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Simultaneous and differential determination of drug and metabolite using the same antibody: difloxacin and sarafloxacin case

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Abstract

To differentiate close structurally related fluoroquinolones (FQs) difloxacin (DIF) and its active metabolite/degradant sarafloxacin (SAR) in animal tissues, rabbit polyclonal antibodies were generated to BSA-EDA-SAR. Using the same antibody two indirect competitive enzyme-linked immunosorbent assay (ELISA) formats with different specificities, for SAR-selective and SAR/DIF-group determination were constructed. Depending on the coated gelatin-SAR antigens which were prepared in active ester or formaldehyde condensation methods, the cross-reactivity of DIF changed from 1.9% to 100%, respectively, and remained <1% for the other FQs. The limit of detection, half-inhibition concentration (IC_{50}), and dynamic range (IC_{20}-IC_{80}) for the SAR-selective ELISA were found to be 0.018, 0.38, 0.037-4.43 ng/mL, and were 0.18, 2.8, 0.3-30 ng/mL for SAR and DIF as equal analytes in group assay. An approach based on parallel analysis of samples in both tests allowed differential detection of SAR and DIF in chicken and turkey muscles. Recovery from fortified tissue samples ranged from 70 to 118% and confirmed the suitability of the developed screening test for measuring and differentiation of these antibiotics about their MRL level in food matrices.

Keywords: Difloxacin; sarafloxacin; differential immunoassay; selectivity; group-specificity; poultry muscles;

1. Introduction

Difloxacin (DIF) and sarafloxacin (SAR) are veterinary fluoroquinolone antibiotics (FQs) extensively used in poultry and aquaculture. Moreover, the administration of DIF is extended to cattle and pigs for the treatment and control of respiratory and intestinal infections [1]. Metabolites of SAR were shown to exhibit weak antimicrobial activity in comparison with that of the parent drug [2]. The primary and major active metabolite of DIF that can be identified in tissues is N-desmethyldifloxacin, also known as SAR [1, 3]. As was reported, DIF degrades to SAR in water under natural sunlight conditions [4]. Thus, since DIF is administered with drinking water in poultry or with feed in fish farming, the both antibiotics can get into animal organism or present in tissues as a result of metabolism. In the similar cases a parent drug and its active metabolite are prescribed to be detected as a sum of both analytes, e.g., enrofloxacin and ciprofloxacin, florphenicol and florphenicol amine or tetracyclines and their 4-epimers. The other examples of structure/activity-related compounds which have identical MRLs are dihydrostreptomycin and streptomycin, sulfonamides and others [5]. Nevertheless, the limitations established for the residues of DIF and SAR as marker substances in the tissues of edible animals differ sufficiently. To minimize risk to consumers, the residual content of DIF in poultry muscles is not allowed to be over 300 μg/kg and the norm for SAR is significantly stricter, 10 μg/kg. This means that two closely related and concomitant analytes should be distinguished during analysis (**Fig. 1**). Identification of both analytes could be realized using high-performance liquid chromatography with ultraviolet, fluorescence or mass-spectrometric detection [6, 7]; however, such techniques are hardly applicable for routine screening of numerous samples because of complicated, laborious, and time-consuming pretreatment.

Alternative methods based on antibody-analyte recognition are known as simple, high sensitive, and high throughput techniques, so they are better suitable for screening purposes. Several immunoassays for FQs have currently been reported. Some of them, group-specific assays, were capable of simultaneous determination of a whole number of representatives. The other immunochemical tests were developed to detect individual FQs from the group [8-14]. Although the certain progress in the sphere of group recognition as well as in selective identification of FQs using immunoassay is achieved, the means for individual determination of SAR as analyte with much stricter regulation have not been found yet. The cross-reactivity between SAR and the other FQs, especially the nearest structural analog DIF, reaches usually of high extent. It makes differentiation difficult. Moreover, the level of sample contamination may be interpreted as noncompliant for one analyte and compliant for another due to 30-fold differences in maximal residue limits (MRLs) between DIF and SAR. So, it seems to be uneasy task to distinguish drug and its metabolite in immunoassay and sometimes it cannot be resolved at all without confirmation methods.

In the present study, an immunochemical approach for high sensitive determination DIF and SAR separately from the other fluoroquinolones is described. Here we demonstrate the possibility of differentiation of related compounds in screening test using one universal antibody reagent. To address this problem, the strategy for antibody recognition of common or distinctive features of target analytes in food matrices depending on assay format is proposed. To our knowledge, the differentiation of parent drug, DIF and its active metabolite, SAR has not been realized in immunoassay before, as well as selective immune techniques for these FQs have not been reported yet.

2. Materials and methods

2.1 Reagents and equipment

Sarafloxacin hydrochloride trihydrate (SAR), difloxacin hydrochloride (DIF), danofloxacin, flumequine, marbofloxacin, oxolinic acid, bovine serum albumin (BSA), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), ethylendiamine (EDA), Freund's complete adjuvant, and horseradish peroxidase were from Sigma (St.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

84 Louis, MO, USA). Dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were from Serva (Heidelberg, Germany).
85 Gelatin (Gel) was purchased from Bio-Rad (Hercules, CA, USA). Ciprofloxacin hydrochloride, enrofloxacin, norfloxacin,
86 pefloxacin, and ofloxacin were purchased from Chimmed (Moscow, Russia). All other chemical reagents were analytical
87 grade.

88 Stock solutions of FQs (1 mg/mL) were prepared using methanol and stored at – 20°C. All the standard solutions
89 were diluted from stock solutions with PBST (phosphate buffered saline buffer containing 0.05% Tween). The coating buffer
90 was 0.05 M carbonate buffer, pH 9.6 (CB). Ready-to-use substrate mixture containing TMB/H2O2 was a product of Biotest
91 Systems (Moscow, Russia).

