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Simultaneous determination of quinolones for veterinary use by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A selective method based on high-performance liquid chromatography with electrochemical detection (HPLC-ECD) has been developed to enable simultaneous determination of three fluoroquinolones (FQs), namely danofloxacin (DANO), difloxacin (DIFLO) and sarafloxacin (SARA). The fluoroquinolones are separated on a Novapack C-18 column and detected in a high sensitivity amperometric cell at a potential of +0.8 V. Solid-phase extraction was used for the extraction of the analytes in real samples. The range of concentration examined varied from 10 to 150 ng g^{-1} for danofloxacin, from 25 to 100 ng g^{-1} for sarafloxacin and from 50 to 315 ng g^{-1} for difloxacin, respectively. The method presents detection limits under 10 ng g^{-1} and recoveries around 90% for the three analytes have been obtained in the experiments with fortified samples. This HPLC-ECD approach can be useful in the routine analysis of antibacterial residues being less expensive and less complicated than other more powerful tools as hyphenated techniques.

1. Introduction

Fluoroquinolones (FQs) are antibacterials mainly used for the control of the urinary tract and respiratory infections. The use of quinolone in food-producing animals can generate microbial resistance and also, it is important to mention that this microbial resistance can be transferred to humans, thus, there is a hazard to human health. For this reason, the European Union has established maximum residue limits (MRL) for quinolone residues in animal tissues, and this is included in the Council Regulation 2377/90 [1]. Danofloxacin (DANO), difloxacin (DIFLO) and sarafloxacin (SARA) are FQs of veterinary use. Sarafloxacin was approved by the U.S. FDA for use in chicken, however, as now have been withdrawn from the market due to concerns about microbial resistance [2]. Nonetheless, it is still important to monitor for the possible presence of residues because it is the principal metabolite of difloxacin [3].

Ultra-performance liquid chromatography coupled with mass spectrometry (UPLC–MS) has been used for the determination of the three analytes, among others, in eels and urine [4,5]. On the other hand, capillary electrophoresis (CE) and generally high-performance liquid chromatography (HPLC) have been used for the simultaneous determination of quinolones. For instance, some methods that use CE coupled with mass spectrometry have been developed for the quantification of several FQs in milk [6] and chicken [7,8]. Likewise, HPLC with several types of detectors, such as, ultraviolet [9–12], fluorescence [13–16] and mass spectrometry [9,11,17–21] has been employed. Focusing on the methods which are developed for the analysis of chicken samples, it can be observed that fluorescence and mass spectrometry are the detection methods most employed.

The analysis of chicken tissue samples has been carried out by CE and HPLC. For instance, CE-MS/MS method has been used [7] to determine eight quinolones of veterinary use obtaining limits of detection (LODs) between 17 and 59 ng L^{-1}. The analytes were extracted from the chicken muscle samples by a pressurized liquid extraction method. Beltran et al. [8] developed a procedure for the determination of SARA, among others, in chicken samples based on a capillary zone electrophoresis method coupled with multivariate calibration methods due that there are a strongly overlapping of the peaks of SARA and another FQ. The detector used was a doped array.

On the other hand, since FQs have intrinsic fluorescence, several papers have been published on their analysis in edible tissues by means of HPLC methods with fluorescence detection. Zhao et al. [15] extracted the fluoroquinolones with phosphate-buffered saline solution and cleaned by solid-phase extraction (SPE). Limits of quantification were in the range 0.3–1.0 ng g^{-1}. Schneider et al. [16] extracted the FQs with a mixture of acetonitrile and 0.1 M citrate, 150 mM $MgCl_2$ at pH 5.0. Good recoveries were obtained with LODs comprising between 0.5 and 5 ng g^{-1}. Yorke *et al.* [22] used an acetonitrile basic solution for the extraction of FQs. Recently; a new spectrofluorimetric method [23] has been developed by our research group for the quantification of DANO and DIFLO in the presence of SARA as interference, in chicken tissue samples. The

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method is based on second-order multivariate calibration, applying parallel factor analysis, to the excitation–emission matrices of these compounds. To solve the high overlapping of the signals and the influence of matrix effects, the standard addition method was used. Both analytes could be analyzed individually and the binary mixture was resolved, with recoveries comprising between 88.7% and 106.6%.

