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Highly sensitive determination of diclofenac based on resin beads and a novel polyclonal antibody by using flow injection chemiluminescence competitive immunoassay

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ABSTRACT

A novel flow injection chemiluminescence immunoassay for simple, sensitive and low-cost detection of diclofenac was established based on specific binding of antigen and antibody. Carboxylic resin beads used as solid phase carrier materials provided good biocompatibility and large surface-to-volume ratio for modifying more coating antigen. There was a competitive process between the diclofenac in solution and the immobilized coating antigen to react with the limited binding sites of the polyclonal antibody to form the immunocomplex. The second antibody labelled with horseradish peroxidase was introduced into the immunosensor and trapped by captured polyclonal antibody against diclofenac, which could effectively amplify chemiluminescence signals of luminol-PIP-H_2O_2. Under optimal conditions, the diclofenac could be detected quantitatively. The chemiluminescence intensity decreased linearly with the logarithm of the diclofenac concentration in the range of 0.1–100 ng mL^{-1} with a detection limit of 0.05 ng mL^{-1} at a signal-to-noise ratio of 3. The immunosensor exhibited high sensitivity, specificity and acceptable stability. This easy-operated and cost-effective analytical method could be valuable for the diclofenac determination in real water samples.

1. Introduction

Diclofenac (DCF), chemically named 2-[(2,6-dichlorophenyl) aminophenyl]-acetic acid, belongs to non-steroidal anti-inflammatory drugs [1] which can relieve pain and inflammation by inhibiting prostaglandin synthesis. Because of its potent analgesic and anti-inflammatory effects, it has been widely used in many treatments, and prepared in a wide range of formulations, containing tablets, capsules, drops, injections, suppositories, gels, ointments, etc. [2]. However, DCF can cause many gastrointestinal problems, such as gastric or duodenal ulceration, hemorrhage and perforation [3]. In addition, several studies have shown that in animal experiments, DCF has certain damage to neurons of the cervical spinal cord [4] and has a direct teratogenic effect on rat embryos [5]. Before more information in humans becomes available, it should be remained alert to use DCF. Due to the universality of DCF, it has been detected in wastewater and natural water bodies all over the world, which is a significantly worrying situation [6,7]. Although DCF can be photodegraded, it still exists in aqueous environment [8]. Due to its good water solubility (5000 mg/L) [9], DCF residues in water are difficult to purge completely by conventional water treatment plants [10]. It is worth noting that the DCF residues in domestic water, such as tap water and drinking water, directly affect daily life of human. Therefore, it is very important and urgent to establish a sensitive and effective method to test DCF residues in order to ensure water safety and safeguard human health.

To date, the reported methods for detecting DCF were mainly chromatographic methods, including high performance liquid chromatography (HPLC) [11], ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) [12], gas chromatography–mass spectrometry (GC–MS) [13], etc. Although these methods have advantages of rapidity and high sensitivity, the drawbacks of expensive instruments, complex pretreatment process and professional operation cannot be ignored. Spectroscopic analysis methods, such as fluorescence spectroscopy [14] and Raman spectroscopy [15], are similarly subjected to the high cost of purchasing and maintaining instruments. Other analytical methods including electrochemisty [16], alkaline-induced salting-out homogeneous liquid-liquid extraction [17] and enzyme-linked immunosorbent assay (ELISA) [18] have also been used for detection of DCF.

Flow injection analysis which played as an alternative for the automation of chemical methods aiming at the development of simple and reproducible automated methodologies emerged in 1975 [19]. This technique is generally based on simple and low cost manifolds with possibility to be adapted to distinct analytical requests [20]. Flow injection chemiluminescence (FI-CL), combining flow injection analysis

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technique with chemiluminescence (CL) which provides a simple, sensitive and low-cost method of detection [21], permits rapid determination [22]. The method has been widely applied to diverse areas like pesticides, environment, food, pharmaceuticals, etc. [23]. Unfortunately, conventional flow injection chemiluminescence method has an obvious shortcoming of poor selectivity [24,25]. Immunological methods, which are based on the specific reaction between the antibody and antigen, exhibit some significant advantages of high sensitivity and specificity, simple sample preparation and low cost [26]. A novel flow injection chemiluminescence immunoassay (FI-CLIA) was developed by combining FI-CL with immunoassay. The merits of the two methods are demonstrated simultaneously.

In this work, we utilized carboxyl beads as solid phase carrier materials to fix more coating antigen. Compared with other materials such as glass microbeads [27,28], nylon, agar, nanoparticles [29,30] and magnetic beads [31], carboxyl beads which can be stored steadily in ethanol aqueous solution for a long time are inexpensive and easy to use. Through the competitive process, the diclofenac in solution competed with the coating antigen immobilized on carboxylic resin beads for the limited binding sites of the antibody to form the immunocomplex. The second antibody labelled with horseradish peroxidase (HRP) introduced into the immunosensor could effectively amplify signals of luminol-PIP-H_2O_2 due to the catalysis of HRP for the chemiluminescence system. Consequently, diclofenac could be detected quantitatively. The immunosensor exhibited high sensitivity and specificity, acceptable stability, simple sample preparation, low cost and automation, indicating a promising method for determination of diclofenac and other small molecular compounds.