92 Immunoenzymatic reactions were carried out in polystyrene 96-well microtiter plates Costar (Corning Inc., Lowell,
93 MA, USA). For incubations ST-3L thermoshaker (ELMI Ltd. laboratory equipment, Riga, Latvia) was used. The absorbance
94 was read using Stat Fax 2100 microtiter plate reader (Awareness Technology Inc., Palm City, FL, USA). Conjugate UV spectra
95 were recorded using scanning spectrophotometer Genesis 10S (Thermo Scientific, Madison, WI, USA).

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97 **2.2 Preparations of immunogen and coating antigens**

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99 **BSA-SAR(ae) and Gel-SAR(ae).** For the synthesis of immunogens and coating antigens an active ester method (ae)
100 was used that conducted according to the procedure described previously [15]. Sarafloxacin hydrochloride (6 mg, 12.6
101 µmol) was dissolved in 0.66 mL of DMSO and supplemented with 4 mg of EDC and 2.4 mg NHS both from 10 mg/mL
102 solutions in DMF and stirred at room temperature for night. Then, solution of activated SAR were dropwise added to BSA (4
103 mg, 0.06 µmol) or Gel (4 mg, 0.025 µmol) in 1.0 mL CB and incubated with stirring for night at room temperature. The molar
104 ratios between BSA or Gel as a carrier and hapten during synthesis were at the range of 1/20 - 1/100. To remove unbound
105 hapten, an exhaustive dialysis against water was followed by.

106 **BSA-EDA-SAR(ae) and Gel-EDA-SAR(ae)**. Protein carriers were firstly cationized, i.e., free carboxylates were
107 substituted with amines [16]. For this purpose, 30 mg (156 µmol) of EDC was dissolved in 1.5 mL water solution of BSA (20
108 mg, 0.3 µmol) and Gel (8 mg, 0.05 µmol) in 1.0 mL of water and mixed using magnet stirrer for 30 min. Then, 1000-fold
109 molar excess of EDA was added to activated proteins and stirred for 2 h at room temperature. Excess of unreacted EDA was
110 removed by exhaustive dialysis against water.

111 The following procedure of hapten coupling using method of active ester was repeated as described above. The
112 ratios between cationized proteins and SAR were from 1/10 to 1/100.

113 **BSA-SAR(f) and Gel-SAR(f)**. The principle of Mannich condensation in the presence of formaldehyde (f) is
114 described in [17]. Briefly, solution of SAR (15 mg/mL) in DMSO was added at 25-, 50-, 75-, and 100-fold molar amount in
115 relation to proteins BSA (4 mg, 0.06 µmol) and Gel (4 mg, 0.025 µmol) in 0.5 mL of water. After the mixtures were prepared,
116 0.2 mL portions of 37% formaldehyde were added and stirred for night at room temperature. After extensive dialysis, the
117 obtained conjugates were equilibrated with glycerol and stored till usage at – 20°C.

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119 **2.3 Immunization schedule and immune response monitoring**

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121 Chinchilla rabbits (2.0–2.5 kg) were obtained from the Scientific and Production Centre for Biomedical
122 Technologies (Elektrogorsk, Russia), kept and treated in accordance with the guideline for the care and use of laboratory
123 animals [18]. All the experiments with animal were approved by the Ethics Committee of Mechnikov Research Institute for
124 Vaccines and Sera.

125 Conjugates BSA-SAR(ae) and BSA-EDA-SAR(ae) were injected subcutaneously to rabbits on 8-12 points on the
126 back. First immunization was performed with 100 µg of immunogens in complete Freund's adjuvant emulsion. The
127 following injections with the same doses of conjugates in physiological saline (without adjuvant) were carried out monthly

and one week after each booster injection, a portion of blood was taken from marginal ear vein. The resultant sera were tested in ELISA using immobilized homologous and heterologous hapten conjugates. Anti-hapten immune response was evaluated in competitive assay format using SAR, DIF and analogues as free haptens.

2.4 ELISA procedure

Optimal concentrations of conjugated antigens and antibodies were firstly determined using chessboard titration method. The pairs of immunoreagents in concentrations which permitted the absorbance level of reaction of 0.8 - 1.2 were chosen and then used in competitive indirect ELISA.

Microplates were coated with synthesized conjugates in optimal concentration. The solutions were prepared in CB, added 0.1 mL per well, and incubated for night at 4°C. After thrice-repeated washing with PBS containing 0.05% Tween 20 (PBST), the wells were filled with 0.1 mL of standard solution of FQs (1000–0.01 and 0 ng/mL) and 0.1 mL of antiserum in working dilution in PBST supplemented with 1% BSA. After incubation for 1 h, the wells were washed again and incubated with 0.1 mL of goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase. After washing, a substrate solution was added 0.1 mL per well. The enzymatic reaction was stopped 30 min later by adding 0.1 mL of 1 M sulfuric acid. The absorbance was registered at 450 nm.

The antibody binding rate was calculated as a percentage $B/B_0 \times 100$ between the absorbances for each concentration (B) and 0 ng/mL of analyte (B_0). Standard curves were plotted using OriginPro 8.0 software and served for assessment of assay parameters: sensitivity taken as half-inhibition concentration (IC_{50}), limit of detection (IC_{10}) and dynamic range (IC_{20} - IC_{80}) [19]. For each analogue, IC_{50} values were determined and cross-reactivity was found as a ratio $IC_{50\ SAR}/IC_{50\ ANALOGUE}$ expressed in percent.

Among the tested pairs of antigen-antibody those ones which performed more sensitive SAR determination were selected for the following experiments.