HPLC coupled with mass spectrometry has been used for the confirmatory and quantitative analysis of FQs. On the one hand, Clemente et al. [9] used an experimental design in order to optimize the eluents for the SPE using Isolute Env+ cartridges. On the other hand Bailac et al. [10] developed an extraction methodology using Isolute Env+ cartridges and eluting the FQs with triflouroacetic acid (TFA) and acetonitrile.

Finally, a comprehensive review on the analysis of quinolone antibacterials was presented by Hernández-Arteseros et al. [24] which covered most of the methods described for the determination of quionolone residues in edible animal products up to 2002. The review includes a summary of the most relevant information about the analytical procedures.

To the best of our knowledge, no method has been described to date for determining those three fluoroquinolones in chicken muscle using high-performance liquid chromatography with electrochemical detection (HPLC-ECD). Since fluoroquinolones are electroactive compounds an analysis by HPLC that uses the electrochemical properties of fluoroquinolones is proposed. In this work, a method is developed which takes advantage of liquid chromatography and the selectivity and sensibility of the electrochemical detection technique for effective detection and quantification of three fluoroquinolones simultaneously in chicken muscle.

2. Experimental procedure

2.1. Chemicals and reagents

Stock 2.5×10^{-3} M solutions of danofloxacin (Fluka), sarafloxacin hydrochloride (Riedel de-Häen) and difloxacin hydrochloride (Fort Dodge Veterinaria S.A., Girona, Spain) were prepared in 50-mL volumetric flasks, by weighing the appropriate amount and dissolving in 0.5% aqueous acetic acid. These solutions were stored in dark bottles at 4 °C, remaining stable for at least 1 month. Working solutions were prepared by appropriate dilution of the stock solutions with Milli-Q water (Millipore, USA). Isolute Env+ cartridges (International Sorbent Technology, Hengoed, Mid Glamorgan, UK) were used for solid-phase extraction. Those cartridges are filled with a copolymer of hydroxyl-polystyrene and divinylbenzene, because of that they have a very strong non-polar (hydrophobic) phase and are extraordinarily appropriate for the extraction of high polar analytes which are not retained in C_8 or C_{18} phases. The pH was measured by a pH meter Crison PH25 (Barcelona, Spain). For all experiments analytical grade chemicals and solvents were used.

2.2. Apparatus

The chromatographic equipment is composed of a 420 two piston HPLC pump from Kontron Instruments, a 7125 Rheodyne sample injector equipped with a 20 μL loop, a Novapak C-18 column (3.9 mm × 150 mm, 60 Å, 4 μm) from Waters, a Coulochem II electrochemical detector equipped with a ESA model 5021 conditioning cell and a ESA model 5011 dual analytical cell protected by ESA filters containing 0.2 μm porous graphite filter elements. The high sensitive analytical cell contains, in series, two porous graphite working electrodes together with associated reference (Pd/H_2) and counter electrodes.

The working electrodes are a large surface area coulometric electrode and a high efficiency amperometric electrode, more than seven times as efficient as conventional amperometric electrodes (70% vs. 5–10% efficiency). The conditioning cell contains a single porous graphite coulometric electrode.

The data acquisition and treatment is controlled from an Intel Pentium II PC equipped with the PC Integration Pack software package CSW32 from DATAAPEX (Prague, Czech Republic).

2.3. HPLC operating parameters

The mobile phase was 0.05 M sodium perchlorate–2% acetic acid:acetonitrile (80:20, v/v). It was filtered through a 0.45 μm nylon membrane filter and degassed in ultrasonic bath before being used. The flow rate was adjusted to 0.60 mL/min and the system was equilibrated for at least 10 min prior to injection of the prepared sample or standard.

The conditioning cell was set at +0.4 V and the electrodes 1 and 2 of the analytical cell were set at +0.4 and +0.8 V, respectively. The selected sensitivity in the PC Integration Pack was 500 nA full scale (1 V).

