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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.

The PAb-R,S and PAb-S antibodies were isolated from the PAs-R,S and PAs-S antisera, respectively. The samples were stored at 4 °C.

2.6. Competitive ELISA of fluoroquinolones

Ofloxacin conjugates of BSA or OVA at a concentration of 0.5 μg mL^{-1} in PBS were immobilized from a volume of 100 μL in wells of a microplate at 4 °C overnight, after which the microplate was washed with PBS containing 0.05% Triton X-100 (PBST). Then 50 μL of R,S-OFL, S-OFL, or R-OFL in PBST were added to wells of the microplate followed by the addition of 50 μL of the specific antiserum in 1:100,000 dilution in PBST. The microplate was incubated at 37 °C for 1 h and then washed with PBST. Then 100 μL of the immunoperoxidase conjugate (1:6000 dilution in PBST) were added, and the microplate was again incubated at 37 °C for 1 h. After washing the peroxidase activity was determined. For this purpose, 100 μL of a 0.4 mM TMB solution in 40 mM sodium citrate buffer, pH 4.0, containing 3 mM H_2O_2, were added, and the microplate was incubated at room temperature for 15 min. The reaction was terminated by adding 50 μL of 1 M H_2SO_4, and then OD_{450} was measured.

The plots of the optical density (y) against the antigen concentration in the sample (x) were fitted using the four-parameter logistic function $y=(A-D)/(1+(x/C)^B)+D$ with the Origin 7.5 software (OriginLab, Northampton, MA, USA). The C parameter value corresponds to the antigen concentration inhibiting the binding of antibodies by 50% (IC_{50}). The antigen concentration causing 10% inhibition (IC_{10}) was calculated using the same function, and it was considered as the detection limit of the assay [38].

2.7. Preparation of colloidal gold

Colloidal gold was prepared according to the protocol described in [39]. A 1% $HAuCl_4$ solution (1.0 mL) was added to deionized water (97.5 mL). The reaction mixture was brought to boiling, and then a 1% sodium citrate solution (1.5 mL) was added with stirring. The reaction mixture was boiled for 25 min, cooled, and stored at 4–6 °C.

2.8. Transmission electron microscopy

A solution of CG preparation in chloroform was dropped onto a grid (300 mesh, Pelco International, USA) coated with polyvinyl formal support film. The images were obtained with a CX-100 electron microscope at accelerating voltage of 80 kV and magnification of 3,300,000. They were digitalized with the Image Tool software [40]. The measurements were carried out by Dr. I.V. Safenkova, Institute of Biochemistry, Russia.

2.9. Synthesis of antiserum–CG conjugates

The preliminary characterization of the conjugation was performed according to the recommendations [41] for the conjugation of immunoglobulins. A CG solution with $OD_{520}=1.0$ (1.0 mL portions) was added to solutions (0.1 mL) of antiserum in water (in dilutions from 1:100 to 1:4000). The mixtures were stirred and incubated at room temperature for 10 min. Then a 10% NaCl solution (0.1 mL) was added to each sample, the mixtures were stirred for 10 min, and OD_{580} was measured.

Prior to conjugation to CG, the antiserum was dialyzed against a 1000-fold excess of 10 mM Tris–HCl buffer, pH 8.5, for 2 h at 4 °C. Then 0.2 M K_2CO_3 was added to the CG solution ($OD_{520}=1.0$) until pH 8.5 was reached, and the solution was spiked with an antiserum solution at a desired concentration. The mixture was incubated with stirring at 20–22 °C for 30 min, and then BSA was added to the final concentration of 0.25%. The CG particles with immobilized antiserum were separated from unbound proteins by centrifugation at 8000g for 30 min. After the removal of the supernatant, the precipitate was resuspended in PBS containing 0.25% BSA. If long-term storage of the product was necessary, NaN_3 was added to the final concentration of 0.02%.

2.10. Synthesis of IgG–CG conjugate

The preliminary characterization of the conjugation was performed as described above for the antisera.

