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Validation of an analytical procedure for the determination of the fluoroquinolone ofloxacin in chicken tissues

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Abstract

A novel analytical procedure was developed for the determination of the fluoroquinolone ofloxacin in chicken kidney, liver, muscle and fat plus skin tissues. The procedure involved a preliminary extraction with 0.15 *M* HCl followed by solid-phase extraction clean-up. The purification step was performed using a polymeric sorbent coated cartridge. Ofloxacin was analyzed by reversed-phase HPLC using UV detection at 295 nm. The mobile phase used was water–acetonitrile–triethylamine (83:14:0.45, v/v, pH 2.30). The use of triethylamine and the acidic pH modulated the retention of ofloxacin and avoided chemical tailing. The amine modifier and acetonitrile content of the mobile phase were optimized to provide the best selectivity. A flow-rate of 1 ml/min was used and ofloxacin eluted at ~5.1 min. HPLC analysis of the tissue samples was performed in 12 min. The procedure was validated according to the International Conference on Harmonisation guidelines across the concentration ranges (100 μg/kg–1.7 mg/kg for kidney and liver tissues and 50 μg/kg–1.0 mg/kg for muscle and fat plus skin tissues). The linearity, the intra- and inter-day accuracies and precisions were determined. The limits of quantification were 50 μg/kg for muscle and fat plus skin tissues and 100 μg/kg for liver and kidney tissues. The procedure was specific and the accuracy values were lower than 20% at the limit of quantitation of spiked samples. The recovery values ranged from 80 to 100%. The limits of detection were established at 60 μg/kg for liver and kidney tissues and at 25 μg/kg for muscle and fat plus skin tissues. Finally, ofloxacin was found to be stable in acidic conditions. The developed procedure is simple, sensitive, accurate and adapted to routine sample analyses such as those carried out for residue depletion studies.

Keywords: Ofloxacin

1. Introduction

The antibacterial agents of the quinolone family are active against a wide range of Gram-negative organisms but lack activity against Gram-positive cocci. A new generation of quinolone drugs commonly known as the fluoroquinolones present an enhanced activity against Gram-positive bacteria due to the presence of an amino functional group and a fluorine atom [1,2]. They act by inhibiting the DNA gyrase resulting in bacterial death [3,4]. These quinolone derivatives include ofloxacin (9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido [1,2,3,-de]-1,4-benzoxazine-6-carboxylic acid) which has a piperazinyl group and an oxazine ring. These functional groups make oflox-

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acin very effective in treating urinary and respiratory tract infections [1,5].

The use of fluoroquinolones as veterinary medicinal products is regulated in the European Union (EU) and their distribution in target tissues of food-producing animals is controlled to guarantee consumer safety [6]. The presence of these antimicrobial agents in animal food could induce pathogen resistance in humans [6–8]. The use of ofloxacin as a veterinary drug is not authorized in the EU and the maximum residue limits have still not been established for the drug. Like many other fluoroquinolones, ofloxacin is noted for its extensive penetration into body tissues and fluids after administration [5]. It is therefore of great interest to develop analytical procedures capable of determining accurately animal tissue concentrations of ofloxacin, to evaluate the withdrawal period of the drug in administered animals proceeding from a residue depletion study. These analytical procedures may also be used for the inspection of ofloxacin in meat. Analytical procedures have been developed for the determination of ofloxacin in various biological fluids but little work has been published regarding ofloxacin analysis in animal tissues [9–23]. Horie et al. extracted fluoroquinolones including ofloxacin from only chicken muscle and liver tissues [16]. In the multiresidue method developed by Rose et al., only egg and turkey muscle were analyzed for ofloxacin [23]. The above mentioned works lacked any information about the performance of their procedure for ofloxacin analysis in kidney and fat plus skin tissues [16,23]. Ion exchange or C_{18} solid-phase extraction (SPE) were used for sample clean-up and UV or fluorescence detection was used for detecting ofloxacin in samples [1,9–21,23]. In general, most of the methods reported in the literature consisted of many experimental steps and therefore were time-consuming and not suitable for routine analysis.