2.4. Procedure for determination of fluoroquinolones in chicken tissue samples

Chicken tissue samples were lyophilized and defatted (by Soxhlet procedure) prior to isolation of fluoroquinoles. Later, 1 g of lyophilized chicken muscle was spiked with the appropriate amount of analytes and later was extracted twice (5 min) with 15 mL of 0.3% meta-phosphoric acid:acetonitrile (75:25, v/v) at pH 3, and the mixture was stirring magnetically. The solution was centrifuged at 4000 rpm for 10 min and the supernatant decanted was filtered through a 0.45 μm nylon filter. It was diluted with 35 mL of water and the resulting solution was passed through a SPE Isolute Env+ cartridge, which was previously conditioned with methanol, water and 50 mM phosphoric acid (pH 3). Afterwards, the cartridge was cleaned with 1 mL of water and the analytes were eluted with 2.5 mL of 2% trifluoroacetic acid:acetonitrile (75:25, v/v) and 1 mL of acetonitrile. The eluted solution was evaporated with heat (50 °C) and under N_2. Finally, the residue was redissolved in 1 mL of mobile phase.

Samples were filtered through 0.45 μm nylon filter membranes and degasified in ultrasonic bath before their injection (20 μL) in the chromatographic system. Three chromatograms per sample were collected and the mean of both height and peak area were used as analytical signal.

3. Results and discussion

3.1. Selection of electrode potentials

Firstly, the optimization of the acetonitrile contents in the mobile phase was investigated. 30% of acetonitrile gave the best results with a resolution of 1.5. Secondly, the influence of pH was studied. For that, different mobile phases containing acetonitrile:water (30:70, v/v) were prepared and the acidity of the media was varied with acetic acid between 0.1% and 5%. The best resolution values were found with 2% of acetic acid. Finally, the influence of sodium perchlorate in the resolution was studied between 0 and 100 mM. No influence could be observed, however, 50 mM of sodium perchlorate was selected as electrolyte for the following experiments.

The hydrodynamic curves of the fluoroquinolones were obtained to select the appropriate potential values to detect them in the used system. The potentials in the conditioning cell and in

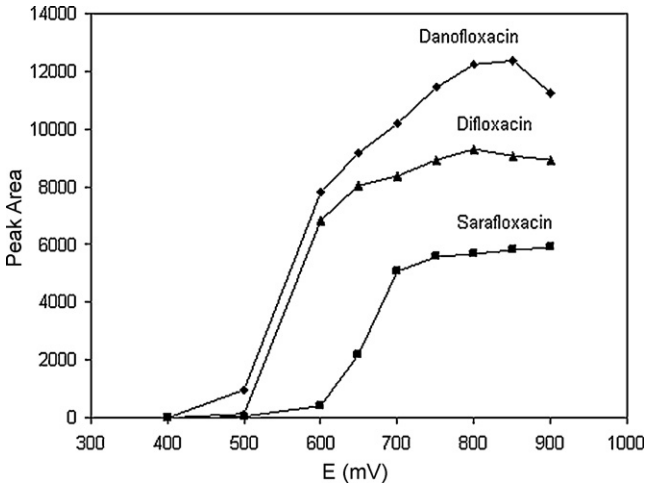

Fig. 1. Hydrodynamic curves of danofloxacin, difloxacin and sarafloxacin in the porous graphite electrode of the coulometric detector (electrode 2). Mobile phase 0.05 M sodium perchlorate–2% acetic acid:acetonitrile (70:30, v/v).

the electrode 1 were set at +0.3 V whereas the potential in the electrode 2 is varied, taking different values between +0.4 and +0.9 V. In Fig. 1 a plot of the results obtained is shown. The sensitivity was fixed in 1 μA. According to these results, a potential of +0.8 V was selected for electrode 2, due to the noise level increases with the baseline increase. The results obtained using both, peak height and peak area, were similar.