Prior to conjugation, IgG was dialyzed as described above and spiked with a CG solution at a desired concentration ($OD_{520}=1.0$, pH 8.5). Subsequent steps of the synthesis are the same as those described for the antisera.

2.11. Preparation of immunochromatographic test strips with antiserum–CG conjugate

Immunoreagents were immobilized on the membranes of the test system using an IsoFlow automated dispenser. The antiserum–CG conjugate was immobilized on a pad in a dilution corresponding to $OD_{520}=2.0$ (32 μL per centimeter of the pad width). The test zone was formed using the R,S-OFL–BSA conjugate (0.5 mg mL^{-1} in 0.2 M carbonate buffer, pH 9.6), the control zone using GARI (0.06 mg mL^{-1} in PBS). The solutions were stabilized, and 2.0 μL of the solution were applied per centimeter of the working membrane. The pads and working membranes thus prepared were dried in air at 20–22 °C for at least 20 h. The multimembrane composite was assembled and then cut into 3.5-mm-wide strips with an Index Cutter-1 automated guillotine cutter. The test strips were hermetically packed in laminated aluminum foil bags containing silica gel as the desiccant with the use of a FR-900 miniconveyor. The cutting and packing were carried out at 20–22 °C in a special room with a relative humidity under 30%. The packed test strips were stored at 20–22 °C.

2.12. Preparation of immunochromatographic test strips with PAb-S–CG conjugate

Immunochromatographic test systems were prepared with the use of the PAb-S–CG conjugate according to the method described in Section 2.11 for antiserum conjugates with the following differences. The PAb-S–CG conjugate was immobilized on a pad in a dilution corresponding to $OD_{520}=2.0$ (32 μL per centimeter of the pad width). The test zone was formed using the R,S-OFL–BSA conjugate (0.25 mg mL^{-1} in 0.2 M carbonate buffer, pH 9.6).

2.13. Preparation of milk, pork, and poultry samples for immunochromatographic assay

Cow's milk with a fat content of 1.5% and 3.5% was purchased in retail stores. Ofloxacin was added, and these mixtures were used without additional sample preparation. Chicken meat and pork purchased in retail stores were homogenized with a home meat grinder. A 0.2 M acetate buffer, pH 5.6 (0.5 mL), and ofloxacin were added to the minced meat (0.5 g). The mixture was incubated with stirring for 10 min, and the precipitate was separated by centrifugation at 4000g for 10 min. The supernatant was subjected to the assay.

2.14. Immunochromatographic assay and data processing

The assay was performed at room temperature. The bag was opened, the test strip was taken out, and its lower end was dipped into an aliquot of the sample (50 μL) for 1 min and then placed on a horizontal surface. Within 10 min after the beginning of the

assay, the result was checked. The digital image of the test strips was obtained with a CanoScan LiDE 90 scanner, and the integrated intensities of the color in the test and control zones were calculated as described previously [39].

2.15. Reference ELISA assay

RIDASCREEN® Quinolones (R-Biopharm AG, Germany) kits were used as the reference assay of ofloxacin in milk, pork, and poultry samples according to manufacturer's instruction.

3. Results and discussion

3.1. Preparation and characterization of immunoreagents

The quality of immunoreagents was pre-characterized by ELISA. To achieve the lowest detection limit, the conditions of ELISA were optimized by varying the dilution of antisera and the type and amount of the hapten–protein conjugate immobilized on a solid phase. Under the optimized conditions, the lowest IC_{50} values for the detection of S-OFL were achieved with the use of the PAs-S antiserum (0.3–0.4 ng mL^{-1}), and for the detection of R,S-OFL, with the use of the PAs-R,S antiserum (0.2–0.5 ng mL^{-1}). Fig. S1 in Supplementary material presents the corresponding curves.

