Trakia Journal of Sciences, Vol. 8, No. 1, pp 64-69, 2010
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Available online at:
http://www.uni-sz.bg

ISSN 1313-7050 (print)
ISSN 1313-3551 (online)

Original Contribution

DEVELOPING AND VALIDATION OF METHOD FOR DETECTION OF QUINOLONE RESIDUES IN POULTRY MEAT

N. Stoilova*, M. Petkova

CLVCE (Central Laboratory of Veterinary Control & Ecology), Department VMP-Analysis, Sofia, Bulgaria

ABSTRACT
The objective of this study was to present simple, rapid and applicable HPLC-method with fluorescence detection for determination of quinolone-residues in chicken muscle.
Presence of antibacterial residia gives reise to risk for health, because of chance by disease, allergy or cancer effect.
The residues of ciprofloxacine, enrofloxacine, norfloxacine, danfloxacine, difloxacine, sarafloxacine, oxolinic acid, nalidixic acid and flumequine are extracted with acetonitril.
The extract was cleaned with an SPE - procedure with HLB cartridges.
Elute was analyzed via Zorbax Eclipse XDB liquid chromatography column.
It was used gradient program for eluting and time table program because of the different wave lengths of excitation and emission.

Key words: quinolone, extraction, SPE, HLB-cartridges, detection, validation

INTRODUCTION

The quinolones are a group of antibiotics which are used in human and veterinary medicine.
They have high activity against gram-positive and gram-negative bacteria. Their activity is shown by inhibiting of DNA-guirase in a bacterial cell [1], thus destroying it.
The use of quinolone-antibiotics has resulted in the presence in food with animal origin.
The European Union has established maximum residue limits for those types of chemicals in different type of animal tissues and food with animal origin. The aim is to protect the human health against potential harmful residues [2, 3].
That make necessary to develop and validate sensitive and selective multi-residue screening method for identification and quantification of those type antibiotics.
There are articles about multi-residue analysis of quinolones with HPLC, LC/MS, GC, CE including SPE, LLE and some other analytical techniques for extraction, clean-up, preconcentration before instrumental analysis [2]. But there are few articles which represent methods for extraction and determination of quinilones in chicken tissues [3, 4, 5].
The aim of this work is to present rapid, easy, selective method for extraction and simultaneous determination of several quinolones, representatives of those types of antibiotics in chicken-tissues: norfloxacin, ciprofloxacin, enrofloxacin, danofloxacine, difloxacine, sarafloxacine, oxolinic acid, nalidixic acid, flumequine.
The present work describes developing and validation of method for determination, identification and quantification of these synthetic antibiotics in a single analysis step.

*** Correspondence to**: *Nadezhda Stoilova*
CLVCE (Central Laboratory of Veterinary Control & Ecology), Department VMP-Analysis
"Iskarsko shousse" str. 5, Sofia 1528, Bulgaria,
e-mail: nadqstoilova@abv.bg
phone: +35929730362

MATERIALS AND METHODS

Chemicals and reagents:
Acetonitril p.a.;
Methanol p.a.
Acetonitril and methanol – high purity, super gradient Labscan;
Formic acid ≥ 99% Merck Germany;
Sodium Hydroxide p.a.; Deionized water was prepared from ELGA system;

HLB - cartridges (OASIS Waters).
Standards were purchased from Riedel de Haen, Vetranal:
ciprofloxacine, enrofloxacine, norfloxacine, danfloxacine, difloxacine, sarafloxacine, oxolinic acid, nalidixic acid and flumequine

Apparatus
Instrumental analysis was provided with LC System Agilent 1100 Series with degasser, quaternary pump, auto sampler, column heater, fluorescence detector, Chemstation software [6].
The LC- Column and pre-column which are used are Zorbax Eclipse XDB C18.
Mobile phase consist of 0.1%HCOOH (A), Acetonitril (B), Methanol (C);
Gradient eluting program;
Time-table program with λex=280nm, λem=450nm; λex=312nm, λem=366nm
Temperature is 50 °C and injection volume 25µl [6]
Solutions prepared for HPLC were passed trough a 0.45µm membrane filter before to use (Millipore Waters).

Standard solutions
All of the stock standard solutions were prepared by dissolving 5mg of each substance in to volumetric flask in 50 µl 3M Sodium Hydroxide and methanol.
These solutions were stored in refrigerator for couple of months.
The working solutions for HPLC were prepared daily from the stock solutions in mobile phase.