When potential of electrode 2 was fixed, the influence of potential in electrode 1 was studied. In this way, potentials of conditioning cell and electrode 2 were set at +0.2 and +0.8 V, respectively, and potential of electrode 1 was varied, taking different values between +0.2 and +0.7 V. In Fig. 2 a plot of the results of peak area measured in electrode 2 is shown. It can be observed in Fig. 2 that signal decreases when potential of conditioning cell and electrode 1 is higher than +0.6 V, thus a value of +0.4 V was set in order to avoid the oxidation of the fluoroquinolones. The potential of conditioning cell was also set at +0.4 V.

3.2. Selection of flow rate

An aliquot of DANO, SARA and DIFLO (100 ng g^{-1}) was injected in the chromatographic system in order to determine the influence of flow rate on the efficiency of the separation of these compounds. The flow rate was varied between 0.5 and 1.5 mL/min. It could be observed that when mobile rate increases both response in electrode 2 and peak width decreases. Effective number of theoretical plates (N) also varies as mobile rate increases. About capacity factor values (k'), no variation was observed. According to these results, a value of 0.6 mL/min was chosen due to the advantage of shorter times of analysis and acceptable values for effectiveness.

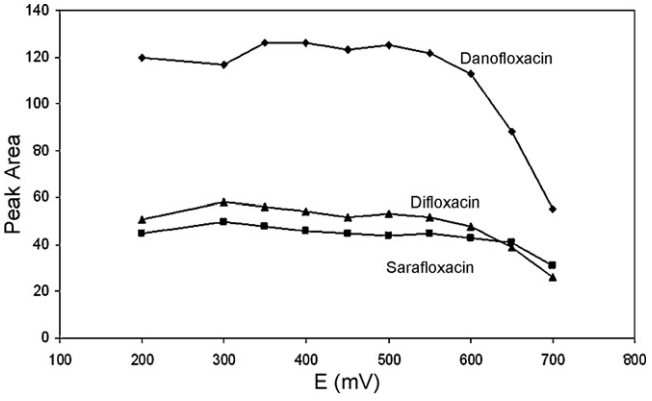

Fig. 2. Influence of conditioning cell and electrode 1 potential on the signal measured at 0.8 V in electrode 2. Mobile phase 0.05 M sodium perchlorate–2% acetic acid:acetonitrile (70:30, v/v).

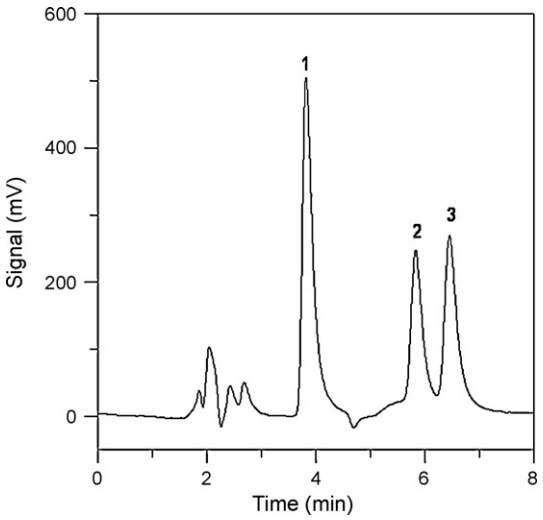

Fig. 3. Chromatogram of standard solution of (1) danofloxacin, (2) sarafloxacin and (3) difloxacin (60 μg g^{-1}). Mobile phase: 0.05 M sodium perchlorate–2% acetic acid/acetonitrile (70:30, v/v). Flow rate: 0.6 mL/min. Potential of electrode 2: +0.8 V.

Table 1
Analytical parameters for the fluoroquinolones chromatographic peaks (AU, area units).

	Danofloxacin	Sarafloxacin	Difloxacin
Slope (AU, ng g^{-1})	169.9	94.1	116.7
Intercept (AU)	290.2	357.9	-70.9
St. Dev. of slope	0.8	2.1	2.5
St. Dev. of intercept	43.6	125.9	139.7
Linearity (%)	99.5	97.6	97.9
RSD (%) (50 ng g^{-1}, n = 11)	3.7	4.8	5.5
Analytical sensitivity[a] (ng g^{-1})$^{-1}$	1.43	0.29	0.32
R^2	0.9996	0.9895	0.9915
LOD (Winedfordner-Long) (ng g^{-1})	0.8	4.0	3.6
LOD (Clayton, $\alpha = \beta = 0.05$)[b] (ng g^{-1})	1.6	8.3	7.4

[a] Analytical sensitivity = $(m/S_r)^{-1}$; where m is the slope and S_r the standard deviation of regression (Ref. [32]).
[b] α is the probability of false positive and β the probability of false negative.