Since the working range of the competitive immunoassay is determined by the equilibrium constant of the antigen–antibody interaction, competitive ELISA and ICA can be characterized by the same working ranges under the optimized conditions. Previously, we observed this situation for atrazine [42] and ampicillin [43]. However, the disappearance of the color in the test zone observed on visual inspection of the results of the competitive immunochromatographic assay corresponds to the lower plateau of the calibration curve rather than to the upper plateau, which is considered as the expected detection limit in ICA. In our experiments, the calibration curve for ELISA reaches the lower plateau at an ofloxacin concentration of 30 ng mL^{-1} (see Fig. S1). This value corresponds to the maximum residue limit of ofloxacin in milk and meat established by the European Union and Russia [13–15]. Based on these results, we used these antisera and ofloxacin–protein conjugates for the preparation of immunochromatographic test systems.

The specificity of two antiserum samples prepared with the use of S-OFL and R,S-OFL conjugates as immunogens was compared (Table 1). The antisera were characterized by considerable cross-reactivity toward S-OFL, R-OFL, R,S-OFL, and garenoxacin, and low cross-reactivity toward other FQs. None of the characterized antibiotics of other groups taken at concentrations below 10 μg mL^{-1} reacts with antisera.

3.2. Preparation and characterization of reagents for immunochromatography

The electron microscopy study showed that the particle shape of the resulting CG is nearly spherical and the preparations are highly homogeneous. The average lengths of the longest and shortest axes of the CG particle are 37 ± 8 and 30 ± 5 nm, respectively. Therefore, the average diameter of the particles is 34 nm, which corresponds to the recommendations [43] for the optimal size of CG for immunochromatography (30–40 nm).

The conditions for the conjugation of antisera to CG were chosen based on the photometric data characterizing the aggregation of the product of this reaction at a high ionic strength. Based on the concentration dependences (Fig. 1A and B), the PAs-R,S and PAs-S antisera were taken for conjugation in an amount 10% to 15% higher than the point at which OD_{580} reaches the plateau, as recommended in [38], i.e., at a protein concentration of 13 μg per milliliter of the colloidal solution. It should be noted that the immobilization of immunoglobulins isolated from the antiserum was characterized by the similar concentration dependence (see Fig. 1C). An excess of the unreacted protein was removed with the supernatant by centrifugation of the conjugate.

3.3. Development of competitive ICA of ofloxacin

To achieve the lowest detection limit of ICA, we optimized the test system, including the choice of the reagents, their concentrations, and conditions for the immobilization on the control, test, and starting zones of the test strip.

We examined the ability of the synthesized antibody–CG conjugates to interact in the immunochromatographic test system. For the PAs-S–CG conjugate, the color intensity in the absence of the analyte was three to five times higher than that for the PAs-R, S–CG conjugate (Table 2). Hence, subsequent experiments were performed with the PAs-S–CG conjugate.

Table 1
Specificity of antisera against R,S-OFL and S-OFL according to ELISA data.

Quinolones	Antisera			
	PAs-S		PAs-R,S	
	Immobilized hapten–protein conjugates			
	R,S-OFL–OVA	S-OFL–OVA	R,S-OFL–BSA	S-OFL–BSA
	IC_{50}, ng mL^{-1} (Cross-reactivity, %)			
R,S-OFL	0.6 (67%)	0.9 (30%)	0.5 (100%)	0.2 (100%)
S-OFL	0.4 (100%)	0.3 (100%)	2.5 (20%)	0.2 (100%)
R-OFL	2.8 (14%)	3.8 (8%)	0.2 (250%)	0.5 (40%)
Garenoxacin	1.7 (24%)	2.5 (12%)	0.1 (500%)	1.1 (18%)
Enrofloxacin	200 (0.2%)	430 (0.07%)	> 1000 (< 0.01%)	> 10,000 (< 0.001%)
Pefloxacin	230 (0.2%)	320 (0.1%)	> 1000 (< 0.01%)	> 1000 (< 0.01%)
Danofloxacin	> 10,000 (< 0.001%)	> 10,000 (< 0.001%)	> 10,000 (< 0.001%)	> 10,000 (< 0.001%)
Moxifloxacin	> 10,000 (< 0.001%)	> 10,000 (< 0.001%)	> 10,000 (< 0.001%)	> 10,000 (< 0.001%)
Nalidixic acid	> 10,000 (< 0.001%)	NI	NI	NI
Sarafloxacin	> 10,000 (< 0.001%)	> 10,000 (< 0.001%)	NI	NI
Enoxacin	NI	> 10,000 (< 0.001%)	> 1000 (< 0.01%)	> 10,000 (< 0.001%)
Cinoxacin	NI	NI	NI	NI

NI – no interaction.

