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DEVELOPMENT OF FLUORESCENCE POLARISATION AND ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR DANOFLOXACIN DETECTION IN MILK

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ABSTRACT

Two selective immunotechniques based on heterologous approach were proposed and chacterized to detect danofloxacin, a priority veterinary contaminant, in milk. The first technique is fluorescence polarisation immunoassay (PFIA), where a danofloxacin tracer was synthesised with 4-aminomethyl fluorescein, with polyclonal antibodies obtained for a danofloxacin conjugate with cationised bovine serum albumin. The developed system has a detection limit for danofloxacin equal to 13 ng/mL and does not show any cross-reactivity with other 24 tested fluoroquinolones. The second assay format is enzyme-linked immunosorbent assay (ELISA) for danofloxacin that is based on the application of the same antibodies and a heterologous solid-phase clinafloxacin conjugate with ovalbumin. The limit of danofloxacin detection for the given ELISA is 0.5 ng/mL, and its strong selectivity to danofloxacin has been also confirmed. The possibility of quantitative control of danofloxacin content in milk has been shown for the developed ELISA technique; the opening percentage was in the range from 83 to 130%.

Key words: Fluorescence polarisation immunoassay, Enzyme-linked immunosorbent assay, Fluoro-quinolones, Danofloxacin, milk.

INTRODUCTION

The increased importance of antibiotics control in foodstuffs is caused by their wide use in animal husbandry that include not only therapy but also preventive measures (i.e., their addition to forage)[1]. The widespread use of antibiotics results in the development of resistant forms of microorganisms[2,3], which poses a serious threat to human health.

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Quinolones represent a class of synthetic antimicrobial agents currently featuring four generations and more than 40 compounds, and their list is regularly replenished with new members[4,5]. All substances of this class inhibit bacterial DNA gyrases and topoisomerases IV that define their bactericidal effect[6,7]. Their biological action also includes influence on the RNA of bacteria, bacterial protein synthesis and membrane stability[8,9]. Fluoroquinolones dominate among used quinolones. They are applied to treat infections caused by gram-negative pathogens and in the cases of multiresistant strains. Currently the following representatives of fluoroquinolones are mainly used for veterinary purposes: ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumekvin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, orbifloxacin and pefloxacin.

Many countries have introduced maximal residue levels (MRL) for fluoroquinolones in foods. Thus, in the European Union the sum of ciprofloxacin and enrofloxacin in milk should not exceed 100 ng/mL[10]. In Russia the same MRL is stated for total content of ciprofloxacin, enrofloxacin, norfloxacin, ofloxacin and pefloxacin[11].

