Korean J. Food Sci. An.
Vol. 33, No. 2, pp. 198~204(2013)
DOI http://dx.do.org/10.5851/kosfa.2013.33.2.198

ARTICLE

The Application of Parallux™ System for Multi-Detection of (Fluoro)quinolone Class Antibiotics Residues in Raw Bovine Milk

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Abstract

This study aimed to apply the Parallux system to detect (fluoro)quinone antibiotics residues in raw bovine milk. The immunogen enabled the generation of a specific antiserum with a titer of 1/40,000. The Parallux™ kit using the antibody displayed IC_{50} value of 10 to 150 ppb for (fluoro)quinolone antibiotics. Parallux™ kit was also sensitive for the detection of incurred (fluoro)quinolone at Korean Maximum Residual Levels in raw bovine milk as the result of dose response test. Cross reactivities of the antibody with the common (fluoro)quinolones were determined to be norfloxacin, 100%; enrofloxacin, 100%; ciprofloxacin, 100%; danofloxacin, 100%; nalidixic acid, 40%. Lower detection limit (LOD) values of the Parallux™ kit in raw bovine milk were determined to be norfloxacin, 4 ppb; enrofloxacin, 5 ppb; danofloxacin, 5 ppb; ciprofloxacin, 5 ppb and nalidixic acid, 10 ppb. The Parallux™ kit was run 8 times with five different concentrations of norfloxacin to determine the coefficient of variation (CV, %) of intra-assay, which was between 2.7% and 11.8%. To confirm the precision among kit batches for the inter-assay, five different batch kits were tested with 2 different concentration of norfloxacin. The CVs of the inter assay were 4.2% at 50 ppb, and 7.2% at 10 ppb norfloxacin, respectively.

Key words: (fluoro)quinolone, Parallax™ system, ELISA, immunoassay, cross activity

Introduction

Quinolones are often used in livestock in case of pulmonary, urinary and digestive infections. Among the quinolones derivatives, (flouro)quinolones are subgroup of widely used quinines in agriculture and aquaculture (Fierens *et al*., 2000). (Fluoro)quinolones are widely used for the prevention and treatment of various diseases in animal husbandry and aquaculture, as well as in human (Hermandez *et al*., 2002). The quinolones and (fluoro) quinolones comprise a series of synthetic antibacterial agents with great clinical relevance and are derived from nalidixic acid, a derivative introduced for the treatment of urinary tract infections (Fierens *et al*., 2000). The original quinolones were mainly used for treating Gram negative bacterial infections. But they were later modified in order to become effective agents for treating both Gram positive and negative infections (Hermandez *et al*., 2011). They have been reported the potential of these compound residues in foodstuffs of animal origin (Yorke and Froc, 2000). (Fluoro) quinolones are among the most commonly prescribed antimicrobials and are an important risk factor for colonization and infection with (fluoro)quinolone-resistant Gram-negative bacilli and for *Clostridium difficile* infection (Werner *et al*., 2011). They prescribed anti-microbial agent worldwide target bacterial type II topoisomerase gyrase (Boyd *et al.,* 2009). These compounds act directly on bacterial DNA, thus residues of these drugs in animal tissues are an important issue. They act via inhibition of DNA-gyrase, abolishing its activity by interfering with the DNA rejoining reaction. (Gigosos *et al.,* 2000).

In parallel to the exposure to low levels of these compounds, an increase of resistant human pathogens constituting a public health hazard, primarily through the increased risk of treatment failures, has been observed. Therefore, the chemical variety of (fluoro)quinolones and the possibility of trace level residues in food made it nec-

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essary to develop a sensitive multi-residue screening method (Fierens *et al*., 2000). Norfloxacin is one of the first modern (fluoro)quinone antimicrobial agents featuring a fluorine atom in position 7 of the quinine nucleus (Chang *et al*., 2007).

Traditional approaches for the detection of antibiotic residues include microbial inhibition tests, immunoassays, and chromatographic method. Most determination technique is based on a liquid chromatography (LC) separation. Gas chromatography (GC) and high-performance thin-layer chromatography (HPLC) are used in only a small proportion of the methods reviewed. Some analytical techniques, such as luminescence or immunological methods, are used without prior chromatographic separation (Gigosos *et al*., 2000). Only immunochemical methods based on enzyme linked immunosorbent assay (ELISA) are reported (Bucknall *et al.,* 2003; Holtzapple *et al*., 1997; Snitkoff *et al.,* 1998; Watanabe *et al*., 2002). These methods are difficult to prepare samples and take a long time to obtain results.