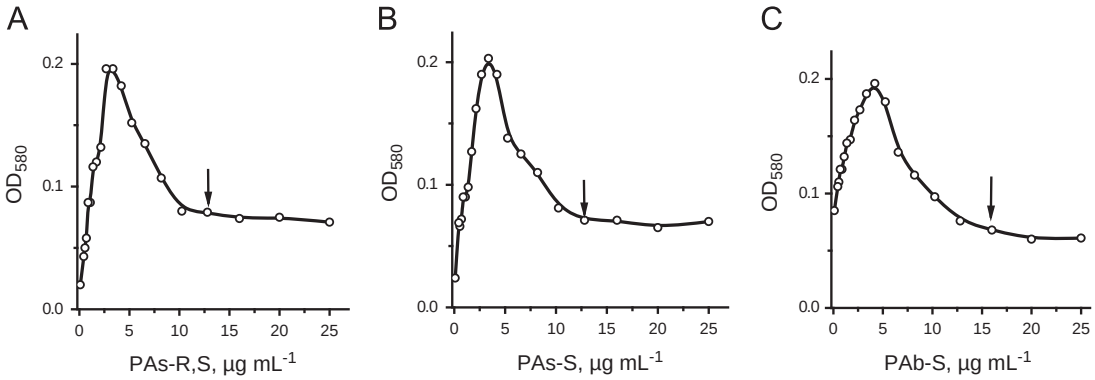

Fig. 1. Concentration dependences of the optical density of colloidal gold at 580 nm after the addition of different concentrations of the anti-ofloxacin antiserum PAs-R,S (A), PAs-S (B), and the immunoglobulin fraction PAb-S (C).

Table 2
Comparison of the characteristics of ICA of ofloxacin for different combinations of antisera and hapten–protein conjugates.

Immobilized hapten–protein conjugate	Antiserum–CG conjugate			
	PAs-R,S–CG		PAs-S–CG	
	IC_{50}, ng mL^{-1}	Signal amplitude, arb. units	IC_{50}, ng mL^{-1}	Signal amplitude, arb. units
R,S-OFL–BSA	0.5 ± 0.1	19	1.7 ± 0.2	94
S-OFL–BSA	5.0 ± 0.1	19	3.1 ± 0.3	54

Different anti-rabbit IgG antibodies were immobilized on the control zone, and their binding to the PAs-S–CG conjugate in the ICA was compared. At saturating concentrations of antibodies, the color intensity of the control zone was 105 and 127 arb. units for goat antibodies GARIss and GARI, respectively, and 92 arb. units for sheep antibodies SARIss. Although the differences are not too large, we chose GARI antibodies for subsequent use because they provide the best binding of the marker. The optimal concentration of anti-species antibodies for immobilization on the control zone was 0.06 mg mL^{-1}, which corresponds to the point at which the concentration dependence of the binding of the antiserum–CG conjugate reaches a plateau.

For the test zone of the test strip, we compared the situation for four different hapten–protein conjugates immobilized on the membrane. Fig. 2 shows the calibration curves for ICA obtained for the immobilization of the R,S-OFL–BSA, R,S-OFL–OVA, S-OFL–BSA, and S-OFL–OVA conjugates. For similar working ranges, the color intensity of the test zone in the absence of the analyte was 92, 79, 54, and 42 arb. units, respectively. Based on this fact, we chose the immobilization of the R,S-OFL–BSA conjugate on the test zone.

To control the threshold for the differentiation between positive and negative samples, the immobilization was performed by varying the concentration of the R,S-OFL–BSA conjugate. As can be seen from Fig. 3, a decrease in this concentration from 1.0 to 0.5 mg mL^{-1} resulted in a decrease in the detection limit of ofloxacin by a factor of 3 (from 100 to 30 ng mL^{-1}). A further decrease in the concentration to 0.25 mg mL^{-1} led to a substantial decrease (by half) in the amplitude of the calibration curve. Hence, we took the 0.5 mg mL^{-1} concentration of the R,S-OFL–BSA conjugate for immobilization, because it gives the required detection limit of ofloxacin with a sufficient color intensity of the test zone and a low reagent consumption.