2.5 Sample preparation, matrix effect estimation and recovery experiments

Tissue samples from private organic-farm hens (antibiotic-free) were minced and grinded. The tubes with 2g-portions of homogenate were refilled with PBST to 10 mL mark, intensively vortexed, and allowed to extract for night at 4°C. Then, the samples were centrifuged for 10 min at 3000 rpm, supernatants were diluted 2, 5, 10, and 20 times with PBST and tested in ELISA.

Matrix effect on antibody binding and calibration curve slope was examined. For this, antibiotic standards were prepared in extracts, analyzed, and compared with standards in assay buffer. Factor of extract dilution diminishing and avoiding the matrix effect was determined and used for the following sample pretreatment.

For recovery estimation the chicken and turkey muscle homogenates were spiked with SAR and DIF at several concentrations around their MRLs, 10 and 300 µg/kg, respectively. Then, the fortified blank samples analyzed and recovery rate was determined as a ratio between the measured and the fortified concentration.

3. Results and discussion

3.1 Preparation of hapten conjugates for immunization and coating plates

DIF and SAR have closely related structures differed only in N-substituent in piperazin cycle (**Fig. 1**). That's the reason that most of antibodies generated against these or other FQs as reported before were capable to recognize both DIF and SAR almost equally or with near cross-reactivity (**Table 1**).

To distinguish these FQs using immunoassay, the antibodies should be directed against distinctive moiety of molecule, i.e. N-substituent in piperazin cycle. Therefore, the most appropriate manner to prepare immunogen for this purpose is to synthesize conjugate using the carboxyl group of hapten for linking with protein. Such attempts were made, however, demonstrated high cross-reactivity between SAR and DIF [8, 11]. As was shown earlier, the cross-reactivity profile of assay may be significantly modified and converted from selective to group-specific using the same polyclonal antibody [20, 21]. This switching of assay specificity depended on sub-repertoire of antiserum antibodies which was involved in binding to specific coating antigen. Thus, we followed this approach to regulate assay specificity.

For preparation of immunogen, an active ester (ae) method was used as a reliable procedure to expose target epitope. However, it cannot be absolutely ruled out the possibility of interaction between SAR's secondary amine and protein carboxyls during synthesis procedure and incubation of mixture of reagents. The other kind of conjugates based on cationized (c) proteins was used to avoid this suspicion. Available COOH-groups in these proteins were blocked and additional amines were introduced instead. An alternative method was formaldehyde condensation; its effectiveness for conjugation of the other fluoroquinolone, ciprofloxacin was demonstrated previously [22].

The formation of conjugates was confirmed by spectral characteristics combined the features typical for SAR (main peak at 277 nm and additional peak at 320 nm) and BSA-carrier (**Fig. 2**). It should be noted that the efficiency of coupling was different in various procedures even under the same reagent ratio. For example, for BSA-SAR(ae), BSA-EDA-SAR(ae) and BSA-SAR(f) prepared at 100-fold molar excess of SAR over BSA the hapten load was equal to 12.3, 5.8 and 1.7 mol/mol. Nevertheless, all the BSA-conjugates prepared using the above mentioned methods and analogical conjugates based on Gel carrier was immunochemically active.

3.2 Immunization

Conjugates BSA-SAR(ae) and BSA-EDA-SAR(ae), which were synthesized at 1/50 and 1/100 molar ratio between carrier and hapten, possessed almost equal SAR density on the carrier (about 1:6), so they were chosen as comparable immunogens. Blood samples from immunized animals were taken monthly and sera were tested for immune response intensity. Both types of antisera actively bound to homologous antigens, however the binding of anti-BSA-EDA-SAR(ae) antibodies to Gel-EDA-SAR(ae) coating antigen could not be inhibited by free SAR. Homologous and heterologous formats of assay for anti-BSA-SAR(ae) showed almost the same sensitivity. Thus, the study of sensitivity and intensity of the immune response for both group of animals during the immunization course was carried out using Gel-SAR(ae) as coating antigen. The most active sera obtained from replicate rabbits were chosen for the following examinations. Comparative study showed that the titer of antisera had increased by the second bleeding (after the third immunogen administration) and it could ensure a sufficiently high level of assay sensitivity (**Fig. 3**). The anti-BSA-SAR(ae) antibodies had an advantage in binding activity towards Gel-SAR(ae), however, the lower IC_{50} index was registered when using anti-BSA-EDA-SAR(ae). This fact can be explained by effect of spacer heterology, lack of spacer arm in coating conjugate, although hapten structure and conjugation bond were homologous to both immunogens. So, for the following experiments antisera #2 were applied.

3.3 Cross-reactivity examination

To achieve differentiation between DIF and SAR in immunoassay, we primarily used the immunogens, design of which permitted the generation antibodies against distinctive moiety of the molecules. Then, we compared the influence of C2-spacer (EDA) in immunogen structure on assay specificity and found that both antibodies recognized SAR and DIF in a similar manner. However, the anti-BSA-EDA-SAR showed the better distinguishing of SAR and DIF, as 100% and 1.9% vs 100% and 11.7% for anti-BSA-SAR, respectively. Owing to EDA-modification of BSA, more distant hapten position on the

carrier and well-defined orientation might facilitate the better focus of immune response on distinctive epitope and, therefore, the better selectivity for SAR.

It is known that cross-reactivity profile of analytes may be significantly changed when heterologous antigen is immobilized. Thus, the behavior of FQs as cross-reactants was examined in heterologous assay format using more selective antibody to BSA-EDA-SAR. As a confirmation the above thesis, the specificity of assay towards SAR and DIF modified critically when heterologous coating antigen, Gel-SAR(f) was used **(Fig. 4**).