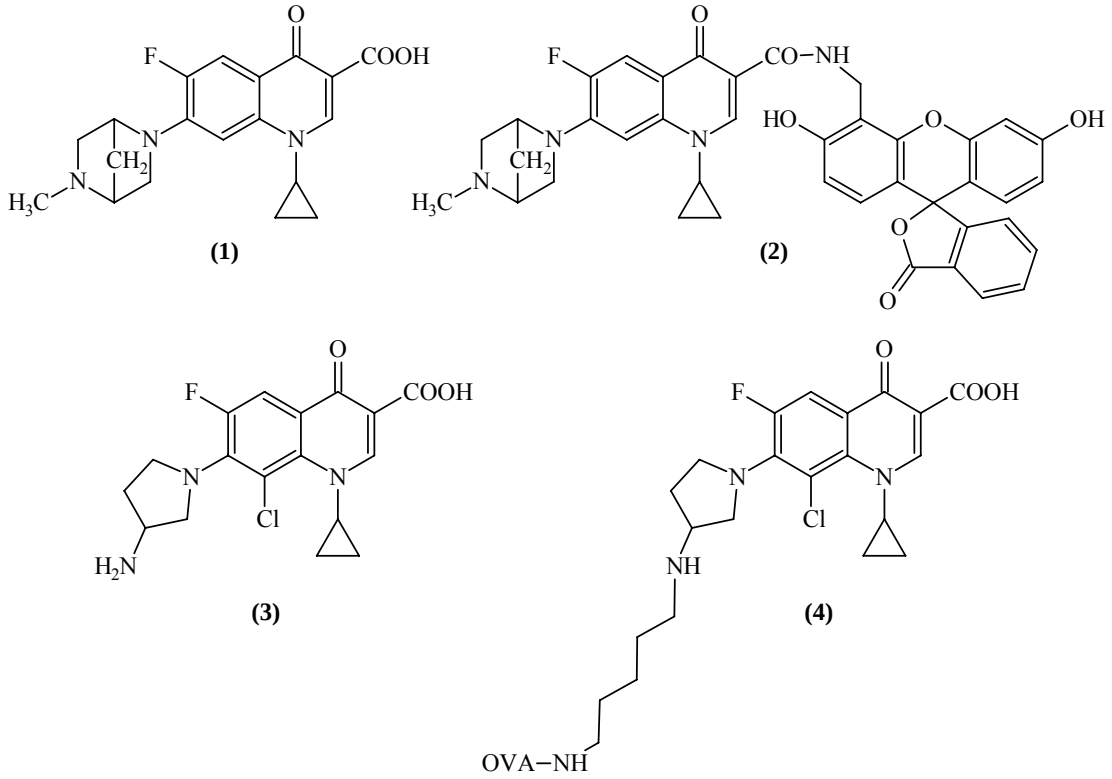

Fig. 1: Structure of main analytes and conjugates used in the study: danofloxacin (1), DAN-4AMF tracer (2), clinafloxacin (3), and CLI-NH-C5-NH-OVA conjugate (4)

Besides, MRLs are stated for certain individual fluoroquinolones. The content of marbofloxacin in milk should not exceeded 75 ng/mL, flumekvin – 50 ng/mL, and the strictest limitations are stated for danofloxacin (Fig. 1, 1)- no more than 30 ng/mL. Due to this, analytical techniques for fluoroquinolones both with wide and individual specificity are necessary, and individual detection of danofloxacin is a task of a special interest. The presented paper describes our development of heterologous immunoassays, namely ELISA and FPIA, for danofloxacin, studying their analytical parameters and applicability for milk control.

EXPERIMENTAL

Reagents

All chemicals used in this investigation were of analytical grade. Danofloxacin (DAN), ofloxacin (OFL), levofloxacin (LEV), garenoxacin (GAR), pefloxacin (PEF), gatifloxacin (GAT), clinafloxacin (CLI), sarafloxacin (SAR), lomefloxacin (LOM), tosufloxacin (TOZ), sparfloxacin (SPA), difloxacin (DIF), pazufloxacin (PAZ), marbofloxacin (MAR), moxifloxacin (MOX), rufloxacin (RUF), norfloxacin (NOR), ciprofloxacin (CIP), enrofloxacin (ENR), pipemidic acid (PIP), nalidixic acid (NAL), oxolinic acid (OXO), orbifloxacin (ORB), enoxacin (ENO), nadifloxacin (NAD), flumequine (FLU), bovine serum albumin (BSA), ovalbumine (OVA), casein, 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), ethylenediamine hydrochloride, triethylamine, sodium borohydride, glutardialdehyde, 4-aminomethylfluorescein (4-AMF), 3,3′,5,5′-tetramethylbenzidine (TMB) and Tween-20 were Sigma-Aldrich products. Complete and incomplete Freund's adjuvants were produced by Becton Dickinson, USA. Peroxidase-labelled anti-rabbit immunoglobulins were from the Gamaleya Institute of Microbiology and Epidemiology, Russia. All other chemicals (salts and solvents of analytical grade) were from Khimmed, Russia.

Instrumentation

Microplate photometer EFOS 9305 made by Sapphire JSC MBP, Russia was used for photometric measurements in ELISA. The measurements were made with a wavelength of 450 nm. Fluorescence polarisation was measured in photo-check mode using the TDxFLx analyser from Abbott Laboratories, USA.

Synthesis of cationised BSA (cBSA)

BSA carboxyl groups were modified using ethylenediamine as described in our previous work[12]. 60 mg (0.88 μmol) of BSA was dissolved in 5 mL of distilled water with the addition of a 0.5 mL solution containing 16.8 mg (88 μmol) EDC and 10.2 mg (88 μmol) NHS with vigorous stirring. This mixture was incubated for 15 min. After this, solution of

13.0 mg (88 μmol) ethylenediamine hydrochloride was poured into the obtained preparation of the activated BSA and 10 mL of 50 mM carbonate buffer pH 9.5 (CB) with 150 μL triethylamine were added. This mixture was incubated for 5 hrs with vigorous stirring. The suspension obtained in this manner was dialysed against 8 changes of distilled H_2O and 2 changes of CB for 5 days at 4°C. The resulting solution was divided into aliquots and stored at -20°C.

Synthesis of protein conjugates with fluoroquinolones

We used two methods of synthesis to provide possibility of heterologous immune assays. The first one was described by Chusri et al.[13] It consisted in using EDC and NHS to activate the carboxyl group at the 3rd position of the quinolone nucleus. Danofloxacin conjugate was synthesised with cationised BSA for immunisation and DAN, CLI, GAR, ENO, NOR, ENR, CIP, MOX and PIP were conjugated with OVA. 14.7 μmol of corresponding fluroquinolone was dissolved in 1.0 mL DMF containing 30 μmol of EDC and 30 μmol of NHS. The mixture was incubated for 2 hrs with stirring. A solution of 0.147 μmol of protein was prepared in 8 mL of CB with the addition of 50 μL of triethylamine; the mixture was incubated for 1 hr at + 4°C. After this two obtained solutions were mixed, namely a solution of fluoroquinolones was added to the protein solution dropwise under constant stirring. The mixture was incubated and stirred for 5 hr at room temperature in the dark. The synthesised conjugate was purified from low molecular weight substances by means of dialysis against distilled water with 2 changes per day for 5 days, with dialysis being performed the last 2 times against 50 mM potassium phosphate buffer, pH 7.4, with 0.1 M NaCl (PBS). The final preparation was divided into aliquots and stored at -20°C.