The development of analytical procedures designed for the extraction and purification of drugs from animal tissues is a major challenge, especially when the molecule presents acidic and basic groups [24,25]. Ofloxacin is an amphoteric drug due to the presence of a piperazinyl and a carboxylic acid group in its structure [26]. These groups are responsible for chemical tailing during the high-performance liquid chromatography (HPLC) analysis and are also involved in pH-dependent interactions between the drug and the components of the tissue matrix [24,25]. This latter fact hinders the extraction and purification of the drug. Furthermore, different tissues differ in their content of interfering polar and apolar constituents [27] and it is often difficult to apply the same procedure to each target tissue, e.g., liver, kidney, muscle and fat+skin tissues. Moreover, the procedure must be sensitive and accurate when it is designed for drug residue analysis. In this study, we developed an analytical procedure suitable for the determination of the fluoroquinolone ofloxacin in chicken kidney, liver, muscle and fat+skin tissues. The validation of the procedure was carried out according to the International Conference on Harmonisation (ICH) guidelines [28]. The linearity, accuracy, the inter- and intra-day precision of the procedure were evaluated. The efficiency of the analytical procedure was assessed by the calculation of absolute recovery values.

2. Experimental

2.1. Chemicals

Ofloxacin and triethylamine (TEA) were purchased from Sigma (Madrid, Spain). Hydrochloric acid (2 *M*) and 85% orthophosphoric acid were obtained from Panreac (Barcelona, Spain). HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from SDS (Peypin, France). Analytical-grade Na_2HPO_4 was obtained from Merck (Darmstadt, Germany). HPLC-grade water was used (Cidasal, Barcelona, Spain). Chicken kidney, liver, muscle, and fat+skin tissues were purchased from a local supermarket.

2.2. Preparation of standards stock and working solutions

Stock solutions of 2 μg/μl of ofloxacin were prepared in 0.2 *M* HCl. The working solution of 10 ng/μl was prepared by diluting 10 μl of the stock solution to 2 ml with 0.2 *M* Na_2HPO_4 (pH 9.0). The working solution of 1 ng/μl was prepared by

diluting 100 μl of the 10 ng/μl solution up to 1 ml with 0.2 *M* Na_2HPO_4 (pH 9.0).

2.3. Sample pretreatment

Raw chicken tissue was transferred to a centrifugation tube and extracted with 7 ml of 0.15 *M* HCl. Sample masses were 300 mg for kidney and liver analyses, 400 mg for muscle analyses and 500 mg for fat plus skin analyses. The tissue was homogenized using an Ultra-Turrax T25 (IKA, Germany) at speed 5 for 20 s. The homogenized extract was centrifuged at 4400 *g* for 10 min at 4°C. The extraction step was repeated twice and the supernatants were pooled. In the case of the muscle tissue, the extract was left at 35°C for 15 min and then centrifuged at 4400 *g* for 20 min at 4°C. The pellet was discarded.

2.4. Sample clean-up by solid-phase extraction

SPE sample clean-up was automated by using the vacuum manifold Vac-Elut SPS 24 (Varian, Harbor City, CA, USA). An SPE cartridge coated with 60 mg of polymeric sorbent (Oasis; Waters, Milford, MA, USA) was conditioned with 2.5 ml of MeOH and 2.5 ml of HPLC-grade water. The final extract (14 ml) was applied onto the cartridge. When the extract loading was completed, the cartridge was washed consecutively with 3 ml of HPLC-grade water, 3 ml of 0.2 *M* Na_2HPO_4 (pH 9.0) and 5 ml of HPLC-grade water. The cartridge was subsequently dried by air aspiration. Ofloxacin was eluted with 3.5 ml of MeOH. The eluate was evaporated to dryness under a nitrogen stream. The dry residue was redissolved in 200 μl of 0.2 *M* Na_2HPO_4 (pH 9.0). The test tube was vortex-mixed for 30 s and then centrifuged at 4400 *g* for 5 min at 4°C. The supernatant was transferred to an injection vial and 30 μl was injected into the HPLC system.