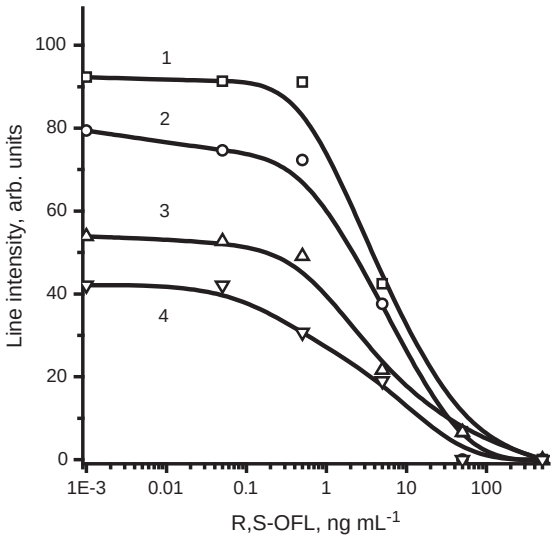

Fig. 2. Dependence of the calibration curve for ICA of ofloxacin on the type of the hapten–protein conjugate used for immobilization on the test zone of the test strip. (1) R,S-OFL–BSA; (2) R,S-OFL–OVA; (3) S-OFL–BSA; (4) S-OFL–OVA. The concentration of the ofloxacin–protein conjugates is 0.5 mg mL^{-1}.

3.4. Determination of analytical characteristics and testing of the developed immunochromatographic test system

Based on the results of optimization, we prepared test strips and performed experiments on the ICA of ofloxacin in standard solutions, milk, and meat.

Fig. 4 shows the results of the testing of samples containing different concentrations of ofloxacin in PBS. The disappearance of the color in the test zone corresponds to the MRL of ofloxacin (30 ng mL^{-1}). The accuracy (standard deviation) of the concentrations detection is $\pm$ 5%.

The immunochromatographic system is characterized by high specificity toward R,S-OFL (cross-reactivity taken as 100%), S-OFL (104%), and garenofloxacin (74%), moderate specificity toward R-OFL (12%) and low specificity (lower than 0.1%) toward other FQs (Table 3). No reactions were observed for antibiotics of other groups used at concentrations of up to 10 μg mL^{-1}.

A comparison with the system in which the IgG fraction isolated from antisera was used showed that the calibration curves for the CG conjugates with IgG and antiserum can almost completely coincide at particular concentrations of the R,S-OFL–BSA conjugate immobilized. These concentrations are 0.25 and 1.0 mg mL^{-1} in the assays using the antibody conjugate and the antiserum conjugate, respectively, reflecting a smaller amount of

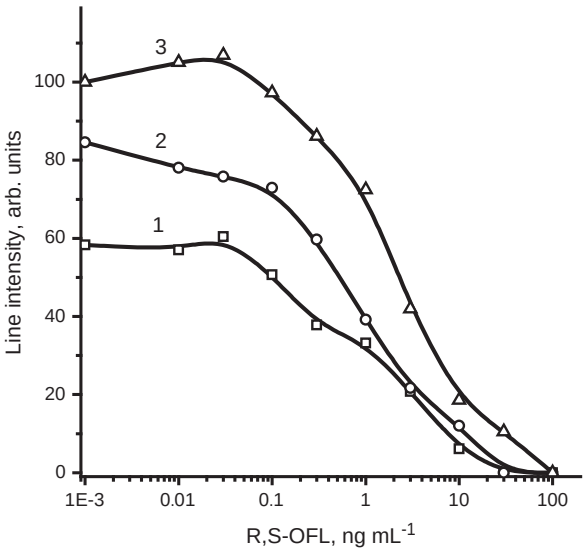

Fig. 3. Dependence of the calibration curve for ICA of ofloxacin on the concentration of the R,S-OFL–BSA conjugate used for immobilization on the test zone of the test strip. Curves 1–3 correspond to the concentrations of the R,S-OFL–BSA conjugates equal to 0.25, 0.5, and 1.0 mg mL^{-1}.

immobilized immunoglobulins when working with the native antiserum. Fig. S2 in Supplementary material presents the corresponding curves.

