for 5 min. The mixture solution was centrifuged for 20 min at 12,000 rpm. The supernatant was transferred to another 1.5 mL vial and evaporated to dryness under N_2. The residue was reconstituted in 250 μL 20 mM borate solution and kept at 4 °C before analysis.

2.3. Immunoreaction

To carry out the immunoreaction, 20 μL different concentrations of P or serum sample, 20 μL 5.0×10^{-7} M HRP-P and 20 μL 2.5×10^{-7} M Ab were mixed and diluted to 100 μL with phosphate buffer (pH 7.4), and incubated for 35 min at 37 °C. The whole procedure is illustrated in the following equations:

$$\text{HRP-P} + \text{P} + \text{Ab} - \text{HRP-P-Ab} + \text{HRP-P} + \text{P-Ab} + \text{P}$$

where Ab is anti-P antibody added at a limited amount, HRP-P was added at a fixed amount, and P is free progesterone. HRP-P competes with P in the solution for binding to a limited amount of Ab. The concentration of P in the solution is directly proportional to that of free HRP-P, but inversely proportional to that of the HRP-P-Ab. Therefore, P in the sample can be measured by monitoring the CL intensity from free HRP-P after separation from the immunocomplex.

2.4. MCE-CL procedure

The microchannels were rinsed sequentially with 0.1 M NaOH, water and electrophoresis buffer for 10 min each. Prior to electrophoresis, all reservoirs were filled with the electrophoretic buffer. Vacuum was applied to the reservoir BW in order to fill the separation channel with the electrophoretic buffer. Then, the electrophoretic buffer solutions in reservoir S and reservoirs R were replaced by sample solution and oxidizer solution, respectively. Sample injection was performed by applying 450 V to S for 15 s with SW grounded, whereas B was set at 280 V, BW was set at 350 V, and R was left floating. For MCE separation, 2500 V was applied to B and 1500 V was applied to both S and SW with BW grounded. At the same time, 500 V was applied to R. The analytes were transported into the separation channel toward BW and then were mixed with the oxidizer solution at the junction of the oxidizer introduction