Sample preparation procedures
The sample preparation stage in detail is described in our previous work [6]. It includes extraction of analyte from the chicken muscle with 10ml acetonitril; centrifugation and evaporation of organic layer under stream of nitrogen. Re-dissolve the dry residue in to 5 ml 0.02M Ammonium Acetate with pH=9.0. After this extraction, analytes are pre-cleaned and pre-concentrated with SPE-procedure with HLB cartridge. Activation and equilibration of the sorbent with volumes from methanol and 0.02M Ammonium Acetate with pH=3.0. After that- load the sample, washing impurities with a volume from de-ionized water, dry and elute analytes with 10ml 0.2% HCOOH in to acetonitril. Evaporate the sample to dryness and dissolve it in to 1ml mobile phase.
Before extraction the samples were contaminated with standards. The standards are added at the desired level; the samples were homogenized and stored in refrigerator camera. Poultry muscles are from samples, according National Monitoring Program for Control of the Contaminants, which came in to the CLVCE. We keep samples according requirements, which are described in to the SOP.
The chromatographic conditions and extraction procedures were determinate for those types of quinolones during method-development about determination of quinolone residues in fish tissues [6]. Determination of the right sample preparation steps: extraction from matrix with organic solvent, concentration by evaporation, cleaning procedures and centrifugation.
According Commission Decision 657/2002/EU [9] the validation steps include: preparation of the spiked chicken-muscles samples at different levels, homogenization of the contaminated samples and storing them in refrigerator at -20°C.

RESULTS AND DISCUSSION

The structure of quinolones includes 1-substituted-1.4- dihydro-4-oxopyridine-3-carboxilic part with aromatics and/or hetero-aromatic ring [7]. It is well known that this group of chemicals has a difference in pKa values between the acidic (oxolinic acid, nalidixic acid, flumequine) and amphoteric (norfloxacin, ciprofloxacin, enrofloxacin, danofloxacine, difloxacine, sarafloxacine) molecules of the quinolones [8].

In this case we use an organic solvent for extraction of analytes. We made extraction more easy for application, because we use the possibilities to take the compounds from the matrix (chicken, pheasant and duck muscle), on the base time to contact between analytes and organic solvent and the big difference between the sample weight (1g) and the volume from the solvent (acetonitril 10ml), which we use. By this way we make the procedure less harmful and cost effective.
We use a time-table program because of the difference in the wave-lengths for excitation and emission for amphoteric and acidic quinolones respectively.

Zorbax-Eclipse XDB C18 liquid-chromatography column was selected because of double end-capping of the silanols. That reduces the interaction between the silanol groups from stationary phase of the column and quinolone-molecules.

The preparation of the samples for instrumental analysis was performed according **table 1.**

Determination of the validations parameters: concentration range, selectivity and sensitivity (with real blank samples and spiked samples), linearity (with standard solutions and spiked samples in the same concentration range as the standard solutions), accuracy, repeatability, reproducibility, CC_{α} (Decission limit), CC_{β} (Detection capability).

Each sample was injected in triplicate in to the chromatographic system **fig.1 A, B**:

fig.1 (A) Chromatogram of standard solution of quinolones - level 0.5MRL;

fig.1 (B) Chromatogram of spiked samples (chicken muscle) - level 0.5MRL.

The results for each one of the determinate quinolone-residues are summarized and represented in to the **table2** (Validation characteristics of the method).

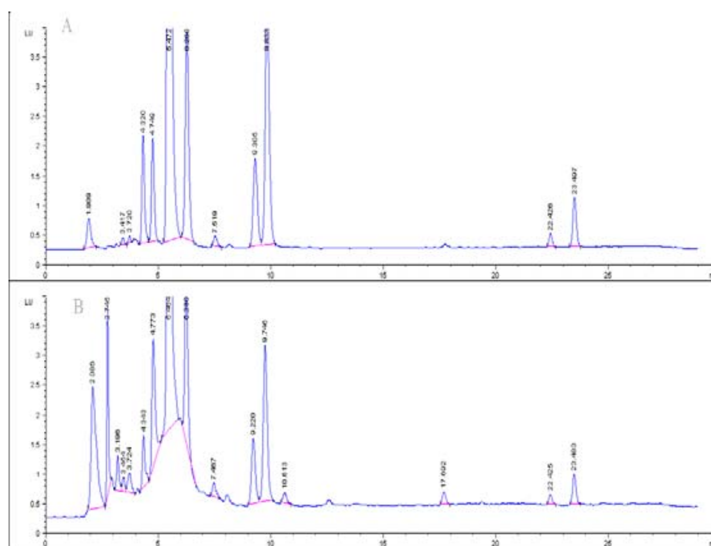

Fig.1 (A) Chromatograms of standard solution of quinolones - level 0.5MRL;
(B) Chromatograms of spiked samples (chicken muscle) - level 0.5MRL.