2.5. Determination of ofloxacin by reversed-phase high-performance liquid chromatography

Ofloxacin determination was performed using a HPLC system consisting of a Waters 510 pump, a Spark-Holland Promis II autoinjector (Spark-Holland, Emmen, The Netherlands) and a Spectroflow 783 UV–Vis detector (ABI Analytical Kratos Division, Ramsey, NJ, USA). A 125 mm×4 mm I.D. LiChrospher $100C_{18}$ HPLC column (5 μm) from Merck (Darmstadt, Germany) was used. A 4 mm×4 mm I.D. LiChrospher $100C_{18}$ guard column (5 μm) was coupled to the HPLC column. The detection wavelength was set at 295 nm. The mobile phase used was water–ACN–TEA (83:14:0.45, v/v). pH was adjusted to 2.30 with 85% H_3PO_4 before adding ACN. The flow-rate was 1 ml/min. HPLC analysis of the samples was performed in 12 min.

2.6. Validation

2.6.1. Spiking

Kidney and liver tissues were spiked with 30, 60, 100, 250, 500 ng of ofloxacin. Muscle tissue samples were spiked with 20, 50, 100, 250, 400 ng of ofloxacin. Fat+skin tissue samples were spiked with 25, 50, 100, 250, 500 ng of ofloxacin. The lowest quantities were the respective limit of quantification (LOQ) for each tissue. The LOQ is defined as the lowest concentration of the analyte which can be detected and quantified with a precision (relative standard deviation, RSD) of not more than 20% and a deviation not more than 20% from the nominal value for accuracy [29]. Five replicates of the LOQ sample were analyzed. Three replicates were analyzed for the samples spiked with higher amounts.

2.6.2. Linearity, accuracy, precision and specificity

The validation of the analytical procedure was performed over 3 days. The linearity of the analytical procedure was evaluated by plotting the detector response (peak height) versus the nominal concentration of ofloxacin present in the tissue sample. The processing of chromatograms, the calculation of correlation coefficients (*r*) and of values for the calibration curve slope and intercept were performed using the Millenium[32] chromatography manager (Waters). The concentration ranges studied were 100 μg/kg–1.7 mg/kg for kidney and liver tissues, and 50 μg/kg–1.0 mg/kg for muscle and fat plus skin tissues. The precision (RSD) of the analytical procedure was evaluated by determining the intra- and inter-day RSDs. The intra-day precision is referred as the repeatability of the assay while the inter-day precision is referred as the intermediate precision of

the assay [28]. The accuracy of the analytical procedure is the extent to which the test results generated by the procedure and the true value agree [28,29]. The accuracy is expressed as the relative error of measurement (RE, %):

$$\text{RE}\,(\%) = \frac{(\text{Mean calculated concentration} - \text{true concentration})}{\text{true concentration}} \cdot 100$$

The intra- and inter-day accuracies were evaluated. The specificity of the procedure for each tissue was evaluated by analyzing daily non-spiked tissue samples proceeding from different sources.

2.6.3. Evaluation of the absolute recovery

Peak heights were used for recovery calculations. After extraction of the analyte from the matrix and injection into the HPLC system, the amount of ofloxacin recovered from the spiked sample was determined by comparing the response (peak height) of the extract with the response of the external standard dissolved in the reconstitution solvent (200 μl of 0.2 *M* Na_2HPO_4, pH 9.0). For each analyte concentration, duplicate reference samples were prepared and the mean response was used in order to assess the concentration of the analyte in the extract.