Therefore, this study carried out to develop of generic antibody against quinolone antibiotics and the screening method for the multi-determination of quinolone antibiotics in raw bovine milk using the Parallux™ system. Parallux™ (a solid-phase fluorescence immunoassay) is an analytical system for detection of antibiotics residues in raw bovine milk. To detect (fluoro)quinolone antibiotics in raw bovine milk using the Parallux™ system, polyclonal anti-sera against norfloxacin were generated in rabbits using a norfloxacin-bovine serum albumin (BSA) conjugate as an immunogen.

Materials and Methods

Materials

Norfloxacin, 1-(3-dimethlaminopropyl)-3-ethyl carbodiimide (EDC), BSA, Freund's Adjuvant complete, Freund's Adjuvant incomplete, Protein G Sepharose 4 fast flow and Sephadex G-25 were purchased from Sigma-Aldrich(USA); N,N,N,N-tetramethyl benzidine substrate (TMB) and stop solution were from KPL (USA); Anti-rabbit Immunoglobulin G-Horseradish Peroxidase (IgG-HRP) was from Santa Cruz Biotechnology(USA); Monofunctional NHS-ester Cy5 dye was from GE Healthcare Life Sciences(USA); Protein assay kit and Keyhole limpet hemocyanin (KLH) were from Pierce; Rabbits were from MJ Ltd. (Korea); 96-well microplate for ELISA was from Greiner bio-one (Germany).

Antibiotics conjugation

Norfloxacin-BSA immunogen

The method of conjugation was based on a variation of the carbodiimide, a zero-length cross linker, reaction described by Preston and Hencin (1986). One milligram EDC was added to 2 mg Norfloxacin in MES buffer and the mixture was added to 2 mg BSA in MES buffer. The conjugation mixture was stirred for 2 h at room temperature. Conjugate was separated from small molecular weight reactants by gel filtration chromatography on a column of Sephadex G-25 equilibrated with 20 mM phosphate buffered saline (PBS).

Norfloxacin-KLH conjugates

The method was based on another variation of the carbodiimide. Two milligram norfloxacin, 4 mg EDC and 8 mg sulfo-NHS in 0.5 mL DMF were stirred for 30 min at room temperature. After incubation, the reactants were added to 2 mg BSA in MES buffer. Conjugate was purified by gel filtration chromatography on a column of Sephadex G-25 equilibrated with 20 mM PBS.

Generation of polyclonal antibody

Polyclonal antisera were obtained from rabbits. Rabbit anti-sera to norfloxacin were prepared by intramuscular inoculation of rabbits with 1 mL norfloxacin-BSA emulsified in an equal volume of Freund's Adjuvant complete. After 2 wk, rabbits were given a booster injection of antibiotics conjugate mixed Freund's Adjuvant incomplete. Two weeks later after booster, rabbits were bled and the anti-sera were separated by storing the blood samples overnight at 4°C followed by centrifugation at 2,500 rpm at 4°C for 10 min. Separated sera were stored at -80°C until use.

Titration

Norfloxacin-KLH conjugate was diluted at concentrated of 10 μg/mL in coating buffer (50 mM carbonate buffer, pH 9.6). Hundred microlitre of diluted conjugate was added to each well of 96-well microplate. After incubation for 2 h at 37°C, the plate was washed 3 times with PBS containing 0.05% Tween 20. In 200 μL of blocking solution, 1% skim milk was added to each well and incubated for 1 h at 37°C. Anti-sera were diluted from 1,000-fold to 256,000-fold in PBS containing 0.3% skim milk and applied to each well. The anti-sera were incubated for 1 h at 37°C and 3 times washed with PBS containing 0.05% Tween 20. Diluted anti-rabbit IgG-HRP was added

to antigen bound anti-sera. After 1 h incubation, substrate was added and incubated for 20 min at room temperature. The reaction was stopped by stop solution. The absorbances were obtained at 450 nm.