Different sensitivities have been assayed to obtain the chromatograms. A value of 500 nA was set in the sensitivity in detector 2 due to the good signal/noise ratio obtained. Fig. 3 shows a chromatogram corresponding to a standard of danofloxacin, sarafloxacin and difloxacin (60 ng g^{-1}) obtained in the mentioned conditions.

3.3. Analytical figures of merit

Under the optimum experimental chromatographic conditions, a linear relationship between peak height or peak area and concen-

Table 2
Regression parameters of the intra-day and inter-day calibrations for danofloxacin, sarafloxacin and difloxacin.

	Intra-day		Inter-day	
	Slope	Intercept	Slope	Intercept
Danofloxacin	170.9 ± 0.8	601 ± 22	171 ± 2	248 ± 27
Sarafloxacin	93 ± 2	284 ± 43	86 ± 2	179 ± 18
Difloxacin	112 ± 2	254 ± 12	103 ± 4	41 ± 12

Table 3
Recovery values (R) for the determination of danofloxacin, sarafloxacin and difloxacin in chicken tissue samples. Concentration in ng g^{-1}.

Sample	Danofloxacin			Sarafloxacin			Difloxacin		
	Added	Found	%$R \pm$ SD	Added	Found	%$R \pm$ SD	Added	Found	%$R \pm$ SD
1	32.81	28.15	85.8 ± 5.7	10.94	8.83	81 ± 11	43.75	39.60	90.5 ± 6.0
2	32.81	28.98	88.3 ± 2.2	21.88	18.27	83.5 ± 0.7	65.63	58.15	88.6 ± 1.4
3	10.94	9.85	90.1 ± 5.3	10.94	9.67	88.4 ± 8.8	43.75	39.77	90.9 ± 5.7
4	10.94	9.85	90.0 ± 2.9	21.88	18.96	86.7 ± 1.5	65.63	58.67	89.4 ± 2.4
5	21.88	20.10	91.9 ± 2.3	10.94	9.77	89.3 ± 2.5	65.63	58.33	88.9 ± 3.4

tration of the fluoroquinolone was found for the three compounds in the range examined (10–150 ng g^{-1} for DANO, 25–100 ng g^{-1} for SARA and 50–325 ng g^{-1} for DIFLO), showing the regression parameters of Table 1. For each standard sample, three replicates were made. Peak area was selected as analytical signal to determine these analytes. For a series of 11 measurements of a solution containing 50 ng g^{-1} of DANO, DIFLO and SARA, the relative standard deviation was ±2 for DANO and SARA and ±3 for DIFLO, respectively, and the relative error was 3.8%, 5.3% and 5.4% for DANO, DIFLO and SARA, respectively (95% confidence level).

For the calculation of the detection limits Long and Winefordner [25] and Clayton et al. [26] methods have been used. It is well known that when applying the IUPAC [27] definition only the standard deviation of the blank is considered. However, Long and Winefordner use the propagation of errors approach, which will give values of detection limits consistent with the reliability of the blank measurements and the signal measurements of the standards. Several authors have pointed out the inadequacy of these traditional methods, among them, Clayton et al. affirm that traditional limits of detection have been concerned only with type I errors (error α) or false positive conclusions (i.e., reporting an analyte as present when it is not). The method proposed by these authors allows the analyst to specify both types of errors type I and type II (false negative assertions or error β) rates when defining detection limits. Their studies demonstrated the close agreement between this statistical approach and laboratory practice. As Clayton considers all possible errors described above, the values obtained with this method are higher and probably more realistic than those corresponding to Long and Winefordner method. The obtained values are included in Table 1.

