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Rapid immunochromatographic assay for ofloxacin in animal original foodstuffs using native antisera labeled by colloidal gold

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ABSTRACT

An immunochromatographic assay was developed to detect fluoroquinolone antibiotic ofloxacin based on the competitive binding of ofloxacin and the membrane-immobilized ofloxacin–protein conjugate to colloidal gold-labeled antibodies in the course of the labeled antibodies, and to test sample flow through the membrane. The specific feature of labeling by colloidal gold is that native antiserum is used instead of purified immunoglobulins or specific antibodies. This makes the synthetic procedure easier, with no sacrifice in the detection limit. The proposed test makes it possible to detect down to 30 ng mL^{-1} of ofloxacin, which corresponds to the demands of food safety assessment. The assay time is 10 min. The assay provides reliable information on the ofloxacin content in milk without the sample preparation and in chicken and pork meat with the minimum sample preparation (the separation of the insoluble fraction of the homogenate by centrifugation). The high degree of detection of ofloxacin in foodstuffs by the proposed assay (70–112%) was shown by a comparison with the data obtained with the use of a commercial immunoenzymatic kit.

1. Introduction

Immunochromatography based on membrane test strips is a highly efficient tool for medical diagnosis, food safety and quality control, and environmental monitoring [1,2]. Its principal advantage over other analytical methods is that it is far less labor intensive. All components necessary for the formation of complexes to be detected and their visualization are pre-coated at specific regions of the membranes forming the test strip. Contact with the sample initiates the lateral flow across the test strip, thus providing all subsequent analytical processes.

Labeled antibodies are key reagents in immunochromatography as tools to detect the formed immune complexes, and labeling by colloidal gold (CG) dominates in current immunochromatographic practice [1,2]. The most commonly used method for the preparation of CG–antibody conjugates [3,4] is adsorption immobilization, which does not require the surface modification of CG. The immobilization is performed with purified preparations of antibodies [2,5,6]. However, immunoglobulins account for 15–20% of the total content of proteins in serum, where albumins are dominant components. Albumins are traditionally added to CG–antibody conjugates after immobilization to stabilize the product and prevent its aggregation [2]. The traditional procedure of preparing conjugates includes the sequential execution of two opposite processes, namely, separation of antibodies from ballast proteins and further addition of such proteins to the obtained conjugates. Although starting incubation of CG with purified immunoglobulins increase their loading, lower antibody: CG ratios are often preferable for immunochromatography. The excess of immunoglobulins due to desorption may worsen performance of the assay. Besides, for competitive assays, if the number of immobilized antibodies is increased, more antigen molecules are needed for blocking them, and finally limit of the assay detection is increased [7].

Thus, it seems reasonable to use non-separated antisera as reactants for the one-step synthesis of CG–antibody conjugates. In this case, a sufficient number of antibodies and stabilizing compounds of sera (such as albumin) are immobilized simultaneously on a colloidal support. The given modification of synthetic protocol would simplify the preparation of a test system and exclude the risks of the antibodies' partial destruction (aggregation, denaturation, etc.) in traditional multi-step protocols.

The present study aimed to prepare CG–antibody conjugates using native antisera for their application to an immunochromatographic assay of the fluoroquinolone antibiotic ofloxacin in foodstuffs (milk, meat).

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Fluoroquinolones (FQ), piperazine derivatives of the quinolone nalidixic acid, belong to the most promising antibacterial chemotherapeutic agents for the treatment of infections of different etiology and localization in humans and animals [8]. The inhibition of DNA gyrase or topoisomerase II with FQs leads to the suppression of both gram-positive and gram-negative bacteria [9]. The wide use of FQs in veterinary medicine poses significant risks to human health associated with the entry of these contaminants into the food chain (via food of animal origin) and pollutions to the environment [10]. The uncontrolled uptake of FQs into the human body can lead to the development of resistant forms of microorganisms and cause pathological states, such as dysbacteriosis, allergic reactions, suppression of the activity of some enzymes, and so on [11,12]. Taking the above into consideration, the maximum residue limits (MRL) for FQs in milk, poultry, bovine, and porcine products allowed are 30–300 $\mu g\ kg^{-1}$ in Europe and Russia, 100–400 $\mu g\ kg^{-1}$ in China, 50–200 $\mu g\ kg^{-1}$ in Japan, and 200 $\mu g\ kg^{-1}$ in the United States [13–15].

Ofloxacin is one of the most widely used FQs. In present-day medicine and veterinary, both ofloxacin, which is a mixture of stereoisomers S-(−)-ofloxacin (S-OFL) and R-(+)-ofloxacin (R-OFL), and levofloxacin, which contains only S-(−)-ofloxacin, are used. Although the antibiotic activity of S-(−)-ofloxacin is much more pronounced [16,17], the regulations are dictated by MRLs for the total content of FQs [13–15]. In some cases, stereoisomers have substantially different immunogenicity [18–20]. Hence, we compared antisera prepared with the use of immunogens based on the ofloxacin racemate (R,S-OFL) and S-(−)-ofloxacin.

Different liquid chromatography [8,21–24] and proton nuclear magnetic resonance [25] are the most common techniques for the control of FQ contaminants in food. However, these methods require complex expensive apparatus and cannot be used outside centralized laboratories. In this respect, the following immunochemical methods are more efficient: immunoenzymatic assay (ELISA) [26–29], immunofluorescence assay [30], and electrochemical and optical biosensors [31–33]. Immunochromatographic tests for the detection of FQs based on the conjugation of purified antibody preparations to a marker have been described [28,34–36]. These tests will be compared with our test system in Section 3.6.