The results of cross-reactivity examination for the other FQs, which are also applied in the poultry farming and listed as regulated substances in the Russian Federation and the EU [5, 23] are shown in **Table 2**. As can be seen from the data given, the first assay format performed exclusive SAR-selectivity. The rest of the 'poultry' FQs demonstrated negligible cross-reactivity. Owing to antibodies that were capable to distinguish the presence of distinctive N-substituent in piperazin cycle, even the nearest structural analogue and parent drug, DIF expressed weak competition (1.9%). However, the mentioned distinctive fragment was not the only epitope recognized by antibody, since ciprofloxacin and norfloxacin bearing the same piperazin determinant like SAR, remained practically undetectable (<1%). Thus, the immunogen designed to produce distinctive epitope targeting antibody, selection of antibodies with desired specificity and best characteristics allowed immunochemical distinguishing between SAR and DIF (1.9%), unlike the immunoassays reported before (**Table 1**).

In similar challenges, the most pronounced results were obtained for distinction between ciprofloxacin (100%) and closely related FQs, norfloxacin (2.99%), and enrofloxacin (0.99%) [17]. The better specificity for these analytes was achieved when immunizing hapten had distinctive N-substituent in piperazin like enrofloxacin (100%), then cross-reactivity for cipro- and norfloxacin was no more than 0.1%. However, such a high specificity was not absolute; the other FQs such as pefloxacin, ofloxacin, and marbofloxacin also bearing N-substituent in piperazin cycle showed significant cross-reactivity up to 10% [24]. Similarly in the recent work [26], one from rabbit McAbs that was generated after mixed FQs immunization showed the 33-fold difference between DIF (18.25%) and SAR (0.55%) but their differentiation were impossible because of the nearest and higher cross-reactivity of many other FQs. So, an important feature of SAR-selective ELISA described here was free of possible interference from the other FQs. Performance parameters of the assay was measured using the standard curves and appeared to be the following: the limit of detection (IC_{10}) were 0.018 and 1.0 ng/mL for SAR and DIF; sensitivity values (IC_{50}) were 0.38 and 20.5 ng/mL, respectively; SAR dynamic range (IC_{20}-IC_{80}) was 0.037-4.43 ng/mL and possible dynamic range for DIF in this assay was 2.39-319.3 ng/mL.

The second developed assay format (heterologous), called SAR/DIF-group ELISA, was capable to recognize SAR and DIF equally (100%). At the same time it possessed insensitivity towards the other FQs (less than 0.6%). This fact suggests that Gel-SAR(f) bound selectively antibody that were generally focused on 4-fluorophenyl epitope. The characteristics of this assay format for SAR and DIF as equal analytes were 0.18 ng/mL (LOD), 2.8 ng/mL (IC_{50}), and 0.3 - 30 ng/mL (dynamic range).

3.4 Sample pretreatment and matrix effect estimation

Due to matrix interference, the assay of analyte in buffer should often be adapted for measurement of analyte in objects such as animal tissues. As a rule, for matrix effect elimination a special sample pretreatment are applied. This procedure often results in analyte dilution and sample concentration step is sometimes required because of the limit of assay detectability. Therefore, to estimate the measurable range of the developed method, to determine permissible level of extract dilution, and on the other hand to find necessary conditions for better differentiation between DIF and SAR, we constructed the following diagram for selective and group assay formats (**Fig. 5**). The dynamic range of each assay format for determination of analyte content in tissue samples (w/w) represented IC_{20}-IC_{80} range of analyte prepared in extracts (w/v) multiplied by extraction coefficient 5 (see Section 2.5). From the diagram, it follows that either SAR or DIF could be reliably detected at their MRL using the selective ELISA format at extract dilution from 1/1 to 1/20 because the both MRLs

were within the corresponding working ranges. Thus, SAR-selective assay could not distinguish these analytes because the DIF MRL was 30 times higher than SAR MRL (300 vs 10 µg/kg) that canceled 50-fold advantage of SAR in sensitivity (0.38 vs 20.5 ng/mL).

The second ELISA format was capable of equal SAR-DIF determination, but measurement of their MRL levels depended on the sample dilution. Dynamic range of the group assay allowed measuring separately SAR MRL in undiluted extract; DIF MRL level could be determined separately in extract after 10-20-fold dilution. The dilution approach contributed to set the required for differentiation dynamic range, but, nevertheless, it did not allow identifying of these analytes.

Possible solution of the problem was parallel testing of samples in SAR-selective and SAR/DIF group assay when extract was diluted by a factor of 10 and SAR standard was used as unified marker substance for both assays. In these conditions, SAR-selective assay could not detect DIF MRL, which was outside the dynamic range (grey column, **Fig. 5A**) and SAR/DIF group assay did register only DIF MRL level (**Fig. 5B**). Comparison and analysis of obtained data allowed identifying the analyte. So, for example, DIF+ samples could be found in the group assay format, while SAR+ sample should be positive in the first ELISA.

The degree of matrix effect was estimated using the blank tissue samples. An extraction with assay buffer is an easy, cheap and safe procedure that requires no organic solvents, often is used and gives satisfactory results [27]. Crude extracts of chicken and turkey breast muscles, extracts gradually diluted with PBST 2, 5, and 10 times were used as media for preparation of SAR and DIF standards and compared with standards in assay buffer (**Fig. 6**). The matrix interference on SAR and DIF determination was examined in both ELISA formats. Extracts from chicken and turkey muscles were found to have an equal impact on decreasing of the optical density signal that resulted in a certain shift of standard curves. This effect was almost imperceptible in SAR/DIF assay and more pronounced in SAR-selective ELISA. It was gradually vanished along with diluting of extract, so that no interference was registered when the factor of extract dilution was 10; since the standard curves obtained in assay buffer and ten-fold diluted extract were virtually superimposed.