The second method[14,15] is based on the use of glutardialdehyde as a crosslinking agent. The resulting synthesised conjugate was CLI-NH-NH-C5-OVA. The diagram of the synthetic process is shown in Fig. 2. OVA (0.11 μmol) and CLI (5.6 μmol) were dissolved in 8 mL of distilled water. 230 μL of 0.25% glutardialdehyde was added to the given mixture with vigorous stirring. The solution was incubated for 1 hr at room temperature with constant vigorous stirring. Then 500 μL of 0.22% sodium borohydride water solution was added and incubated for 30 min. The resulting conjugate was purified from low molecular weight substances by dialysis for 5 days (8 times against distilled water, and 2 last times against PBS). The final preparation was divided into aliquots and stored at -20°C.

Synthesis of fluorescein-labelled derivative (tracer) of DAN

4-AMF labeled danofloxacin was synthesised as described previously[16] using the molar ratio of danofloxacin EDC: NHS: 4-AMF = 2 : 4 : 4 : 1. DAN (2.8 μmol), EDC (5.4 μmol), and NHS (5.4 μmol) were mixed to activate the fluoroquinolone carboxyl group,

with the resulting solution dissolved in 1.0 mL of DMF. The mixture was incubated and stirred for 2 hrs at room temperature in the dark. The obtained activated fluoroquinolone was added to the solution of 4-AMF (1.4 μmol) in 0.3 mL of DMF with 50 μL of triethylamine. The mixture was incubated and stirred for 24 hrs at room temperature in the dark. The product of the synthesis was purified using the thing layer chromatography (TLC, see[16]) in chloroform : methanol mixture having volume ratio 4:1. The TLC fractions were tested by FPIA (see below), and the selected fractions were stored at + 4°C.

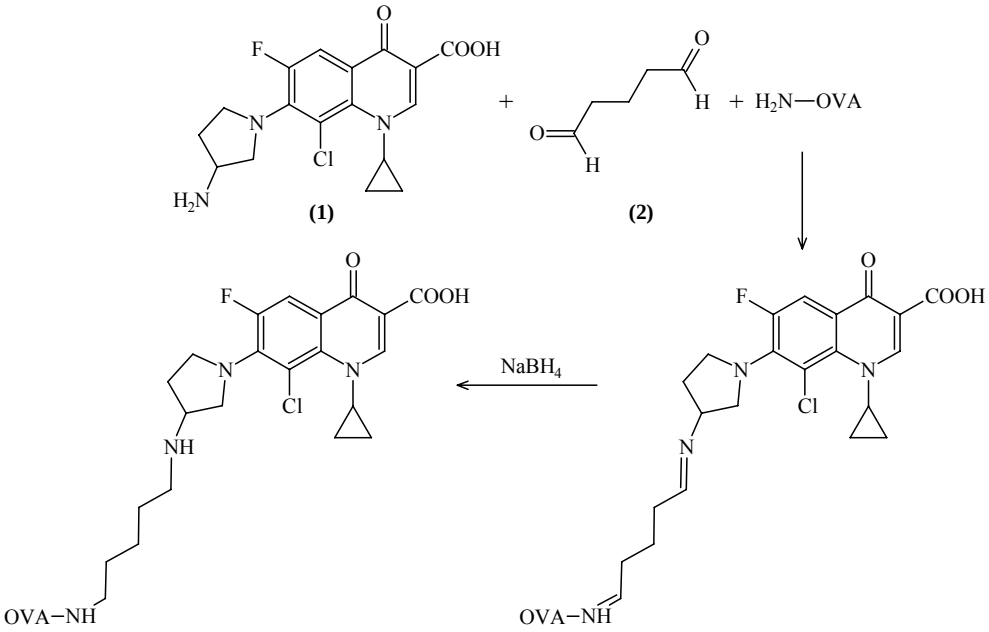

Fig. 2: Scheme of the CLI-NH-C5-NH-OVA conjugate, 1 – clinofloxacin, 2 – glutaraldehyde

The structure of the synthesised DAN-4AMF tracer was confirmed by the mass spectrometry (HPLC-MS). The mass spectrum was obtained using Triple Quad LC/MS liquid tandem mass spectrometer of Agilent (ionisation method : electrospray).