CONCLUSIONS

It was develop and validate liquid chromatography method with fluorescence detection for simultaneous determination of quinolone-residues in chicken muscle tissues.

Higher analytical recovery can be obtained for the quinolones by increasing the time of contact between tissue and organic solvent during extraction. There are used modern analytical techniques on the sample-preparation stage as SPE with HLB cartridges. Because of the difference in molecule structure and pKa values of the quinolone, there is a good to use HLB-cartridges for cleaning and pre-concentration of the quinolone before instrumental analyses step [8, 6].

The separation and detection of the quinolone-residues is achieving using RP/HPLC with fluorescence detection.

The method was validated for each one of the quinolone according Commission Decision 657/2002/EU.

The results which are obtained are satisfactory and lower than the MRL.

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Table1: *Implementation plan for the sample preparation in different days: Index above means the type of matrixes (1 –chicken muscle; 2-pheasant; 3-duck)*
Index below means a serial number of repetition

Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Ден 12**
0.5 MRL^{1}_{1}	2 MRL^{1}_{1}	0,5 MRL^{1}_{2}	2 MRL^{1}_{2}	0,5 MRL^{1}_{3}	2 MRL^{1}_{3}	0,5 MRL^{1}_{4}	2 MRL^{1}_{4}	0,5 MRL^{1}_{5}	2 MRL^{1}_{5}	0,5 MRL^{1}_{6}	2 MRL^{1}_{6}
0.5 MRL^{2}_{1}	2 MRL^{2}_{1}	0.5 MRL^{2}_{2}	2 MRL^{2}_{2}	0.5 MRL^{2}_{3}	2 MRL^{2}_{3}	0.5 MRL^{2}_{4}	2 MRL^{2}_{4}	0.5 MRL^{2}_{5}	2 MRL^{2}_{5}	0.5 MRL^{2}_{6}	2 MRL^{2}_{6}
0.5MRL^{3}_{1}	2 MRL^{3}_{1}	0.5MRL^{3}_{2}	2 MRL^{3}_{2}	0.5MRL^{3}_{3}	2 MRL^{3}_{3}	0.5MRL^{3}_{4}	2 MRL^{3}_{4}	0.5MRL^{3}_{5}	2 MRL^{3}_{5}	0.5MRL^{3}_{6}	2 MRL^{3}_{6}
MRL^{1}_{1}	2.5 MRL^{1}_{1}	MRL^{1}_{2}	2.5 MRL^{1}_{2}	MRL^{1}_{3}	2.5 MRL^{1}_{3}	MRL^{1}_{4}	2.5 MRL^{1}_{4}	MRL^{1}_{5}	2.5 MRL^{1}_{5}	MRL^{1}_{6}	2.5 MRL^{1}_{6}
MRL^{2}_{1}	2.5 MRL^{2}_{1}	MRL^{2}_{2}	2.5 MRL^{2}_{2}	MRL^{2}_{3}	2.5 MRL^{2}_{3}	MRL^{2}_{4}	2.5 MRL^{2}_{4}	MRL^{2}_{5}	2.5 MRL^{2}_{5}	MRL^{2}_{6}	2.5 MRL^{2}_{6}
MRL^{3}_{1}	2.5 MRL^{3}_{1}	MRL^{3}_{2}	2.5 MRL^{3}_{2}	MRL^{3}_{3}	2.5 MRL^{3}_{3}	MRL^{3}_{4}	2.5 MRL^{3}_{4}	MRL^{3}_{5}	2.5 MRL^{3}_{5}	MRL^{3}_{6}	2.5 MRL^{3}_{6}
1.5 MRL^{1}_{1}	$Blank^{1}_{1}$	1.5 MRL^{1}_{2}	$Blank^{1}_{2}$	1.5 MRL^{1}_{3}	$Blank^{1}_{3}$	1.5 MRL^{1}_{4}	$Blank^{1}_{4}$	1.5 MRL^{1}_{5}	$Blank^{1}_{5}$	1.5 MRL^{1}_{6}	$Blank^{1}_{6}$
1.5 MRL^{2}_{1}	$Blank^{2}_{1}$	1.5 MRL^{2}_{2}	$Blank^{2}_{2}$	1.5 MRL^{2}_{3}	$Blank^{2}_{3}$	1.5 MRL^{2}_{4}	$Blank^{2}_{4}$	1.5 MRL^{2}_{5}	$Blank^{2}_{5}$	1.5 MRL^{2}_{6}	$Blank^{2}_{6}$
1.5MRL^{3}_{1}	$Blank^{3}_{1}$	1.5MRL^{3}_{2}	$Blank^{3}_{2}$	1.5MRL^{3}_{3}	$Blank^{3}_{3}$	1.5MRL^{3}_{4}	$Blank^{3}_{4}$	1.5MRL^{3}_{5}	$Blank^{3}_{5}$	1.5MRL^{3}_{6}	$Blank^{3}_{6}$