Indirect antigen competitive ELISA assay

Recently, enzyme-linked immunosorbent assays (ELISAs) have been developed due to the increasing interested in the identification or quantification of various drug residues in biological sample (Duan and Yuan, 2001). (Fluoro)quinolones-BSA immunogen was made using a method based on the reaction. To obtain the inhibition percent of several concentrations of norfloxacin, norfloxacin-KLH conjugate was coated into microplate and blocked by 1% skim milk solution. During incubation, norfloxacin stock solution was prepared in 0.1 N NaOH. The stock solution of antibiotics was diluted in PBS containing 0.3% skim milk. Purified antibodies were diluted to concentration of 1 μg/mL in PBS containing 0.3% skim milk. Diluted antibody and each concentration of antibiotics added to each well simultaneously. Diluted anti-rabbit IgG-HRP was added to antigen bound antibodies. After 1 h incubation, substrate was added and incubated for 20 min at room temperature. The reaction was stopped by stop solution. The absorbances were obtained at 450 nm. Percents of inhibition were obtained by absorbance of each concentration of antibiotics divided by absorbance of antibody only.

Purification of anti-norfloxacin antibody

Anti-sera were diluted to be 1:5 with binding buffer and filtrated by 0.45 μm syringe filter. The anti-sera were loaded onto Protein G Sepharose 4B Fast Flow column equilibrated with IgG binding buffer. After washing with binding buffer, the bound immunoglobulin was eluted by elution buffer and collected peak. Purified antibodies in phosphate buffer were stored at -80°C until use.

Antibody conjugation with Fluorescence (Cy5)

To conjugate antibody with Cy5, the purified anti-norfloxacin in phosphate buffer was exchanged with 50 mM carbonate buffer, pH 9.2 and concentrated for 5 mg/mL of concentration. Cy5 solution in DMF was added to antibody solution and incubated for 1 hour at room temperature. After incubation, antibody conjugated Cy5 was purified by sephadex G-25 column equilibrated with 50 mM borate buffer, pH 7.9 and stored at -80°C until use.

Assessment of antibody sensitivity

The competitive Parallux™ system was used to determine the sensitivity of the polyclonal antibody generated. The Parallux system is a solid-phase fluorescence immunoassay (SPFIA)-based test, designed for milk analysis, which is very easy to perform and yields results within 5 min. Different types of test cartridges are available, each containing 4 capillary channels for detection of 1-4 different analytes, irrespective of cross-reactions. The test itself, including mixing of the sample with the antibody, immunological binding and reading, takes less than 5 min (Okerman *et al.,* 2003). The optimum antibody dispensed into tray and antibiotics conjugate coated into capillary were predetermined by titration results.

Assessment of antibody specificity

Cross-reactivity studies were carried out in supernatants of centrifuged homogenates spiked with (fluoro)quinolones (Bucknall *et al*., 2003). The percentage cross-reactivity values for other (fluoro)quinolone antibiotics were determined by comparing the concentration of analyte required to produce a B/Bo = 50%. IC_{50} was calculated as the concentration of analyze that caused a 50% reduction in binding of the fluorescence labeled antibody.

The cross-reactivity value for each drug was calculated as follows:

$$\% \text{ Cross-reactivity} = \frac{IC_{50} \text{ for norfloxacin}}{IC_{50} \text{for potential cross-reactant}} \times 100$$

Reproducibility

The Parallux™ assay is a competitive solid-phase fluorescence immunoassay intended for use as a rapid detection method for Penicillin residues in raw bovine milk. The results of the Parallux™ system are given as the S/C ratio of the sample inhibition value/cut-off inhibition value, the sample inhibition value = [1 − (sample signal/negative control signal) × 100.

The cut-off inhibition value is lot and capillary-specific. And it is included in the barcode that must be scanned before each test. The S/C ratio > 1.0 is recorded as positive by the processor (Kim *et al*., 2006).

Intra-assay

The Parallux™ system test assay was run 8 times with five different concentrations of norfloxacin spiked in raw bovine milk.

Inter-assay

Batch-to- batch variability of kits of norfloxacin were

assayed. Two raw bovine milk samples containing different concentrations of norfloxacin were tested with five different batch kits. Each test was run 8 times.

Results and Discussion

Antibody titers

All of the rabbits immunized with the norfloxacin-BSA conjugate produced immune responses to the antibiotics portion of immunogen. In order to quantitate the antibody response of the rabbits to norfloxcin, we performed ELISA tests as described above using norfloxacin-BSA as the antigen bound to the microtiter plates.

The titers of anti-sera against norfloxacin were confirmed to determine most optimum antibody for parallux™ system by indirect ELISA. Produced four anti-sera were diluted from 1,000 to 256,000-fold and applied to microwells coated norfloxacin conjugated KLH. The absorbance of each anti-serum was obtained as a described method (Fig. 1).