In accordance with the stated above aim, the presented study included reactants testing for ofloxacin immunodetection, obtaining and characterization of CG–antibody conjugates using native anti-ofloxacin antisera, their application to immunochromatographic assay (ICA), and comparison with traditional CG–IgG conjugates.

2. Materials and methods

2.1. Chemicals and materials

Ofloxacin racemate, S-(−)-ofloxacin (levofloxacin), R-(+)-ofloxacin, garenoxacin, pefloxacin, danofloxacin, moxifloxacin hydrochloride, enrofloxacin, enoxacin, cinoxacin, nalidixic acid, and sarafloxacin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bovine serum albumin (BSA), chicken egg albumin (OVA), ampicillin, penicillin G, chloramphenicol, streptomycin sulfate, kanamycin, tetracycline hydrochloride, rifampicin, 3,3′,5,5′-tetramethylbenzidine (TMB), N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), Triton X-100, and sodium azide were from Sigma (St. Louis, MO, USA). Neomycin, cephalexin, ciprofloxacin, and gold chloride hydrate were from Fluka (Buchs, Switzerland). Dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF), and Tween-20 were from MP Biomedicals (Santa Ana, CA, USA). Goat and sheep anti-rabbit immunoglobulins (GARIss and SARIss, respectively) and goat anti-mouse immunoglobulins (GAMIss) were from Imtek (Moscow, Russia). Goat anti-rabbit immunoglobulins (GARI) were from Arista Biologicals (Allentown, PA, USA). Peroxidase-labeled anti-rabbit immunoglobulins were from the Gamaleya Institute of Microbiology and Epidemiology (Moscow, Russia). All other chemicals (salts and solvents of analytical grade) were from Khimmed (Moscow, Russia).

Solutions of CG and its conjugates with antibodies were prepared using deionized water (MilliQ, Millipore, Bedford, MA, USA, 18.2 MΩ cm at 25 °C).

Stock solutions (1–5 mg mL^{-1}) of R,S-OFL, S-OFL, R-OFL, garenoxacin, pefloxacin, danofloxacin, enrofloxacin, enoxacin, cinoxacin, sarafloxacin, rifampicin in DMSO, nalidixic acid, moxifloxacin hydrochloride, kanamycin, neomycin, tetracycline hydrochloride, streptomycin sulfate, cephalexin, ciprofloxacin in 50 mM phosphate, pH 7.4, containing 0.1 M NaCl (PBS), ampicillin, penicillin G in 50 mM sodium citrate buffer, pH 6.4, and chloramphenicol in ethanol were prepared immediately before analysis.

Mdi Easypack (Advanced Microdevices, Ambala Cantt, India) kits of membranes were used for immunochromatography. Costar microplates 9018 (Corning, NY, USA) were used for ELISA.

2.2. Apparatus

UV spectra were recorded on a Biochrom Libra S60 spectrophotometer (Biochrom, Cambridge, UK). The ELISA was carried out using a WellWash 4 MK 2 washer (Thermo Electron Corporation, Shanghai, China) and a Zenyth 3100 microplate reader (Anthos Labtec Instruments, Wals, Austria). Transmission electron microscopy of CG particles was performed with a CX-100 microscope (Jeol, Tokyo, Japan). Test strips were manufactured with an IsoFlow dispenser (Imagene Technology, Hanover, NH, USA), an Index Cutter-1 (A-Point Technologies, Gibbstown, NJ, USA), and an FR-900 mini-conveyor (Wenzhou dingli packing machinery, Wenzhou, China).

2.3. Preparation of immunogens and coating antigens

R,S-OFL or S-OFL was coupled to proteins using the NHS ester method [37] with some modifications. FQ (4 mg, 12.5 μmol) was dissolved in a mixture of 0.5 mL solution of DCC (50 μmol mL^{-1}) in anhydrous DMF and 0.5 mL solution of NHS (50 μmol mL^{-1}) in anhydrous DMF. The reaction mixture was incubated overnight at room temperature. A 0.4 mL solution of this activated hapten was then added dropwise with shaking to a solution of BSA (17 mg, 0.25 μmol) or OVA (15 mg, 0.25 μmol) in 2 mL of cold 50 mM carbonate buffer (pH 9.6) with 50 μL DMF. The resulting mixture was incubated at 4 °C overnight. The conjugate solution was dialyzed against six changes of distilled water for three days. The obtained conjugate was divided into aliquots (0.5 mL), freeze dried, and stored at 4 °C until use.

2.4. Antiserum production

New Zealand white male rabbits (10 weeks, supplied by the Guangdong Medical Laboratory Animal Center, PR China) were immunized with immunogen by hypodermic injection at 3-week intervals. Bleeds were taken from the rabbits on the eighth day after the fifth immunization. Immunization with the R,S-OFL and S-OFL conjugates gave the PAs-R,S and PAs-S antisera, respectively. The antisera were divided into aliquots (1 mL) and stored at −20 °C until use.

2.5. Isolation of IgG fraction

Immunoglobulins (IgG) were purified by precipitation of the antiserum with 50% ammonium sulfate repeated three times.