Day 13	**Day 14**	**Day 15**	**Day 16**	**Day 17**	**Day 18**	**Day 19**	**Day 20**
0.5 MRL^{1}_{7}	2 MRL^{1}_{7}	0.5 MRL^{1}_{8}	2 MRL^{1}_{8}	0.5 MRL^{1}_{9}	2 MRL^{1}_{9}	0.5 MRL^{1}_{10}	2 MRL^{1}_{10}
0.5 MRL^{2}_{7}	2 MRL^{2}_{7}	0.5 MRL^{2}_{8}	2 MRL^{2}_{8}	0.5 MRL^{2}_{9}	2 MRL^{2}_{9}	0.5 MRL^{2}_{10}	2 MRL^{2}_{10}
0.5MRL^{3}_{7}	2 MRL^{3}_{7}	0.5MRL^{3}_{8}	2 MRL^{3}_{8}	0.5MRL^{3}_{9}	2 MRL^{3}_{9}	0.5MRL^{3}_{10}	2 MRL^{3}_{10}
MRL^{1}_{7}	2.5 MRL^{1}_{7}	MRL^{1}_{8}	2.5 MRL^{1}_{8}	MRL^{1}_{9}	2.5 MRL^{1}_{9}	MRL^{1}_{10}	2.5 MRL^{1}_{10}
MRL^{2}_{7}	2.5 MRL^{2}_{7}	MRL^{2}_{8}	2.5 MRL^{2}_{8}	MRL^{2}_{9}	2.5 MRL^{2}_{9}	MRL^{2}_{10}	2.5 MRL^{2}_{10}
MRL^{3}_{7}	2.5 MRL^{3}_{7}	MRL^{3}_{8}	2.5 MRL^{3}_{8}	MRL^{3}_{9}	2.5 MRL^{3}_{9}	MRL^{3}_{10}	2.5 MRL^{3}_{10}
1.5 MRL^{1}_{7}	$Blank^{1}_{7}$	1.5 MRL^{1}_{8}	$Blank^{1}_{8}$	1.5 MRL^{1}_{9}	$Blank^{1}_{9}$	1.5 MRL^{1}_{10}	$Blank^{1}_{10}$
1.5 MRL^{2}_{7}	$Blank^{2}_{7}$	1.5 MRL^{2}_{8}	$Blank^{2}_{8}$	1.5 MRL^{2}_{9}	$Blank^{2}_{9}$	1.5 MRL^{2}_{10}	$Blank^{2}_{10}$
1.5MRL^{3}_{7}	$Blank^{3}_{7}$	1.5MRL^{3}_{8}	$Blank^{3}_{8}$	1.5MRL^{3}_{9}	$Blank^{3}_{9}$	1.5MRL^{3}_{10}	$Blank^{3}_{10}$

Table 2 *– Validation characteristics of the method:*

Com-pound	Linea-rity R^2	Workarea µg/kg	Analyti-cal recovery %(real)	Repeat-ability	Repro-ducibility %	Accuracy %	CCα (Decission limit) µg/kg	CCβ (Detec-tion capabili-ty) µg/kg
Nor-floxacin	0,9881	25÷125	89	4.79	23	19	27.56	27.94
Cipro-floxacin	0,9926	25÷125	70	9	23	19	50.38	50.75
Enro-floxacin	0,9894	25÷125	77	10.5	23	9	50.38	50.75
Dano-floxacin	0,9957	20÷100	75	8.3	23	13	40.31	40.75
Di-floxacin	0,9527	50÷250	48	12	24	47	100.39	100.79
Sara-floxacin	0,9909	5÷25	87	2.06	22	17	10.36	10.72
Oxolinic acid	0,9508	50÷250	123	27	23	14	100.38	100.75
Nalidixic acid	0,9916	25÷125	107	5.6	21	5.7	25.49	25.83
Flu-mequine	0,9951	40÷200	108	19	23	6.4	80.38	80.75

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