3.5 Recovery experiments

The procedure of sample preparation and factor of extract dilution (equal to 10) was predetermined as optimal for avoiding of matrix effect and possible differentiation of DIF and SAR. So, to check these parameters, tissue homogenates were fortified with several concentrations of SAR and DIF around their MRLs, extracted using PBST, diluted 10-fold using this buffer and tested in both assay formats. Calculation of antibiotic content in the samples was performed according to the following formula:

$$C\ (\mu g/kg) = 5 \times 10 \times c\ (ng/mL),$$

where *C* – content (residual concentration) of analyte in chicken/turkey muscle, *5* – coefficient of extraction, and *10* – factor of extract dilution, and *c* – measured concentration. Concentrations of unknown analyte (SAR or DIF) in both ELISAs were determined using SAR-calibration curves. The obtained data confirmed the correctness of the predicted conditions for differentiation SAR and DIF, as can be seen from the **Table 3**.

Both FQs could be detected at their MRL level using the first ELISA in spite of its SAR-selectivity. However, when calibrating with SAR the assay failed to detect DIF (≈1%), while the recovery rate for SAR-fortified samples was adequate (70-118%). The other FQs could not interfere the results since their cross-reactivity was less than DIF showed. For comparison, the critical level of SAR content could not be registered in SAR/DIF group assay. The condition of sample preparation was chosen such way that working range of group assay excluded SAR MRL. Thus, using SAR-calibrator it was possible to quantify SAR in SAR-selective assay and DIF in SAR/DIF group assay.

4. Conclusion

This paper described the development of immunochemical approach for determination and differentiation of fluoroquinolones, DIF and SAR residues in animal tissues as exemplified by chicken and turkey muscles. SAR is main metabolite of DIF, so both antibiotics have very similar structures and differed in single methyl radical. Owing to the immunogen design the polyclonal antibodies were generated to focus on distinctive moiety of the molecules. Using the selection of heterologous coating antigen as an approach to modify assay specificity two ELISA variants were developed based on the same antibody. One assay variant was developed for group (equal) determination of SAR and DIF, another one performed SAR-selectivity. Parallel analysis of samples in two ELISAs based on the same antibody allowed differentiation DIF and SAR in screening procedure before the final certification in confirmatory methods; SAR-noncompliant samples could be selected as positive ones in the first ELISA format, whereas DIF-noncompliant samples were registered only in the second test.

The advantage of screening method described in this paper illustrated by possibility of determination and differentiation of both analytes using one antibody and one calibration. Besides, the developed assay formats may be also useful as independent tests for specific and sensitive determination of SAR or simultaneous summary determination of SAR and DIF in samples with known target analyte or those objects where there is no different regulation for these FQs.

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Table 1

Cross-reactivity characteristics and IC50 values for SAR and DIF in immunoassays of fluoroquinolones.

Assay format	Antibody	Immunogen	IC50, ng/mL	CR, %		Reference
				SAR	DIF	
ic-ELISA	6 McAbs	cBSA-SAR (ae)	7.3-48.3	100	126-216	8
ic-ELISA	4 McAbs	BSA-N-sulfanilyl SAR(ga) BSA-N-ethylamine SAR(ga)	6.5-12.1	100	97-113	9
dc-ELISA	PcAb	KLH-N-hexanoic SAR (ma)	0.21	100	64	10
ic-ELISA	McAb	BSA-SAR (edc)	0.32	100	85.5	11
ic-ELISA	PcAb	BSA-N-ethylamine NOR(ga)	3.3	18	19	12
dc-ELISA	PcAb	cBSA-N-hexanoic NOR(ma)	nd	9	18	13
FPIA	McAb	BSA-N-CIP(ma)	24.16	8.9	7.8	14
ic-CLEIA	PcAb	BSA-PAZ (edc)	221	7.4	10.2	23

ae – active esters method; ga – glutaraldehyde crosslinking; ma – mixed anhydride method; edc – carbodiimide condensation; dc / ic– direct / indirect competitive assay format; CIP – ciprofloxacin; CLEIA –chemiluminescence enzyme immunoassay; ELISA enzyme-linked immunosorbent assay; FPIA – fluorescent polarization immunoassay; nd – not determined; NOR – norfloxacin; PAZ - pazufloxacin.

Table 2

The cross-reactivity profile of FQs used in poultry farming in ELISA formats based on Gel-SAR coating antigens differed in synthesis procedure.

Fluroquinolones	SAR-selective ELISA [Gel-SAR(ae)–coated]		SAR/DIF-group ELISA [Gel-SAR(f)–coated]	
	IC_{50}, ng/mL	CR,%	IC_{50}, ng/mL	CR,%
Sarafloxacin	0.38	100	2.8	100
Difloxacin	20.5	1.9	2.8	100
Ciprofloxacin	41.6	0.91	470	0.6
Enrofloxacin	475	0.08	620	0.45
Norfloxacin	97.4	0.39	1040	0.27
Pefloxacin	1270	0.03	2840	0.1
Danofloxacin	1900	0.02	1420	0.2
Flumequin	>10000	<0.01	2560	0.11
Ofloxacin	>10000	<0.01	>10000	<0.01
Oxolinic acid	>10000	<0.01	>10000	<0.01

Gel-SAR(ae) – coating conjugate prepared using active ester method;

Gel-SAR(f) – coating conjugate prepared by formaldehyde condensation.

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Table 3

Recovery studies of DIF and SAR in fortified blank chicken and turkey muscles using selective and group ELISAs.