Production of polyclonal preparations of anti-DAN IgG

The synthesized cBSA conjugate (see above) was mixed with Freund's adjuvant before each immunization to obtain fresh emissions. The conjugate (0.5-1.0 mg per 1.0 mL of PBS) and the adjuvant (complete one for the first immunisation and incomplete one for the subsequent immunisations) were mixed at equal volume ratio. The male brush rabbits at the age of 3-5 months were immunised every 2 weeks; the emulsion was administered for 5-10 sites subcutaneously along the spine.

Blood sampling was carried out from the marginal ear vein using Green Vac-Tube 0238 vacuum tubes with separating gel and a coagulation activator (SiO_2). During the immunisation, the serum was separated by centrifugation at 1,000 g for 20 min, with IgG fraction separated via a three-stage bedding method with 50%, 40%, 33% ammonium sulfate at + 4°C successively, which was dissolved in PBS. The resulting solution was mixed with glycerol (v:v = 1:1) and stored at -20°C. By this way, two IgG preparations were obtained, namely DAN-110 and DAN-130.

ELISA

Conjugate solutions in PBS were added to the wells of polystyrene 96-well microplate at 100 μL/well. The plate was sealed using adhesive stickers and incubated at + 4°C within 24 hr. It was further washed two times with distilled water (300 μL per well), with the liquid carefully removed from the wells. The plates were dried at room temperature for 2 days and then stored at + 37°C before the assay.

For analysis, 50 μL of standard (analyte) and 50 μL of antibody solution were added to each well. The contents of the wells were stirred, incubated at +37°C for 1 hr, removed and washed 3 times with distilled water (300 μL per well). Horseradish peroxidase-labeled antibody solution was added (100 μL per well) diluted 1:5,000 and incubated at the same conditions for 30 min. The contents of the wells were removed and washed 5 times with distilled water (300 μL).

To detect activity of the bound peroxidase label, a substrate solution[17] containing 0.42 mM TMB and 1.8 mM H_2O_2 in 0.1 M citrate buffer, pH 4.0, was added (100 μL per well). After 15 min incubation at room temperature, the reaction was stopped with 1 M H_2SO_4 (50 μL per well). The absorbency values were measured at a wavelength of 450 nm using a microplate photometer.

PFIA

50 μL of danofloxacin standard solution was mixed with 500 μL of tracer-diluted solution, and 500 μL of the diluted antibody. Dilutions were prepared using a 0.1 M borate buffer with an addition of 0.1% of Tween-20. The working dilution of tracer was selected to reach 2000 ± 100 units units of fluorescence registed by the TDxFLx analyser.

A series of antibodies dilutions with a step of two was prepared. A working solution of a tracer was added, and a fluorescence polarization signal was measured. Graphs on the dilution–signal coordinates were built using the obtained data, and the optimum graph was selected based on the difference between the maximum and minimum signals to achieve the best resolution ability of the system.

Under the selected working dilution of tracer and antibodies, the competitive PFIA of fluoroquinolones has been carried out. The reactants were mixtured, incubated during 5 min at room temperature, and the obtained level of the fluorescence polarization was registered. The fluorescence polarization value was presented as the mP / mP_0 ratio.

Data processing

The plots of the optical density for ELISA of fluorescence polarization for PFIA (y) versus the antigen concentration in the sample (x) were fitted to a four-parameter logistic function using Origin 7.5 software (Origin Lab, USA), where A_1 is the maximum signal value, A_2 is the minimum signal value, p is the slope of the calibration curve, x_0 is the antigen concentration causing 50% inhibition of the label binding (IC_{50}).

$$y = A_2 + \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p}$$

To compare different reactants or assay conditions used, the degree of inhibition for the label binding by a standard concentration of danofloxacin was calculated as

$$DI = (A_0 - A) / A_0 \times 100\%$$

where A_0 is a maximal shift of the registered signal ($A_1 - A_2$), A is an experimantally registered shift for the given conditions.

To characterise cross-reactivity of the assay of compound A to alternate compound B (CR_B, %), the y versus x dependences were fitted by the same approach. The IC_{50} values for compounds A and B ($IC_{50,A}$ and $IC_{50,B}$, respectively) were determined and the value was calculated as the measure of the assay cross-reactivity to alternate compound B.

$$CR_B = (IC_{50,A} / IC_{50,B}) \times 100\%$$

The recovery (R, %) of the analyte, i.e. danofloxacin in our case, was calculated as:

$$R = (x_{exp}/x_{theor}) \times 100\%$$

where x_{theor} is the added concentration of the analyte and x_{exp} the found concentration after the calculation of ELISA/PFIA results using the fitting of the corresponding calibration curve.

Spiked milk samples

Milk samples (3.2% fat) were purchased from the local market. Spiked milk samples were prepared by mixing the analyte stock solutions and pre-tested antibiotic-free milk samples.