2.6.4. Stability

The stability of blank and ofloxacin spiked tissue extracts was evaluated. Two samples from each tissue were homogenized in 0.15 *M* HCl. One tissue extract was spiked with 300 ng of ofloxacin. The blank and the spiked tissue extracts were stored at −20°C for 20 days. After that period, the extracts were thawed and the drug was extracted and purified as described in Sections 2.3 and 2.4. HPLC analysis was carried out as reported in Section 2.5. The recovery values of ofloxacin from spiked tissue extracts were assessed in order to detect any effect that frozen storage had upon the efficiency of the analytical procedure. The chromatograms obtained for the blank and the spiked tissue extracts were compared to monitor the appearance of additional and interference peaks.

3. Results and discussion

3.1. Chromatographic conditions

As a consequence of the presence of acidic and basic functional groups, the fluoroquinolones are prone to chemical tailing due to interactions with stationary phase free silanols [30]. The pH and the organic modifier content of the mobile phase used for HPLC analysis of fluoroquinolones are known to influence the capacity factor of these antibacterial agents [26]. In this work, ofloxacin was analyzed in the cationic form due to the acidic pH of the mobile phase since dissociation constants of ~6.0 and ~8.0 were reported for the carboxylic function and the piperazinyl moiety, respectively [26,31–33]. The combination of the acidic pH (2.30) and the presence of the amine modifier TEA in the mobile phase was necessary to cause the early elution of ofloxacin (~5.1 min) with a good peak shape (Figs. 1 and 2). Besides the acidic pH, TEA also contributed to the reduction of free silanol groups [30,34]. Various proportions of water, TEA and acetonitrile were tested. It was found that the proportion water–TEA–ACN (83:0.45:14, v/v) provided the best results in terms of selectivity when processed samples were analyzed. HPLC analysis of the tissue samples was performed in 12 min (see Figs. 1 and 2). Ofloxacin was detected at 295 nm since it was the maximum observed in the absorption spectra.

3.2. Extraction and purification of the drug from the chicken tissues

Contrasting with the work of Horie et al. [16] and Rose et al. [23], we developed a novel analytical procedure for the determination of ofloxacin in the four target chicken tissues (liver, kidney, muscle, fat+skin) usually required by the European Agency of the Evaluation of Medicinal Products (EMEA) in the case of fluoroquinolone residue depletion studies [35–37]. Ofloxacin was extracted from chicken liver, kidney, muscle and fat+skin in an acidic medium. Under these conditions, ofloxacin is extracted in its cationic form since pK_a values of 7.90 and 8.22 were reported for the piperazinyl moiety [26,32–34]. HCl (0.15 *M*) was found to provide higher recoveries and