				ELISA formats [a]					
Matrix	Extract dilution	FQs	Fortified, µg/kg	SAR-selective			SAR/DIF group		
				Measured, µg/kg	RC[b], %	CV[c], %	Measured, µg/kg	RC, %	CV, %
Chicken muscle	1:10	SAR	5	3.5±0.1	70	2.9	nd[d]	-	-
			10	10.3±0.9	103	8.7	nd	-	-
			20	23.7±1.3	118	5.5	nd	-	-
		DIF	150	nd	-	-	121±11.6	80.7	9.6
			300	2.9±0.4	0.97	13.8	291±49.8	97	17.1
			600	6.8±1.5	1.1	22.0	654±77.0	109	11.8
Turkey muscle	1:10	SAR	5	4.6±1.2	91	26.1	nd	-	-
			10	10.9±1.4	109	12.8	nd	-	-
			20	23.5±4.5	115	19.1	nd	-	-
		DIF	150	nd	-	-	108±12.9	72	11.9
			300	4.5±1.2	1.5	26.7	273±40.8	91	14.9
			600	9.2±1.5	1.5	16.3	560±120	93.3	21.4

[a] Concentrations of SAR and DIF as unknown analytes in both assay formats were calculated using SAR calibration curve;
[b] RC - The average recovery value (n=4);
[c] CV – coefficient of variation;
[d] nd – value was not determined due to the content of analyte was outside the dynamic range of assay;

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Figure legends

Fig. 1 Chemical structures of parent drug difloxacin and its metabolite/degradant sarafloxacin. The number in brackets is MRL value for each drug in chicken/turkey muscle (µg/kg).

Fig. 2 UV-spectrogram of BSA-SAR conjugates prepared at molar ratio 1 to100 between BSA and SAR using active ester (ae) method and formaldehyde (f) as a coupling agent.

Fig. 3 Dynamics of sensitivity of SAR determination (A) and antibody titer (B) during immunization with BSA-SAR(ae) and BSA-EDA-SAR(ae) in competitive indirect ELISA using Gel-SAR(ae) as coating antigen. Each point represents the average of independent replicates (n=3).

Fig. 4 Standard curves for SAR and DIF obtained in competitive indirect ELISA formats based on anti-BSA-EDA-SAR(ae) antibodies and coating antigens prepared using homologous and heterologous conjugation methods, Gel-SAR(ae) and Gel-SAR(f), correspondingly. Error bars represents ± standard deviation for three replicates.

Fig. 5 Relation between sample dilution and dynamic range of SAR and DIF determination in SAR-selective (A) and SAR/DIF group assay (B). Dynamic ranges represent IC_{20}-IC_{80} range for each analyte in corresponding assay format. The factor of extract dilution 1 corresponds to a crude extract prepared accordingly the description in sample pretreatment section, and factors from 2 to 20 mean that extracts were diluted with PBST 2-20 times, respectively.

Fig. 6 The influence of chicken/turkey extracts on determination SAR (A) and DIF (B). Standards of FQs were prepared in PBST and extracts were diluted with PBST and analyzed in SAR-selective (solid lines) and SAR/DIF-group ELISA formats (dash lines). Each curve was produced using the average of five replicates (chicken/turkey n=3/2). Curves for SAR and DIF prepared in PBST and extracts diluted 1/10 are superimposed.

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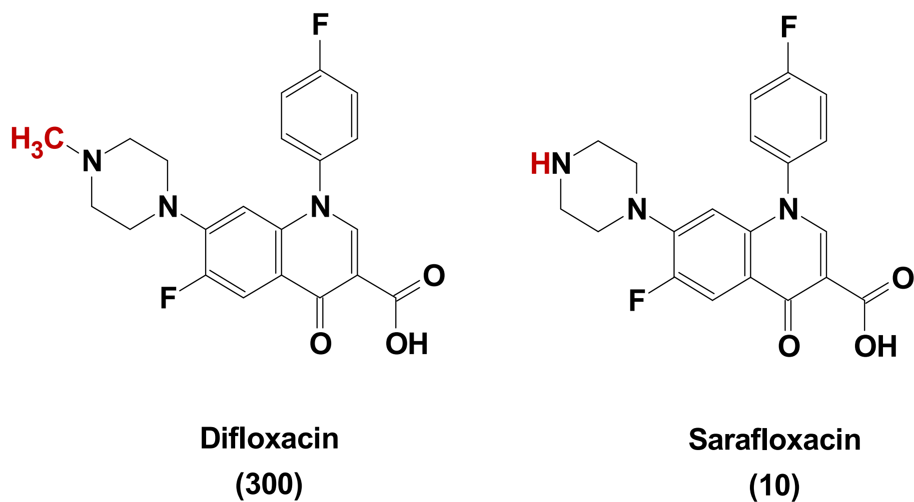

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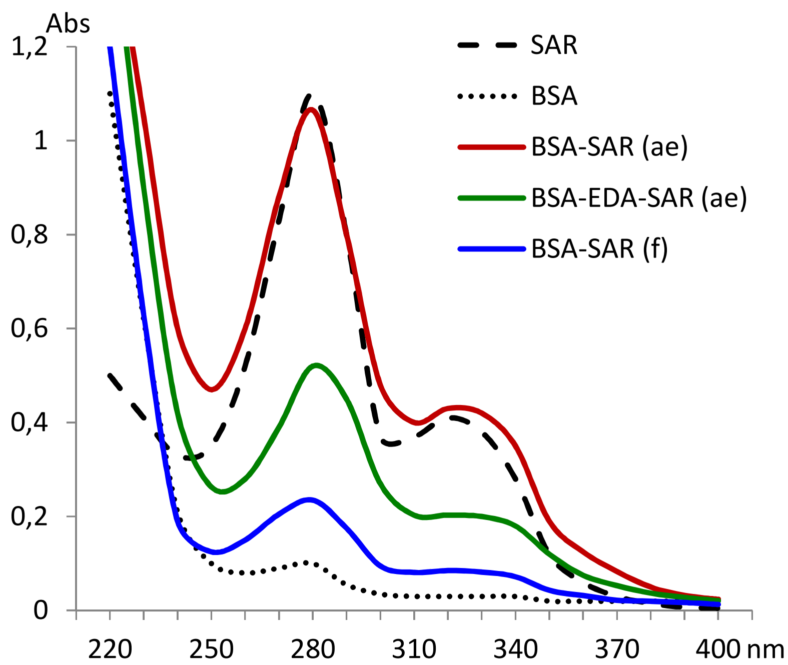