Before the analysic methanol (800 µL) and hexane (200 µL) were added to milk sample (200 µL), mixed by vigorous shaking for 2 min and centrifuged for 10 min at 10,000 g. A water-alcohol layer was sampled, diluted 6 times with PBS and used in the assay. The result of ELISA/PFIA experiments calculated using the corresponding calibration curve was multiplied by a factor of 30 to found the danofloxacin content in the initial samples.

RESULTS AND DISCUSSION

Immuno reagents

Fluoroquinolone-proteins conjugates were analysed by UV-Vis spectrophotometry at wavelengths range from 260 to 400 nm. The maximum protein absorption was observed at 280 nm. The spectra of the conjugates featured an additional absorption maximum at 320-360 nm. Fig. 3 shows the spectrum of clinafloxacin conjugate with ovalbumin. Availability of a peak other than 280 nm indicates the presence of fluoroquinolone groups in the conjugate. Spectra of other conjugates had an additional peak as well.

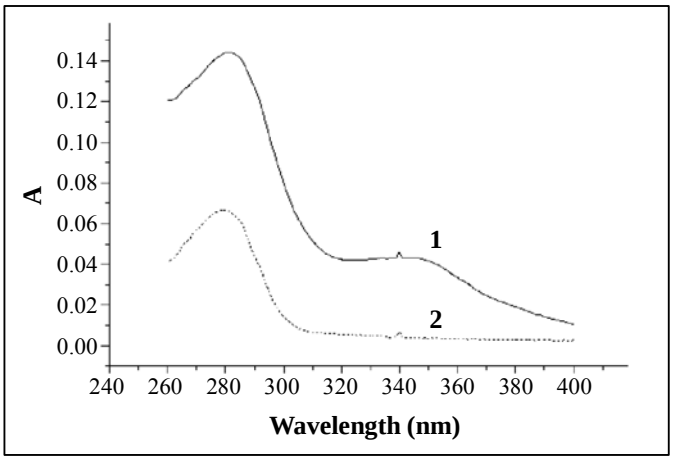

Fig. 3: UV-Vis spectral data for CLI-NH-C5-NH-OVA conjugate (1) and native OVA (2)

The danofloxacin molecule (Fig. 1) has neither secondary nor primary amine groups in the structure; therefore during conjugate synthesis for immunisation, haptene is coupled with a carrier protein via the activated carboxyl group.

Danofloxacin ELISA

The analytical capabilities of the resulting immune reagents were characterised by ELISA. The optimal concentration of conjugates adsorbed in the microplate wells of the is 0.5 µg/mL. For DAN-110 and DAN-130 antibodies (1:1000, 1:2000, 1:4000, 1:8000 dilution) in combination with the homologous coating antigen DAN-OVA, the DI was determined at a danofloxacin concentration of 100 ng/mL (Fig. 4). DAN-110 antibodies were characterised by higher DI.

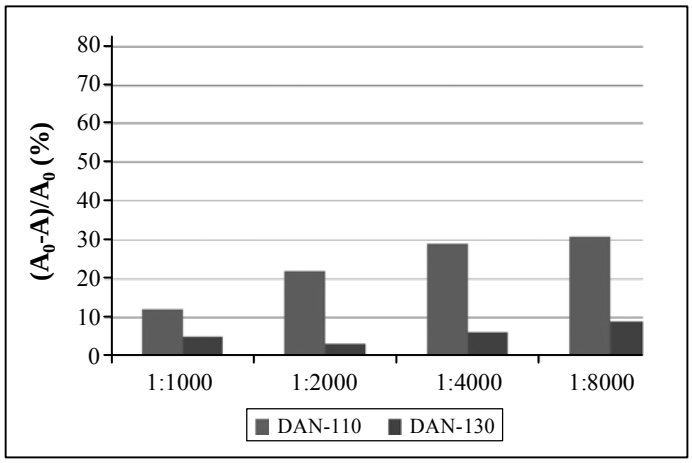

Fig. 4: Degrees of inhibition for DAN-110 and DAN-130 antibodies in homologous ELISAs with the DAN-OVA conjugate

Heterologous combinations were tested using the following conjugates as coating antigens: ENR-OVA, PIP-OVA, NOR-OVA, GAR-OVA, MOX-OVA, CLI-OVA, CIP-OVA, ENO-OVA and CLI-NH-C5-NH-OVA (Fig. 5). It has been shown that the ELISA with a CLI-NH-C5-NH-OVA as a coating antigen has the maximum DI.

The ELISA based on DAN-130 antibodies was characterised by IC_{20} = 3.3 ng/mL, IC_{50} = 24.5 ng/mL and IC_{80} = 152.5 ng/mL. ELISA based on DAN-110 antibodies was characterised by some better parameters: IC_{20} = 0.7 ng/mL, IC_{50} = 4.5 ng/mL, and IC_{80} = 28.4 ng/mL. Fig. 6 shows the calibration curves of the ELISA based on DAN-110 antibodies. The range of detectable concentrations reached 0.7-28.4 ng/mL, with a detection limit of 0.5 ng/mL.