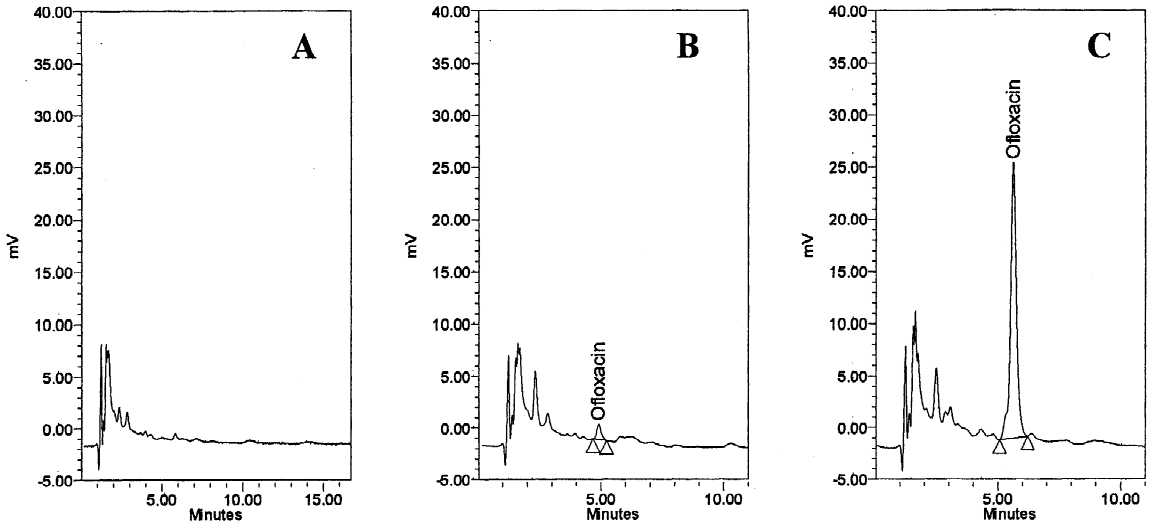

Fig. 1. Determination of ofloxacin in chicken kidney spiked samples. (A) Non-spiked kidney tissue sample; (B) kidney tissue sample spiked with 100 μg/kg of ofloxacin (limit of quantification); (C) kidney tissue sample spiked with 1.7 mg/kg of ofloxacin. △, Indicates peak start and peak end.

recovered less interfering compounds when compared with more concentrated HCl or other tested extractants such as phosphate buffers. Tissue extraction at the isoelectric point (6.97) [19,34] of ofloxacin was found to be not so effective.

Regarding the purification step, liquid–liquid extraction was avoided since this process can generate variable results [19] unless a suitable internal standard is used. We therefore opted for the use of SPE to purify the drug from the primary extract. Oflox-

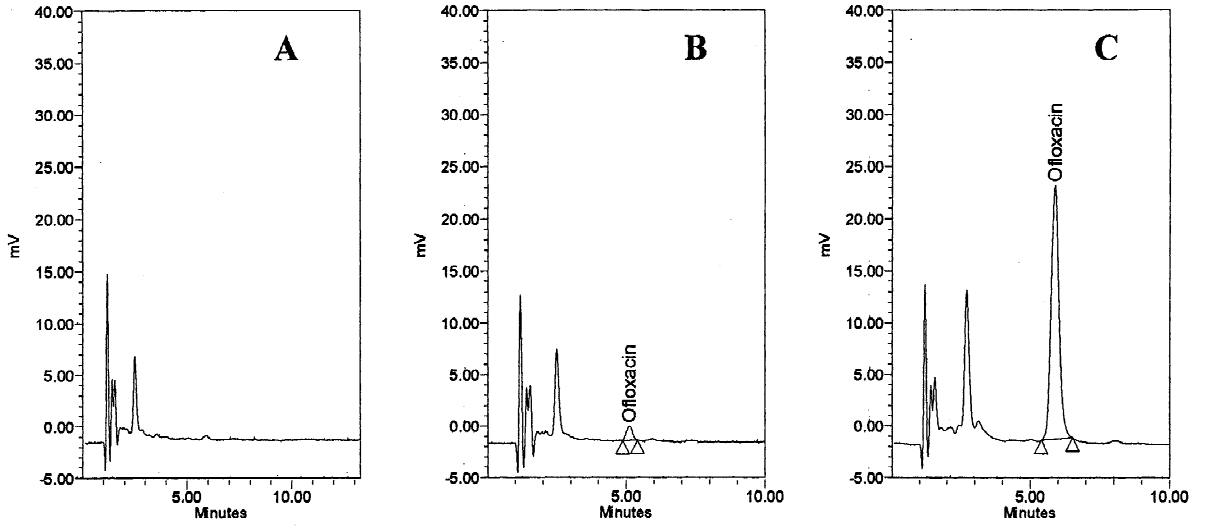

Fig. 2. Determination of ofloxacin in chicken fat plus skin spiked samples. (A) Non-spiked fat plus skin tissue sample; (B) fat plus skin tissue sample spiked with 50 μg/kg of ofloxacin (limit of quantification); (C) fat plus tissue samples spiked with 1.0 mg/kg of ofloxacin. △, Indicates peak start and peak end.