Figure 2

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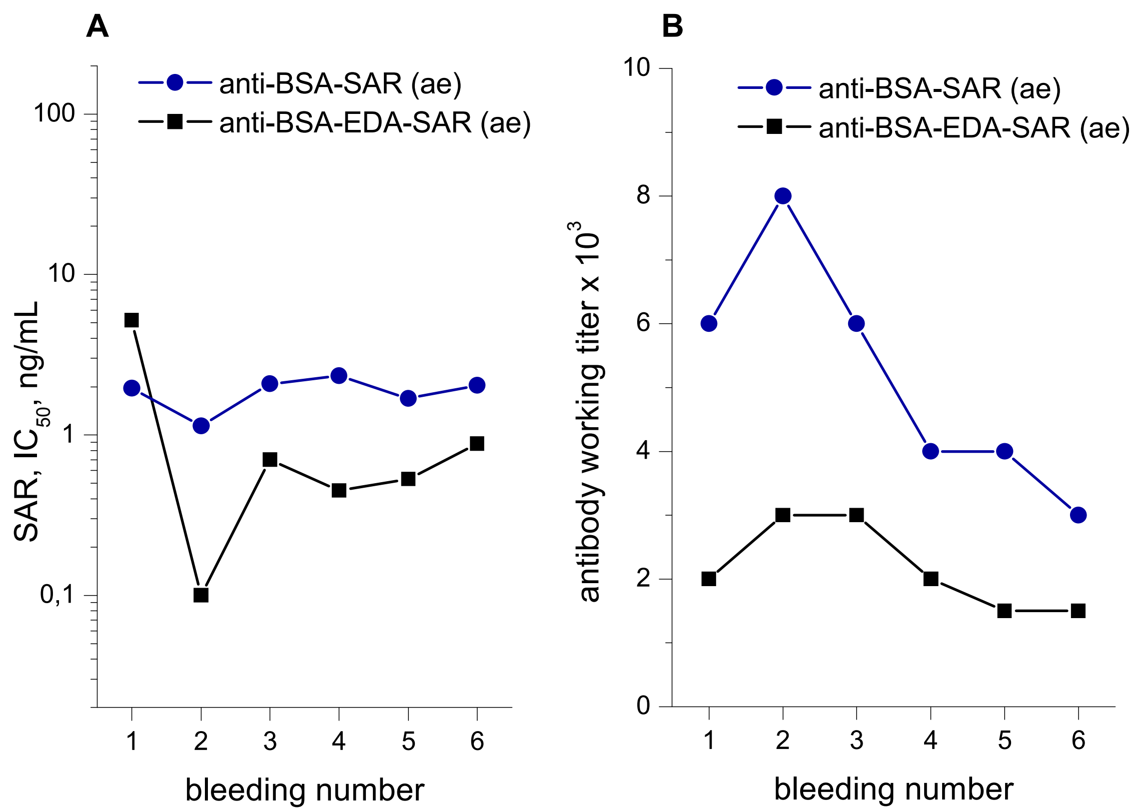

32 Figure 3

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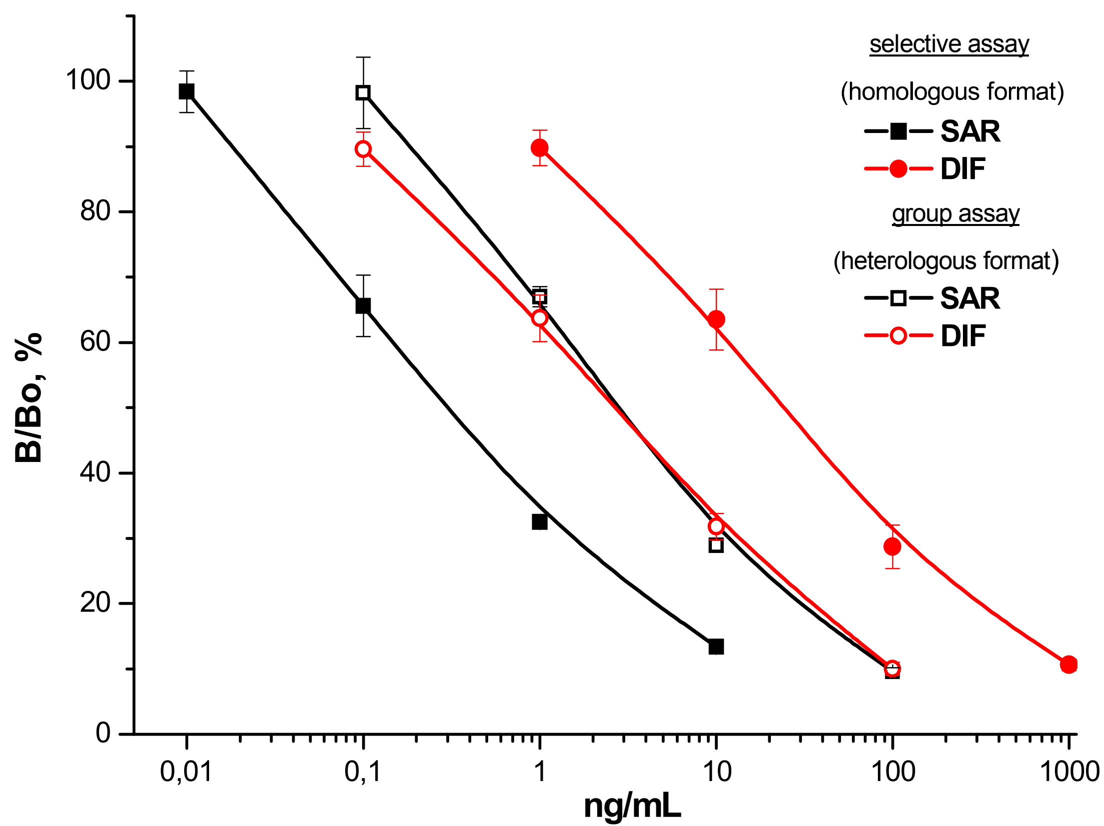