Besides, intra-day calibration and inter-day calibration were performed. In Table 2 the regression parameters are shown. A test for comparing the slopes of calibration curves was made [28]. No significative differences ($p = 0.05$) were found between them.

3.4. Determination of danofloxacin, sarafloxacin and difloxacin in chicken tissues

The proposed method was applied to the determination of danofloxacin, sarafloxacin and difloxacin in chicken tissues. As the signals of the analytes overlap with the front, a modification of the mobile phase was necessary. The new mobile phase was 0.05 M sodium perchlorate–2% acetic acid:acetonitrile (80:20, v/v), so a delay of the retention time of danofloxacin, sarafloxacin and difloxacin was achieved. In Fig. 4 a chromatogram of a chicken tissue sample spiked with danofloxacin, sarafloxacin and difloxacin using the modified mobile phase is shown.

The concentration range studied was assumed according to the MRL concentration ranges for the three analytes: 50–150 ng g^{-1} for DANO (MRL 200 ng g^{-1}) and 200–300 ng g^{-1} for DIFLO (MRL 300 ng g^{-1}) and 50–100 ng g^{-1} for SARA.

In order to test the proposed method, several chicken tissue spiked samples were prepared. The concentration of danofloxacin, sarafloxacin and difloxacin in each sample was according to a Box–Behnken design [29]. Only one requirement was imposed and was that the concentration of SARA has to be always a half of DIFLO because it is its main metabolite.

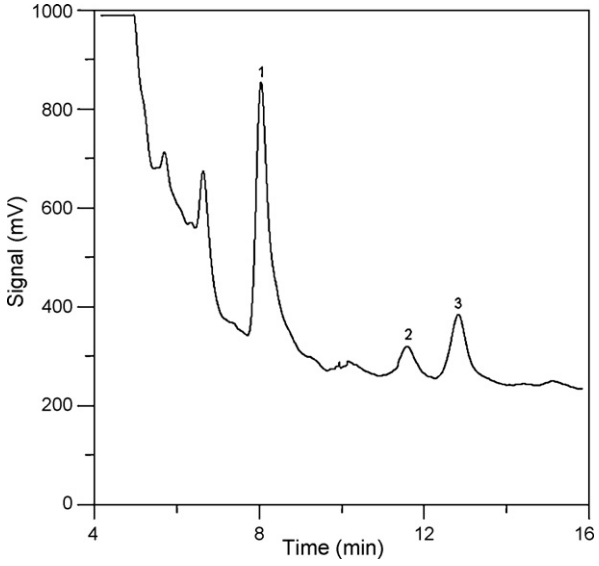

Fig. 4. Chromatogram of a chicken tissue sample spiked with (1) danofloxacin, (2) sarafloxacin and (3) difloxacin (75, 50 and 150 ng g^{-1}, respectively). Mobile phase: 0.05 M sodium perchlorate–2% acetic acid/acetonitrile (80:20, v/v). Flow rate: 0.6 mL/min. Potential of electrode 2: +0.8 V.

Box–Behnken designs [30] are a class of rotatable or nearly rotatable second-order designs (this mean that all non-center samples are located on a sphere) that is able to describe a response surface which has a minimum or a maximum inside the experimental range and its use is indicated to this purpose [31]. It is used to examine the relationship between one or more response variables and a set of quantitative experimental parameters. One of its advantages is that it does not contain combinations for which all factors are simultaneously at their highest or lowest levels, for that these designs are useful in avoiding experiments performed under extreme conditions, for which unsatisfactory results might occur.

Each sample was injected in triplicate. In Table 3 the composition of synthetic samples and the recovery values obtained are shown. As can be seen, the lowest recoveries are found for SARA (81–89.3%), while the recoveries of DANO and DIFLO are similar and comprising between 85.8% and 91.9%.

4. Conclusions

A method to determine the fluoroquinolones danofloxacin, sarafloxacin and difloxacin is proposed by using high-performance liquid chromatography with electrochemical detection. Recovery values obtained are about 90%. Hence, the proposed method in which a coulometric detector is used and detection limits under 10 ng g^{-1} are achieved, is recommended for controlling residues of these fluoroquinolones in chicken tissues.

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