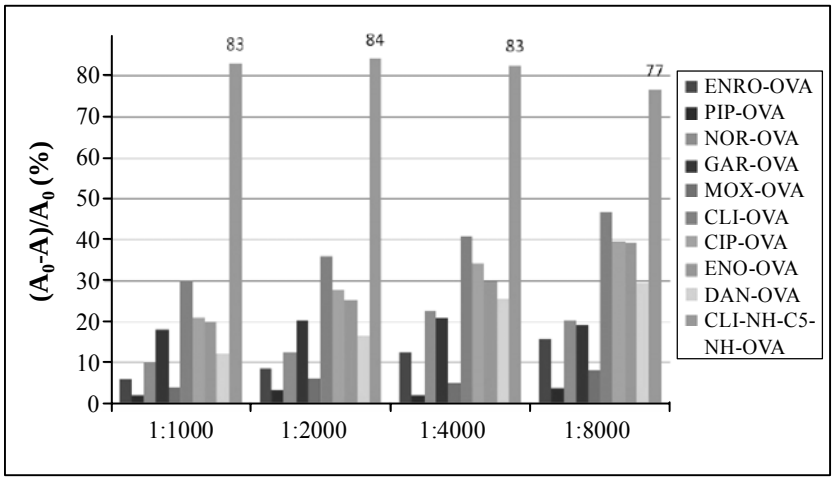

Fig. 5: Degrees of inhibition for DAN-110 antibodies in different ELISA systems

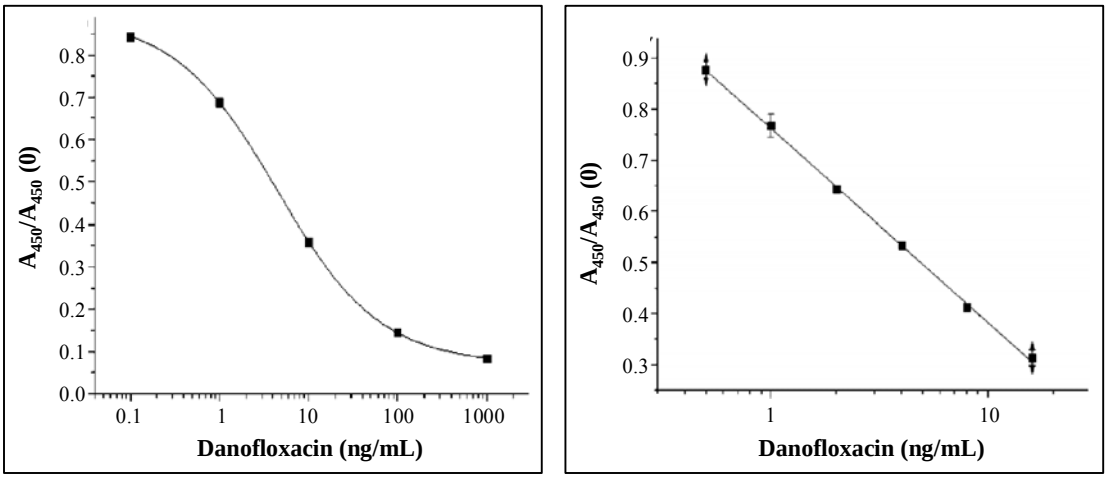

Fig. 6: Calibration curve for DAN ELISA, $A/A_0 = (0.76 \pm 0.01) - (0.38 \pm 0.01) \times \lg(c)$, $R^2 = 0.9997$, n=3

Characterization of the DAN-4AMF tracer

Mass spectrometry of the synthesized DAN-4AMF tracer preparation features three peaks (Fig. 7): 701.2 – peak of the single-charged ion of the DAN-4AMF molecule, 351.2 – peak of the double-charged ion of the DAN-4AMF molecule, 1401.3 – dimer peak in the form of a single-charged ion. This data confirms the structure of the target tracer DAN-4AMF.