Figure 4

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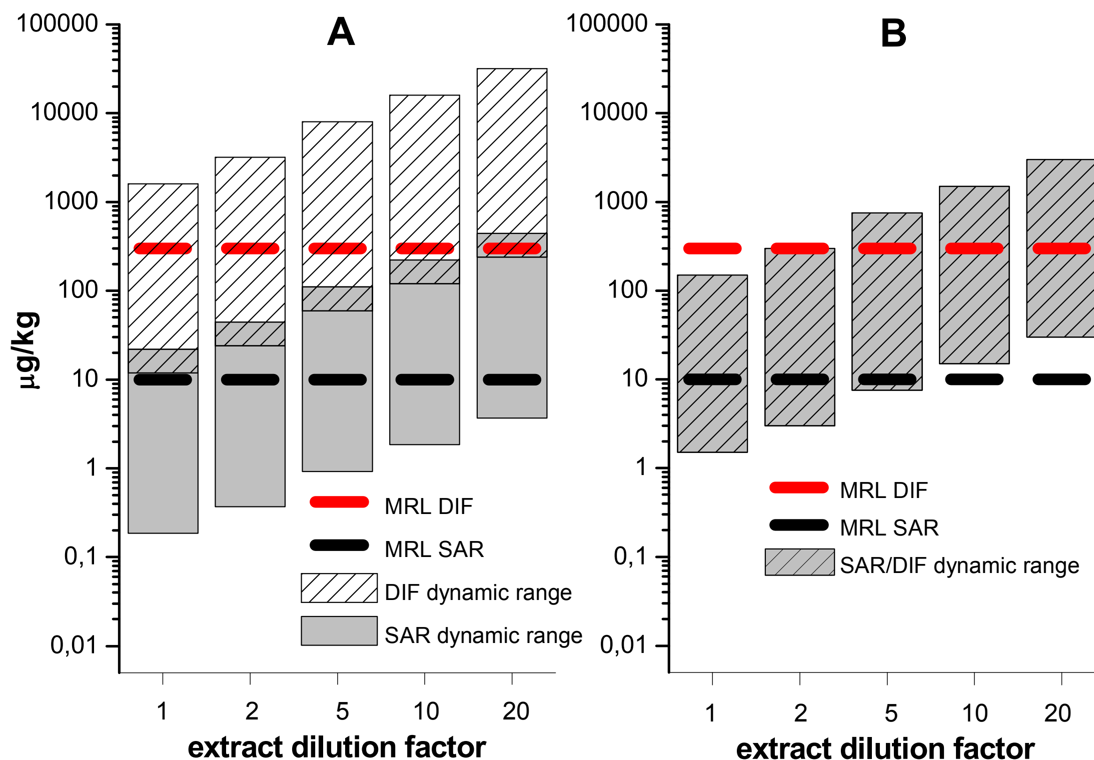

Figure 5

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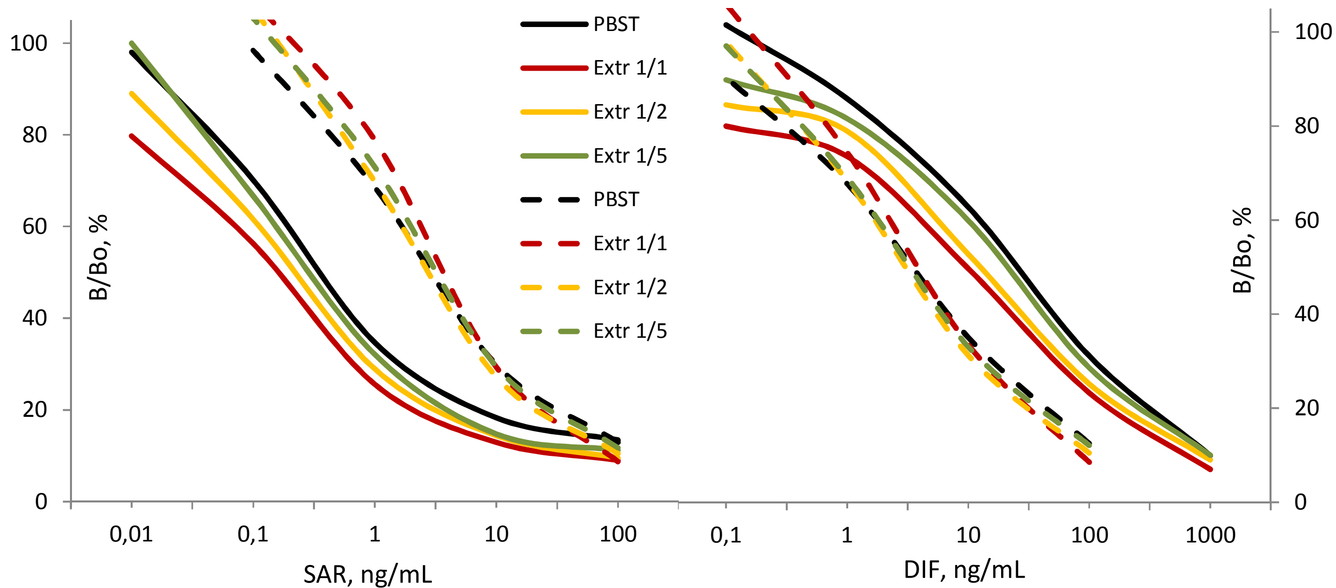

Figure 6

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