The antibody from rabbit 07-1 was the highest titer by ELISA for Parallux™ kit. The antibody prepared with norfloxacin involved mixed anhydric-mediated conjugation through the carboxyl group on the quinolone moiety. The antibodies from rabbit 09-1, 2 and 07-3 were lower titer. And also it showed that absorbance value of antibody from rabbit 07-1 at 450 nm was stable from 1,000 to 8,000-fold dilution and thereafter it decreased.

The norfloxacin from 07-1 showed non-specific binding in tissue homogenates.

Assessment of antibody sensitivity

IC_{50s} for (fluoro)quinolone antibiotics were calculated for antibody sensitivity using Parallux™ system (Table 1).

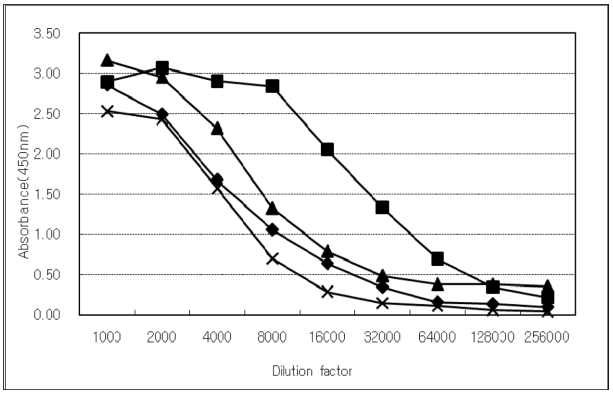

Fig. 1. Determination of titres of anti-sera against norfloxacin (07-1, 07-3, 09-1, 09-2) by indirect ELISA. ■: 07-1, ◆: 07-3, ▲: 09-1, ×: 09-2 serum respectively. Points are means of two replicates.

Table 1. IC_{50} value for (fluro)quinolones by Parallux™ kit

(Fluro)quinolone class	IC_{50}(ppb)
Norfloxacin	10
Enrofloxacin	10
Danofloxacin	10
Ciprofloxacin	10
Orifloxacin	25
Lomefloxacin	25
Enoxacin	25
Spafloxacin	50
Ofloxacin	50
Nalidixic acid	25
Flumequine	100
Levofloxacin	150
Oxolinic acid	150

The results varied widely with a range of values between 10 and 150 ppb. The antibiotics of norfloxcin, enrofloxacin, danofloxacin and ciprofloxacin were more sensitive than other (fluoro)quinolones. The value of IC_{50s} of orifloxacin, lomefloxacin, enoxacin and nalidixic acid were 25 ppb. And the spafloxacin and ofloxacin were 50 ppb, whereas the value of flumequine was 100 ppb. But levofloxacin and oxolinic acid were less sensitive than any other (fluoro)quinolones.

The antibody of (fluoro)quinolone class was also sufficiently sensitive for detection of incurred residues at Korean Maximum Residual Levels (MRL)'s (ppb).

Several (fluoro)quinolone antibiotics were spiked into raw bovine milk to determine dose response using development parallux™ kit. It showed also that the values of % inhibition of norfloxacin, enrofloxacin, danofloxacin, and ciprofloxacin were increased with the antibiotic concentrations. The results of % inhibition by (1 − sample signal/negative control) × 100 were 71; norfloxacin, 74; enrofloxacin, 70; danofloxacin, 69; ciprofloxacin and 53; nalidixic acid at 25 ppb of Korean MRL, respectively (Fig. 2).

The % inhibition of nalidixic acid was significantly

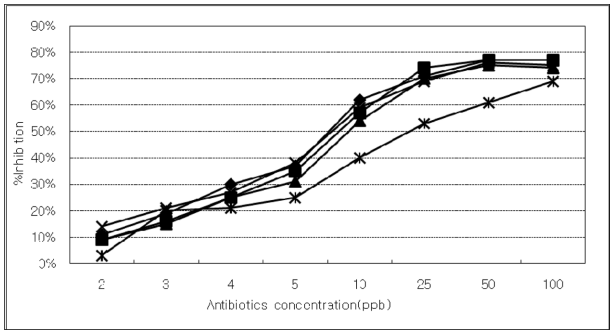

Fig. 2. Dose response curve for (fluoro)quinolone antibiotics spiked into raw bovine milk by Parallux™ kit. ◆: norfloxacin, ■: enrofloxacin, ▲: danofloxacin, ×: ciprofloxacin, *: nalidixic acid. Points are means of two replicates.