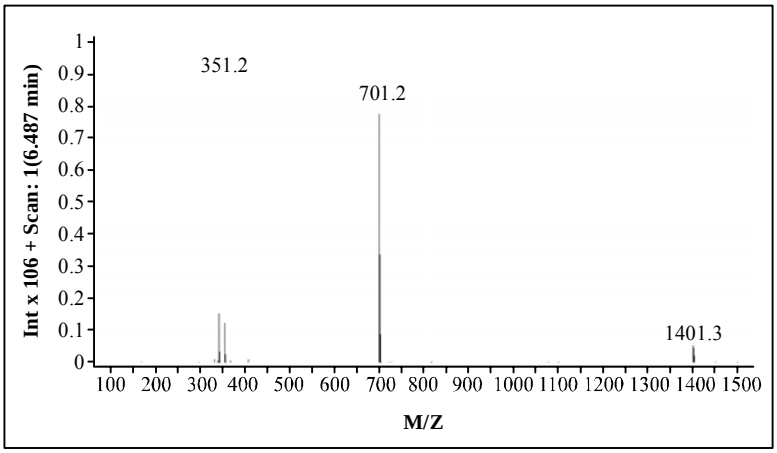

Fig. 7: Mass spectrum of the DAN-4AMF tracer

Danofloxacin FPIA

Danofloxacin-specific DAN-110 and DAN-130 antibodies were compared in terms of their interaction with a DAN-4AMF tracer. Fig. 8 shows a dependence fluorescence polarisation on the antibody dilution when in contact with a DAN-4AMF tracer. As can be seen, a better ability to bind to a tracer is peculiar to DAN-130. DAN-110 antibodies have 10 times worse binding ability in 1:200 dilution; therefore, FPIA was further optimised to determine danofloxacin based on DAN-130 antibodies.

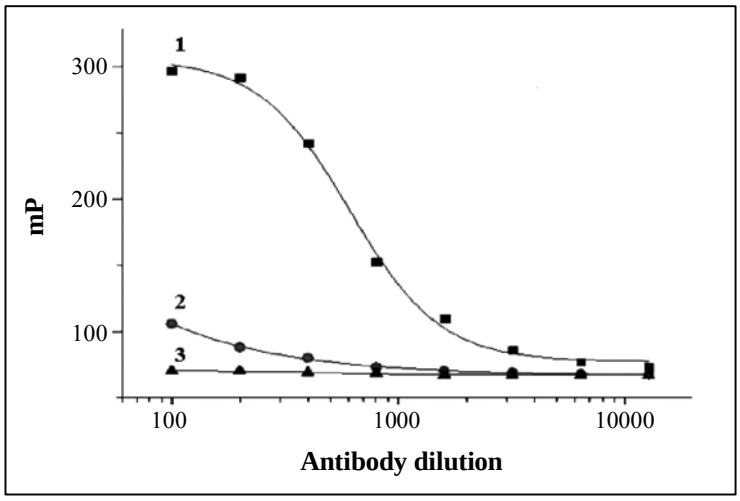

Fig. 8: Fluorescence polarisation dependences from the dilutions of DAN-130 (1), DAN-110 (2) and not immune IgG (3)

Calibration curves of dependence of the fluorescence polarisation upon danofloxacin concentration (Fig. 9a) were obtained. Additional measurements were carried out to clarify the IC_{20} – IC_{80} range (Fig. 9b). The range of detectable concentrations was 16.8-203.4 ng/mL with a detection limit of 13.0 ng/mL, which is worse than the analytical characteristics of ELISA.

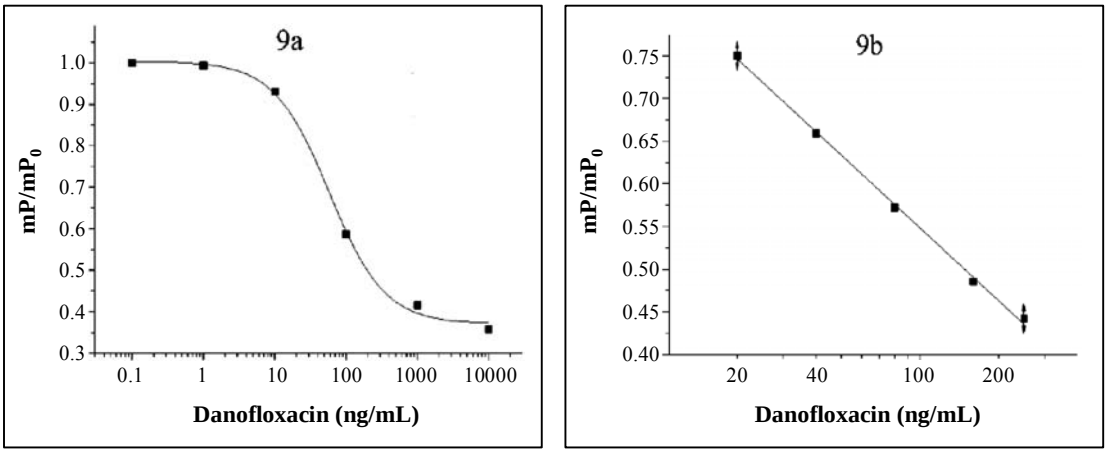

Fig. 9: Calibration curve for DAN PFIA using DAN-130 and DAN-4AMF, $mP/mP_0 = (1.11 \pm 0.01) – (0.28 \pm 0.01) \times \lg(c)$, $R^2 = 0.998$, n = 3

Evaluation of specificity

Twenty-four fluoroquinolones (see their list in the Reagents section) class were tested to evaluate the specificity of the developed methods. Table 1 shows the compounds with cross-reactivity values above 0.1%. Thus confirming high selectivity of the developed assays.