2. Experimental Section

2.1. Reagents and Materials

N-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 99%), ketoprofen and meclofenamic acid were purchased from J&K Scientific Ltd. (Beijing, China). *N*-hydroxysuccinimide (NHS, 97%), *p*-iodophenol (PIP) and dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Casein and diclofenac were purchased from Sigma Co., Ltd. (St. Louis, USA). 5-Hydroxy diclofenac was bought from Toronto Research Chemicals Inc. (Toronto, Canada). Tris(hydroxymethyl)amino-methane (Tris) and hydrogen peroxide (30%) were purchased from Innochem Science & Technology Co., Ltd. (Beijing, China) and Shanghai Lingfeng Chemical Reagent Co. Ltd. (Shanghai, China), respectively. Horseradish peroxidase labelled goat anti-rabbit IgG (HRP-GaRIgG) was purchased from ZhongShan-Golden Bridge Biological Technology Co. Ltd. (Beijing, China). Diclofenac coating antigen freeze-dried powder and antiserum were obtained from Deng's Laboratory at Soochow University (Suzhou, China). All other reagents and chemicals were of analytical reagent grade. Sub-boiling distilled deionized water was used throughout the study. Carboxylic resin beads (diameter: 150 μm; sphericity: >99%; degree of crosslinking: 7%; content of water: 30–40%) were obtained from Nanjing Microspheres Hi-Efficiency Isolation Carrier Co. Ltd. (Nanjing, China).

2.2. Buffers and Solutions

Assay buffer of 0.01 M Phosphate-buffered saline (PBS, pH 7.4) was prepared by NaCl, $Na_2HPO_4 \cdot 12H_2O$, KCl and KH_2PO_4. Washing buffer (PBST) and blocking solution were 0.05% (v/v) of Tween-20 in PBS and 2% of casein in PBS, respectively. Coating antigen stock solution was obtained by 1 mg coating antigen powder dissolved in 1 mL of 0.05 M carbonate buffer (pH 9.6) and then the solution would was diluted with PBS for further use. The stock solution of 1 mg mL^{-1} diclofenac was stored in the dark before use.

The stock solution of 0.01 M luminol was prepared by dissolving 177 mg of luminol in 100 mL of 0.1 M NaOH solution and then placed in the dark for a week before use. The stock solution of 0.01 M PIP was obtained by dissolving 110 mg of PIP in 5 mL of DMSO and then diluted with water to a final volume of 50 mL, followed by stored in the dark. The chemiluminescence substrate solution was composed of 0.6 mM luminol, 0.7 mM PIP and 4 mM H_2O_2. Prior to use, all the stock solutions would be diluted with 0.1 M Tris–HCl buffer solution (pH 8.5) to the desired concentrations.

2.3. Apparatus

IFFM-E luminescence analyzer equipped with an IFFS-A multifunction chemiluminescence detector was from Remex Analytical Instruments Co., Ltd. (Xi'an, China). Polytetrafluoroethylene tubes (0.8 mm i.d.) were used to connect all components in the flow system. Two peristaltic pumps were used to deliver all solutions. The chemiluminescence substrate zone was formed with a 100 μL loop around the injection valve and driven by the carrier to the immunosensor (as shown in Fig. 1A). The chemiluminescence signals were measured with a photomultiplier from Remex Analytical Instruments Co., Ltd. (Xi'an, China) operated at −600 V, and the data of corresponding chemiluminescence intensity was recorded for the quantitative determination. High speed refrigerated centrifuge was purchased from Anhui USTC Zonkia Scientific Instruments Co., Ltd. (Hefei, China). The concentration of antibody in the obtained solution was measured with UV-2300 spectrophotometer from Tech-Com (Shanghai, China). Scanning electron micrographs (SEM) of naked and coating antigen immobilized resin beads were obtained with a VEGA 3SBH Scanning Electron Microscope from TESCAN (Brno, Czech) at an acceleration voltage of 20 kV.

2.4. Purification of Polyclonal Antibody

The obtained antiserum was purified by using a saturated ammonium sulfate method. In summary, under slightly stirring, 4 mL of

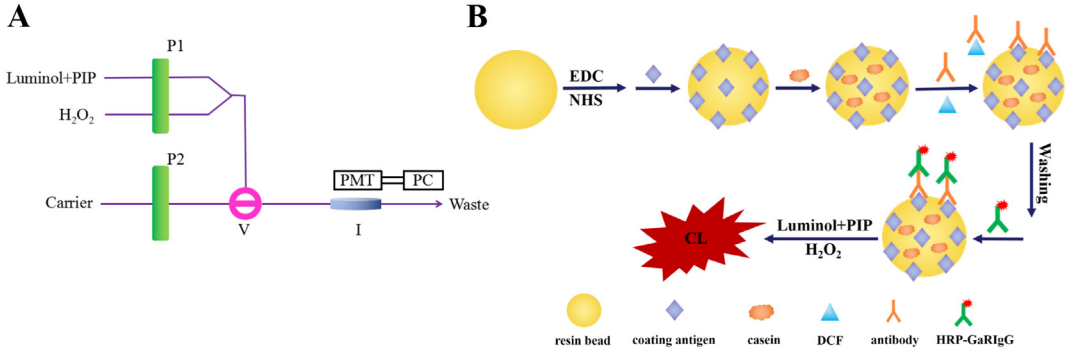

Fig. 1. (A) Schematic diagram of the FI-CLIA detection system: (P1) peristaltic pump, (P2) syringe pump, (V) injection valve, (I) immunosensor, (PMT) photomultiplier and (PC) personal computer. (B) Fabrication process and the detection mechanisms of the CL immunosensor for DCF.