acin was purified from the tissue extracts by the use of a polymeric sorbent (stable in the 1–12 pH range) which supports the loading of the acidic extracts and washing steps at basic pH values. Washing the 60 mg cartridge with 0.2 *M* Na_2HPO_4 at pH 9.0 removed major interfering compounds, especially in the case of the liver and kidney tissues (Figs. 1 and 2). The extraction process and sample clean-up used in this work are not as laborious as those reported by Horie et al. [16] and Rose et al. [23]. The extraction process reported by the latter authors involved a large volume (100 ml) of acetonitrile and metaphosphoric or acetic acid mixture which was evaporated before SPE sample clean-up [16,23]. In the present work, the extract (14 ml) was directly applied onto the SPE cartridge without need for evaporation. Ofloxacin was eluted with 3.5 ml of MeOH while Horie et al. recovered the drug from a 500 mg C_{18} cartridge with 10 ml of MeOH [16]. The lower solvent volumes used in this study for extracting and purifying ofloxacin from the four chicken tissues shortened the working time per sample and contributed to a high throughput and lower cost. Finally, redissolving the dry eluate in 0.2 *M* Na_2HPO_4 (pH 9.0) contributed to minimizing the recovery of interfering compounds and contaminants. As a consequence of the optimized purification processes, the HPLC analysis of the tissue samples only lasted 12 min (Figs. 1 and 2).

3.3. Validation

The described procedure was fully validated for each target tissue according to ICH guidelines [28] and complied with the criteria reported by Shah et al. [29]. The analytical procedure was linear over the concentration ranges tested for the four analyzed tissues and calibration curves had a correlation coefficient higher than 0.99 (Table 1). The procedure was accurate for all the tissues and for all the concentrations tested including the LOQ (Table 1). The accuracy values were not higher than 20% at the

Table 1
Evaluation of the linearity, accuracy and precision of the analytical procedure

Chicken tissue	Calibration curve	LOQ[a]		100 ng		250 ng		400–500 ng[b]	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Linearity, intra-day precision and accuracy									
Kidney	$y=51.35x-388.64$, $r=0.9988$	17.3	5.6	−6.9	3.0	−14.0	6.1	2.4	2.2
Liver	$y=61.40x-172.00$, $r=0.9990$	−1.7	12.9	−9.3	16.8	−0.7	4.7	0.1	3.1
Muscle	$y=836.58x-992.15$, $r=0.9992$	−8.4	14.6	0.8	6.9	−1.7	6.0	0.5	3.3
Fat plus skin	$y=48.30x+70.09$, $r=0.9992$	5.4	3.7	1.8	5.5	−1.2	0.7	0.3	3.5
		50–60 ng[c]		100 ng		250 ng			
		Accuracy	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)		
Inter-day precision and accuracy[c]									
Kidney		−0.1	4.5	−1.8	5.2	−4.7	7.9		
Liver		−0.5	5.3	−4.4	11.0	−1.4	4.7		
Muscle		2.3	6.4	−0.5	5.8	−0.6	3.3		
Fat plus skin		−3.2	5.7	1.7	3.3	−0.5	1.0		

[a] LOQ, Limit of quantification; RSD: relative standard deviation. Five replicates spiked at the LOQ were analyzed. Three replicates were performed for the other amounts. Sample mass was 300 mg for kidney and liver tissues, 400 mg for muscle tissue and 500 mg for fat plus skin tissue. The LOQ was 100 μg/kg for liver and kidney tissues and 50 μg/kg for muscle and fat plus skin tissues. See Section 2 for further details. Reported results are for the day 1 of validation.

[b] The spiking amount was 400 ng in the case of the fat plus skin tissue.

[c] The assay was repeated on three days to obtain inter-day precision and accuracy values. A 50-ng amount of ofloxacin was added to the fat plus skin and muscle tissue samples and 60 ng in the case of the liver and kidney tissue samples.