The developed test system was employed for testing milk at different ofloxacin concentrations. Figs. 5A and 6,1 present the results of the investigation. For all samples under study, it was shown that ofloxacin can be reliably detected at the same minimum detectable concentration, like in the buffer (30 ng mL^{-1}), and with the same accuracy of the quantitative detection ($\pm$5%).

The procedure developed previously for the immunochromatographic detection of ofloxacin in chicken and pork meat [35] involves homogenization, incubation at 80 °C, and removal of the precipitate by centrifugation. We excluded the incubation at high temperature as a sample preparation step, which simplifies the assay as a whole. The color in the test zone was observed at ofloxacin concentrations in the supernatant lower than 30 ng mL^{-1} (which corresponds to its content in the starting meat sample lower than 28.8 μg kg^{-1}) (Figs. 5B, 6,2 and 6,3). This threshold limit value coincides with the results of experiments for a pure ofloxacin solution.

3.5. Comparison of the developed assay with reference ELISA

To compare with current practice, milk samples with a fat content of 1.5% and 3.5%, pork, chicken, and ofloxacin at different concentrations were tested using the manufactured test strips and commercial RIDASCREEN® Quinolones ELISA kits. Since immunochromatographic tests are primarily aimed at qualitative assays (the appearance or disappearance of a color in the analytical zone), a comparison with ELISA was carried out in terms of exceeding the controlled residue level of ofloxacin. A perfect agreement between the qualitative conclusions based on ELISA and immunochromatographic data was observed for all 18 tested milk samples (Table 4).

When considering the ICA with the quantitative detection, it was possible to estimate the correlation between the results obtained with this method and the determination of the ofloxacin content by reference ELISA technique. Our immunochromatographic measurements with a scanner showed that the correlation coefficient of the results of assays was 0.88 for milk samples with 3.5% fat content and 0.85 for milk samples with 1.5% fat content. The degree of detection varied from 70% to 112%. The ofloxacin