Table 2. Dose response values of Parallux™ kit

Conc. (ppb)	Norfloxacin			Enrofloxacin			Danofloxacin			Ciprofloxacin			Nalidixic acid		
	I (%)	S/C ratio	P/N[1)]	I (%)	S/C ratio	P/N	I (%)	S/C ratio	P/N	I (%)	S/C ratio	P/N	I (%)	S/C ratio	P/N
1	-1	-0.03	N	-5	-0.17	N	-1	-0.03	N	3	0.10	N	-2	-0.07	N
2	11	0.37	N	9	0.30	N	9	0.30	N	14	0.47	N	3	0.10	N
3	19	0.63	N	16	0.53	N	15	0.50	N	21	0.70	N	20	0.67	N
4	30	1.00	P	25	0.83	N	25	0.83	N	27	0.90	N	21	0.70	N
5	37	1.23	P	35	1.17	P	31	1.03	P	38	1.27	P	25	0.83	N
10	62	2.07	P	57	1.90	P	54	1.80	P	59	1.97	P	40	1.33	P
25	71	2.37	P	74	2.47	P	70	2.33	P	69	2.30	P	53	1.77	P
50	77	2.57	P	77	2.57	P	75	2.50	P	76	2.53	P	61	2.03	P
100	77	2.57	P	77	2.57	P	74	2.47	P	75	2.50	P	69	2.30	P

[1)]Positive/Negative

lower than other (fluoro)quinolones ($p<0.05$).

The S/C ratio is obtained by sample % inhibition/cut-off % inhibition. The cut-off inhibition value is included in the bar code that must be scanned before each test. The result of the parallux™ system is recorded as positive when S/C ratio >1.0. The detection limit was 4 ppb for norfloxacin, 5 ppb for enrofloxacin, 5 ppb for danofloxacin, 5 ppb for ciprofloxacin, and 10 ppb for nalidixic acid, respectively (Table 2).

The cut-off % inhibition of norfloxacin was 30%, and 35% for enrofloxacin, 31% for danofloxacin, 38% for ciprofloxacin, and 40% for nalidixic acid. The S/C ratio >1.0 is recorded as positive by the processor. S/C ratio of sample inhibition value to cut-off inhibition value was employed as an index to determine the antibiotic residue in milk (Bucknall *et al*., 2003).

Assessment of antibody specificity by Parallux™ kit assay

The percentage cross-reactivity of antibody for (fluoro)quinolone class antibiotics was determined by Parallux™ kit using the antibody against norfloxacin etc. The major cross-linking reaction for the immunogen was taken to be the production, which then reacted with the secondary amino group of the (fluoro)quinolone piperazinyl ring. The lack of specificity for all the (fluoro)quinolones tested in the generic ELISA confirms this, as cross-reactivity occurs with flumequin and nalidixic acid. The antibiotics of norfloxacin, enrofloxacin, danofloxacin and ciprofloxacin were 100% and they showed higher cross-reactivity than the other (fluoro)quinolone antibiotics (Table 3). And the percentage cross- reactivities of the antibody of orifloxacin, lomefloxacin, enoxacin and nalidic acid were 40%. But the percentage cross-reactivities of spafloxacin and ofloxacin were 20%, and the cross-reactivity of flumequine was 10%. The percentage cross-reactivities of levofloxacin and oxolinic acid appeared the lowest value.

Buckman *et al.* (2003) reported that a cross-reaction relative to norfloxacin (100%) of more than 15%. Duan and Yuan (2001) reported that enrofloxacin and norfloxacin had 69.8% and 44.6% cross-reactivity, respectively.

Otherwise, the other class antibiotics showed no cross reactivity. For examples, antibiotics of penicillin G, sulfamethazine, cefalexin, streptomycin, tetracycline, neomycin, spiramycin, lincomycin and chloramphenicol had no cross-reactivity.

The ability to obtain high-affinity antisera is critical for an ELISA procedure. Our experiment indicated the high affinity for (fluoro)quinolone. The result showed that the protein BSA and KLH were excellent carriers. We tried to measure how change antibody in dose during the overall protocol and the interval between every injection affected the immunization effect.

Assessment of reproducibility

The S/C ratio of the sample inhibition value to the cut-off inhibition value was calculated for each test sample run.