Table 1: Comparison of methods specificity

Compound	CR (%)		
	ELISA, for DAN-110 antibodies	FPIA, for DAN-130 antibodies	ELISA[7]
Danofloxacin	**100**	**100**	**100**
Clinafloxacin	11	< 0.1	-
Nadifloxacin	130	< 0.1	-

Cont…

Compound	CR (%)		
	ELISA, for DAN-110 antibodies	**FPIA, for DAN-130 antibodies**	**ELISA[7]**
Ciprofloxacin	38	< 0.1	0.3
Enrofloxacin	25	< 0.1	< 0.2
Garenoxacin	5	< 0.1	-
Enoxacin	5	< 0.1	< 0.2
Gatifloxacin	5	< 0.1	< 0.2
Lomefloxacin	5	< 0.1	< 0.2
Sparfloxacin	6	< 0.1	-
Orbifloxacin	3	< 0.1	-
Pefloxacin	< 0.1	< 0.1	22
Fleroxacin	-	-	21
Ofloxacin	< 0.1	< 0.1	< 0.2
Sarafloxacin	< 0.1	< 0.1	< 0.2

Danofloxacin detection in milk

Based on the optimised ELISA protocol, experiments were performed on danofloxacin determination in dairy products. To perform the "added-found" test, milk samples were used with danofloxacin concentrations of 20, 40, 80 ng/mL. The range of detectable danofloxacin concentrations in milk was 20-200 ng/mL; the results are shown in Table 2. The standard deviation for the repeated measurements did not exceed 5%.

Table 2: Results of "added-found" test for danofloxacin in milk using ELISA

Added danofloxacin (ng/mL)	Qty of samples	Found (ng/mL)	Opening percentage (%)
0	3	Not found	
20	3	25 ± 1	115-130
40	3	50 ± 2	120-130
80	3	78 ± 2	95-100
200	3	170 ± 4	83-87

To test the developed method, 83 samples of milk and dairy compounds were purchased from different manufacturers. All samples met the established maximum permissible levels of danofloxacin in milk – no more than 30 ng/mL.

Immunoassays are intesively developed analytical tools for wide screening control of toxicants in food stuffs due to their simplicity, productivity and low cost[1]. Enzyme-linked immunosorbent assay (ELISA) and fluorescence polarisation immunoassay (FPIA) are the most widely used immunoanalytical formats for quantitative control of target compounds.

Despite of intence development of immunoassays for fluoroquinolones[18], only a few publications are strongly focused on danofloxacin detection. Liu et al.[19] used danofloxacin for synthesis of immunogens and subsequent production of antibodies. The developed homologous test system with IC_{50} = 2.0 ng/mL allowing detecting several fluoroquinolones including pefloxacin and fleroxacin (cross-reactivities were equal to 22% and 21%, respectively). Cao et al.[20] proposed another homologous test system for danofloxacin with better IC_{50} = 0.1 ng/mL, but with cross-reactivity to nine fluoroquinolones.

It is known that the immunoassay selectivity could be improved significantly by the application of its heterologous format, where different derivatives of the target compounds are applied to produce antibodies and to carry out competitive immune interactions[21-23]. This idea has been realized experimentally and confirmed by the obtained data. No cross-reactivity was demonstrated for all other tested fluoroquinolones.

The high specificity of the developed heterologous immunoassays can be explained by the fact that the primary clinafloxacin amine group in the radical of the 7th position of the quinolone nucleus is attached to a protein using glutardialdehyde (Fig. 1). Thus, in this case, the specific target moiety for antibodies against danofloxacin are the radicals in the first and eighth positions of the quinolone nucleus, which reduces the ability of the antibodies to bind to the solid phase conjugate and narrows the detection specificity.

The reached detection limit of 0.5 ng/mL is slightly higher than the detection limit of 0.8 ng/mL for homologous test system described by Liu et al.[19]. The proposed sampling protocol with 30-fold final dilution of the initial milk samples allow controlling the exceeding MRL stated for danofloxacin (30 ng/mL) by the existing regulations.

CONCLUSION

The paper is the first to describe the heterologous combination of reactants for determination of danofloxacin. Two immunotechniques were proposed and chacterized,

namely fluorescence polarisation immunoassay (PFIA) for rapid one-stage preliminary screening and enzyme-linked immunosorbent assay (ELISA) for high-sensitive assay with 2 hr duration. The developed systems are highly selective and do not show cross-reactivity with other fluoroquinolones. The proposed immunoanalytical techniques may be considered as efficient tools for control of danofloxacin contamination in foods due to their sensitivity, selectivity, rapidity, simplicity and low cost.

ACKNOWLEDGEMENT

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