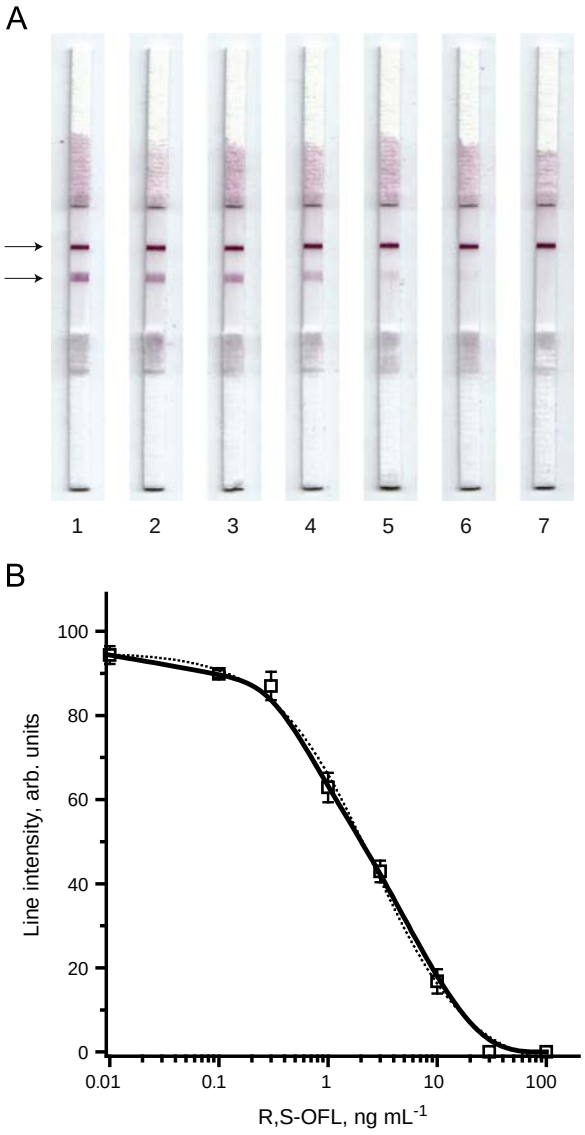

Fig. 4. (A) Immunochromatographic detection of ofloxacin under optimized conditions in PBS. The ofloxacin concentrations in the samples are 0 (1), 0.3 (2), 1 (3), 3 (4), 10 (5), 30 (6), and 100 (7) ng mL^{-1}. Upper arrows indicate the control zones; lower arrows indicate the test zones. B. The calibration curve for the immunochromatographic detection of ofloxacin in PBS. The plots of the test line intensity (y) against the antigen concentration in the sample (x) were fitted (dashed line) using the function $y=(A-D)/(1+(x/C)^B)+D$. $A=-4.72$, $D=94.95$, $C=2.53$, $B=0.98$.

Table 3
Specificity of immunochromatographic detection of ofloxacin with PAs-S antiserum.

Antibiotic	Cross-reactivity, %
R,S-OFL	100
S-OFL	104
R-OFL	12
Garenoxacin	74
Pefloxacin	< 1.0
Danofloxacin	< 0.1
Moxifloxacin	< 0.1
Enrofloxacin	< 0.1
Enoxacin	< 0.1
Cinoxacin	< 0.1
Nalidixic acid	< 0.1
Sarafloxacin	< 0.1

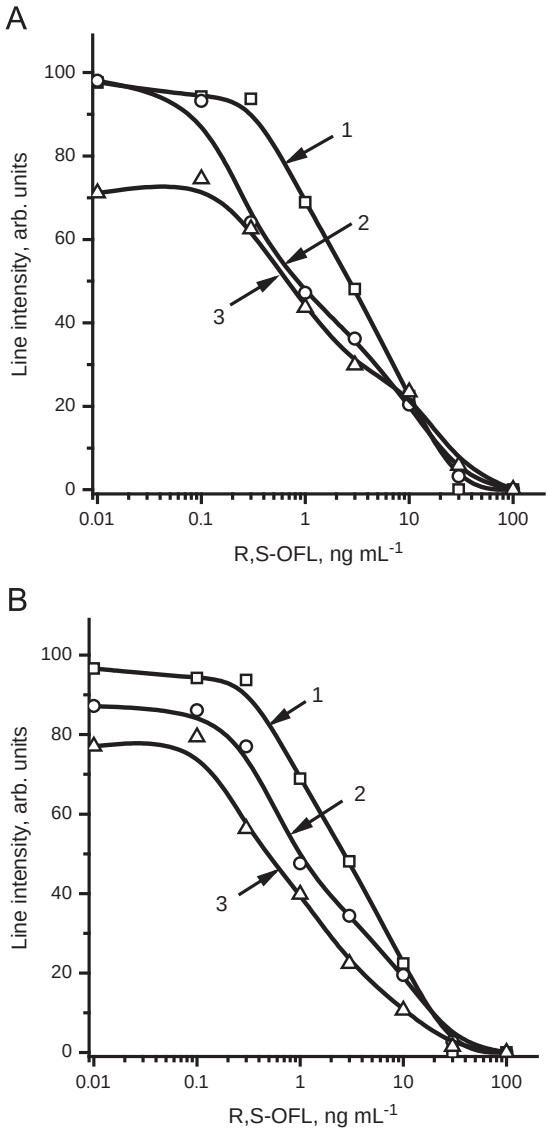

Fig. 5. Calibration curves for the immunochromatographic detection of ofloxacin. (A) Comparison of the curves in PBS (1), milk with a fat content of 1.5% (2), and milk with a fat content of 3.5% (3). (B) Comparison of the curves in PBS (1), chicken meat (2), and pork (3). The meat:buffer ratio used for the extraction is 1:1.