LOQ level of each tissue (see Table 1). Regarding the assay precision, intra- and inter-day RSDs were generally lower than 10% (Table 1). The use of an internal standard for the determination of ofloxacin in these tissues was not necessary as shown by the accuracy and precision values. In general, recovery values were within the range 80–100% (Table 2). Fabre et al. obtained a 70% recovery of ofloxacin from lung [17]. Rose et al. reported recovery values for ofloxacin in turkey muscle ranging from 53 to 69% [23] whereas Horie et al. reported recovery values ranging from 83 to 90% only for the chicken liver and muscle tissues [16]. No information was reported by Horie et al. [16] and Rose et al. [23] about the performance of their procedures for ofloxacin analysis in kidney and fat plus skin tissues.

Recovery values higher than 80% were obtained for the frozen stored ofloxacin-spiked liver, kidney and fat plus skin tissue extracts indicating that frozen storage for 20 days in acidic media did not affect the stability of the fluoroquinolone nor the efficiency of the procedure for these extracts (Table 3). Recovery of ofloxacin was less from the stored spiked muscle tissue extract (see Table 3). Additional or interfering peaks in the blank and spiked tissue extract chromatograms were not observed. It is difficult to explain the lower recovery obtained for the muscle tissue extract. The instability of the drug could be discarded since accuracy and precision values obtained during the validation process did not suggest this.

Table 2
Absolute recovery values for ofloxacin-spiked chicken tissues

Tissue	Amount of ofloxacin (ng)	Absolute recovery[a]	
		Mean (%)	RSD (%)
Kidney	60	78.8	9.0
	100	79.8	10.5
	250	83.8	4.3
Liver	60	96.6	10.7
	100	90.0	10.7
	250	85.8	4.3
Muscle	50	93.6	18.0
	100	88.4	7.5
	250	87.2	4.2
Fat + skin	50	88.6	8.5
	100	92.4	4.9
	250	91.2	1.0

[a] The mean absolute recovery for each level was calculated from three ofloxacin spiked replicates. RSD, Relative standard deviation.

Table 3
Absolute recovery values for frozen stored chicken tissues spiked with 300 ng of ofloxacin

Tissue[a]	Absolute recovery (%)
Kidney	81.1
Liver	88.7
Muscle	68.1
Fat + skin	83.1

[a] The tissue extracts were stored at −20°C for 20 days.

Horie et al. [16] and Rose et al. [23] reported LOQ values of 20 μg/kg for chicken muscle and liver and 50 μg/kg for turkey muscle, respectively. The latter authors used HPLC with fluorescence detection. In this work, UV detection at 295 nm was used for the determination of ofloxacin. This mean of detection reduces the specificity of the analysis and is less sensitive than fluorescence detection. Nonetheless, the procedure gave quantification limits of 50 μg/kg for muscle and fat plus skin tissues and of 100 μg/kg for liver and kidney tissues. It should be noted that the procedure was fully validated for each tissue and over the entire concentration range including the limit of quantification. Limits of detection (LODs) were set at 25 μg/kg for muscle and fat plus skin tissues and at 60 μg/kg for liver and kidney tissues. The veterinary use of ofloxacin is not authorised in the EU and therefore the maximum residue limits (MRLs) have not yet been defined for ofloxacin. The LOQ/LOD values characterizing the described procedure should be sufficient for food screening or for future residue depletion studies involving ofloxacin. As an example, for comparison, MRLs for enrofloxacin and ciprofloxacin in poultry were fixed at 100 μg/kg for muscle and fat plus skin tissues, 200 μg/kg for liver tissue and 300 μg/kg for kidney tissue [35–37]. Nonetheless, if the LOQ/LOD values are not sufficient, lower quantification and detection values could probably be obtained if the present procedure was combined with HPLC using fluorescence detection.

4. Conclusions

The analytical procedure developed in this work for the determination of ofloxacin in four chicken tissues is fast, specific, accurate, precise, reproducible and sensitive although UV detection was used. The same procedure is used for the determination of the fluoroquinolone in chicken liver, kidney, muscle and fat plus skin tissues. Finally, the method can be applied to the analysis of samples proceeding from residue depletion studies or can be used for meat inspection.

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