Table 3. Percentage cross-reactivity values of (fluro)quinolone class by Parallux™ kit in raw bovine milk

(Fluoro) quinolone class	cross-reactivity (%)
Norfloxacin	100
Enrofloxacin	100
Danofloxacin	100
Ciprofloxacin	100
Orifloxacin	40
Lomefloxacin	40
Enoxacin	40
Spafloxacin	20
Ofloxacin	20
Nalidixic acid	40
Flumequine	10
Levofloxacin	6.7
Oxolinic acid	6.7

Table 4. Intra-assay values of Norfloxacin concentration

Norfloxacin concentration (ppb)	Repetition								Mean (S/C ratio)	SD	%CV
	1	2	3	4	5	6	7	8			
100	1.74	1.74	1.78	1.77	1.79	1.85	1.87	1.84	1.80	0.05	2.7%
50	1.74	1.81	1.72	1.84	1.79	1.82	1.83	1.81	1.80	0.04	2.4%
25	1.66	1.64	1.62	1.65	1.64	1.6	1.7	1.64	1.64	0.03	1.8%
10	1.44	1.41	1.40	1.41	1.48	1.43	1.39	1.54	1.44	0.05	3.5%
5	0.87	0.96	0.74	0.96	0.71	0.78	0.75	0.82	0.82	0.10	11.8%

S/C ratio is means of eight replicates.

The cut-off inhibition value is lot-and capillary-specific.

Intra-assay values

The Parallux kit was run through 8 times with five different concentrations of norfloxacin to determine the CV (%) ratio for intra-assay.

Intra-assay values of norfloxacin summarized S/C ratios in the concentration of 5-100 ppb, the CV ratio of intra-assay was between 2.7% and 11.8% (Table 4).

It showed that the S/C ratio of 5 different concentrations (5, 10, 25, 50, and 100 ppb) of norfloxacin spiked in raw bovine milk at 8 times. The mean of S/C ratio of norfloxacin increased with the concentration from 5 ppb to 50 ppb of concentration. The percentage of CV value in norfloxacin at the concentration of 100 ppb was 2.7%. There were no significant differences in percentage of CV value between 100 ppb and 50 ppb concentration of norfloxacin. But in concentration of 50 ppb, the S/C ratio was higher than the 25 ppb and 10 ppb of norfloxacin. In concentration of 10 ppb of norfloxacin, the percentage CV value was 3.5%, and in 5 ppb was 11.8% of CV. The percentages of CV of norfloxacin tend to decrease following to increasing concentration of norfloxacin. By Bucknall *et al* (2003), the detection limits using the ELISA were to 10 ppb for chicken liver and muscle, and 1 ppb for cattle milk. The mean recovery values were 77.3-96.0% for chicken liver, 72.4-92.0% for chicken muscle and ca. 10 ppb for cattle milk (Watanabe *et al*., 2002).

Table 5. Inter-assay values of Norfloxacin concentration in the kit batch number

Kit batch No.	Norfloxacin concentration (ppb)	
	50	10
1	1.80	1.23
2	1.89	1.21
3	1.73	1.43
4	1.96	1.30
5	1.85	1.17
Mean (S/C ratio)	1.85	1.27
SD	0.08	0.09
%CV	4.2%	7.2%

S/C ratio is means of five replicates

Inter-assay values

To confirm the precision among kit batches, two raw bovine milk samples containing of 50 and 10 ppb of norfloxacin were tested with 5 different batch kits. The results of inter-assay for kit batch-to-batch variability of kits for norfloxacin summarized Table 5.

Inter-assay values of norfloxacin in 50 ppb concentration were 4.2% and 7.2% in 10 ppb of norfloxacin, respectively.

Bucknall *et al.* (2003) reported that intra-assay performance data on norfloxacin showed 2.91% and the inter-assay was 7.46%. They described that the antibodies provide the means for a rapid screening procedures, in order to detect a range of (fluoro)quinolones by immunoassay, more suitable for large numbers of samples than existing physico-chemical methods. Duan and Yuan (2001) reported that the test of ciprofloxacin in coefficients of variation for the intra-assay was the range of 3.7-6.3%, whereas those of the inter-assay were 6.9-9.2%.

Our research demonstrated that the norfloxacin antibody plays a major role in (fluoro)quinonlone residue detection. We used norfloxacin-KLH and norfloxacin-BSA conjugate as immunogen and coating conjugates respectively. The Parallux™ kit based on antibody-antigen format using fluorescence as the label was developed. It is a modified method of ELISA for the detection of norfloxacin in biological sample. It was developed a specific and convenient ELISA procedure for the screening of norfloxacin residues in animal edible tissues.

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(Received 2013.1.21/Revised 2013.3.13/Accepted 2013.3.20)