Fig. 6. Immunochromatographic detection of ofloxacin in milk with a fat content of 3.5% (1), chicken meat (2), and pork (3). The left test strip for each matrix corresponds to the absence of ofloxacin, and the right strip corresponds to the presence of ofloxacin at a concentration of 30 ng mL^{-1}.

Table 4
Results of detection of R,S-OFL in milk samples by the developed ICA method.

Added, ng mL^{-1}	Found, ng mL^{-1} (Degree of revealing)	
	Milk with a fat content of 1.5%	Milk with a fat content of 3.5%
50	> 30	> 30
20	20 (100%)	15 (75%)
10	9.5 (95%)	7 (70%)
4	4.5 (112%)	3.5 (70%)
2	1.6 (80%)	1.8 (90%)
1	0.8 (80%)	0.8 (80%)
0.5	0.3 (60%)	0.4 (80%)
0.2	0.2 (100%)	0.2 (100%)
0	0	0

content ranged from 0.2 to 50 ng mL^{-1} ($n=8$ for each experiment). These data show that the developed method is applicable to the quantitative evaluation of the ofloxacin content.

3.6. Discussion

As follows from the ELISA and ICA data, the antisera prepared with the use of immunogens based on R,S-OFL and S-OFL interact with both stereoisomers of ofloxacin, but they differ in the quantitative affinity and specificity parameters. Taking into account these results, a test system for highly sensitive ICA should contain the PAs-S antiserum conjugated to CG and the immobilized R,S-OFL–BSA conjugate. Nevertheless, R,S-OFL, as well as S-OFL and R-OFL, can be detected in all the cases under consideration.

The above-described experiments showed that it is possible to simplify a procedure for the preparation of antibody–CG conjugates using native antiserum for immobilization. Based on the sizes of CG particles used in the study and the geometry of immunoglobulins, the immobilization of purified IgG resulted in the inclusion of 150–200 molecules into the monolayer. Taking into account the ratio of specific to non-specific antibodies in the serum of immunized animals [44], the amount of reactive IgG molecules immobilized on one CG particle may vary from 5 to 20. The co-immobilization of serum albumin leads to a several-fold decrease in this amount. Hence, ICA has an advantage of decreasing the concentration of antibodies, whose binding to the antigen does not block the interaction of the antibody–CG conjugate in the test zone (i.e., the content of an excess of antibodies with respect to CG particles). As a result, the immobilization of non-fractionated serum does not deteriorate the analytical characteristics of the test system and, in some cases (depending on the ratio of immunoreagents and the constants of the immunochemical reaction), can lead to their improvement.

Compared with other methods for the immunochromatographic detection of ofloxacin described in the literature, the method developed in the present study is the most rapid and the least labor-intensive. Thus, in the study [35] samples of chicken meat were homogenized, incubated at 80 °C, and the solid precipitate was again extracted prior to the assay. In the study [34] milk samples were centrifuged at 48 °C to remove the fat layer. In the study [28], ferricyanide and zinc sulfate were

added to precipitate milk proteins, and then centrifugation was performed at 4 °C. An important advantage of the new method is that the assay can be performed at room temperature.

It should also be noted that the use of a scanner as a photometric detector makes it possible not only to document the results of the detection but also to quantitatively estimate the ofloxacin content up to 0.3 ng mL^{-1} (see Fig. 4B).

Due to the rapidity and ease of the detection, this test system can be considered an efficient tool for the high-throughput screening of milk and meat for the ofloxacin content.

4. Conclusions

The results of the present study confirm that it is possible to prepare colloidal gold conjugates not with isolated antibodies but with native antiserum for their use in immunochromatographic assay. It was shown that there is a correspondence between the analytical characteristics of the test systems for ofloxacin prepared with the use of two different conjugates. This methodology can potentially be used to simplify the preparation of test systems for immunochromatographic assay of different compounds.

The developed test system for ofloxacin was shown to be efficient for the detection of this antibiotic in different food matrices and can be considered an efficient tool for the rapid food quality and safety control.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.10.054.

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