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Determination of (fluoro)quinolone antibiotic residues in pig kidney using liquid chromatography–tandem mass spectrometry I. Laboratory-validated method

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Abstract

A new LC–MS/MS method has been developed for the multiresidue determination of 11 (fluoro)quinolone antibiotics (FQs), including acidic and amphoteric species, around their maximum residue level (MRL) in pig kidney. The procedure involves a common sample preparation by solid-phase extraction on disposable extraction cartridges followed by a fast reversed-phase liquid chromatography–tandem mass spectrometry analysis. The method was validated according to the Commission Decision 2002/657/CE. The accuracy of the method was satisfactory with recoveries included in the interval 80–100%. The precision results showed mean repeatability and reproducibility coefficients of 7.4% and 11.8%, respectively. Limits of quantification much lower than the MRLs could be obtained.

Keywords: Fluoroquinolone; Pig kidney; Liquid chromatography–tandem mass spectrometry; Validation

1. Introduction

The widespread use of antibiotics in agriculture has resulted in the presence of these compounds residues in foodstuffs from animal origin. Usually an interval between the last administration of the drug to the animals and the time when treated animals can be slaughtered is established for the production of safe foodstuffs [1]. However, due to the repeated exposure to antibiotic residues, an increase of resistance to human pathogens has been observed [2,3] with dramatic consequences on public health [4–6]. To face this problem, more and more efficient antibiotics have been developed such as the 4-quinolones and their 6-fluorinated piperazinyl derivatives, the (fluoro)quinolones (FQs). These antibiotics are used in human and veterinary medicine in the treatment of respiratory diseases and enteric bacterial infections.

In 1990, the European Union (EU) established safe maximum residue limits (MRLs) for residues of veterinary drugs in animal tissues entering the human food chain (Council Regulation (EEC) No. 2377/90) [7]. Their monitoring in the Member State laboratories of the EU has been requested by the Council Directive 96/23/EC in 1996 [8]. The new EU legislation will strengthen the control of all types of additives in animal feed and in particular will complete the EU's drive to phase out antibiotics as growth promoters [EU Institutions press release, IP/02/1891, 16/12/2002].

In order to support this policy, sensitive multiresidue analytical methods are required [9]. Methods described in literature for the determination of 4-quinolones are most often based on liquid chromatography with UV [10–13], fluorescence [14–19] or mass spectrometric detection [20–23], and capillary electrophoresis [24]. The multiresidue analysis of this group of compounds is often hampered by the differences in pK_a between the acidic and the amphoteric dyes [15,20,25]. Therefore a liquid chromatography–tandem mass spectrometry method has been developed in our laboratory for the simultaneous detection of 11 FQs [26]. The studied FQs were danofloxacine, cinoxacine, ciprofloxacine, enoxacine, enrofloxacine, flumequine, marbofloxacine, nalidixic acid, norfloxacine, ofloxacine and oxolinic acid (Fig. 1) which MRLs are presented in Table 1. For the FQs for which no MRL has been defined yet, 150 $\mu g\,kg^{-1}$ was selected as an average limit. Qualitatively, this method allowed

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Table 1
Maximal residue limits (MRLs) of the (fluoro)quinolones in pig kidney and MRM conditions for their detection in MS/MS (fragment ions in bold were used for quantification)

FQ	MRL ($\mu g\,kg^{-1}$)	Parent ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Fragment ion (*m/z*)
Amphoteric FQs					
Norfloxacine	[a]	320.14	35	15	**276.15**
				25	233.11
Ofloxacine	[a]	362.15	25	25	**261.10**
				20	318.17
Enoxacine	[a]	321.14	35	30	**206.07**
				20	257.14
Marbofloxacine	150	363.15	30	20	**345.20**
				15	320.10
Enrofloxacine	[b]	360.17	35	20	**316.20**
				30	245.20
Ciprofloxacine	[b]	332.14	30	15	**288.13**
				25	245.20
Danofloxacine	200	358.16	35	25	**96.10**
				20	314.20
Acidic FQs					
Cinoxacine	[a]	263.10	35	20	**217.10**
				15	245.20
Flumequine	1500	262.20	35	35	**202.10**
				20	244.20
Oxolinic acid	150	262.14	40	30	**216.10**
				20	244.10
Nalidixic acid	[a]	233.17	40	15	**215.15**
				25	187.10
Internal standards					
Lomefloxacine	[a]	352.20	35	25	**265.18**
				25	308.26
Cincophen	[a]	250.04	45	35	**128.02**
				30	222.17

[a] No MRL has been fixed by the European legislation.
[b] MRL for ciprofloxacine + enrofloxacine = 300 $\mu g\,kg^{-1}$.

the identification of the 11 FQs. However, only the determination of seven amphoteric FQs could be validated whereas that of the four acidic FQs showed poor repeatability and accuracy results therefore requiring further investigations [27].

The present paper shows the optimisation and in-house validation of a new method allowing the simultaneous identification and quantification of these 11 FQs in a single analysis. A new common sample preparation procedure involving a liquid and a solid-phase extraction is described, followed by the multiresidue LC–MS/MS detection of the extracted compounds at MRL and lower. Two internal standards, lomefloxacine and cincophen, are proposed for the quantification of the amphoteric and acidic FQs, respectively. The in-house validation of the method, according to Commission Decision 2002/657/CE [28], is discussed.

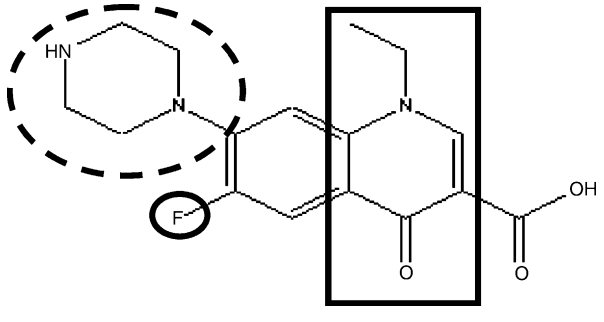

Fig. 1. Chemical structure of norfloxacine showing the 4-oxo-1,4-dihydroquinoline skeleton common to all FQs (▭), the piperazinyl moiety typical for amphoteric FQs (-.-) and the fluorinated dye (⬭).

2. Experimental

2.1. Reagents

The FQs standards norfloxacine, enoxacine, enrofloxacine, ciprofloxacine, ofloxacine, lomefloxacine hydrochloride, cinoxacine, oxolinic acid, nalidixic acid, flumequine and 2-phenyl-4-quinoline carboxylic acid (cincophen) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Danofloxacine mesylate was provided by Pfizer (Groton, CT, USA) and marbofloxacine was from Vetoquinol (Lure, France).

All reagents and water used are of analytical purity and suitable for HPLC. Methanol Chromosol for HPLC (99.9%) was from Riedel de Haën. Acetonitrile hypergrade for liquid chromatography (LC–MS) (Lichrosolv, 99.9%), ammonium hydroxide suprapur 25%, ammonium acetate 98% and acetic

acid suprapur 96% were from Merck (Darmstadt, Germany). Formic acid 98% was from Fluka.

Pig kidney samples were provided by the University of Gent, Faculty of Veterinary Medicine (Ghent, Belgium). These samples reacted negatively to a microbiological test for quinolones based on the detection of an inhibition zone in a culture media [29].

2.2. Standard solutions preparation

A 100 $\mu g\,ml^{-1}$ standard stock solution was prepared for each FQ and for each internal standard. Ten mg of standard were first dissolved in 2 ml of ammonia 2 M and placed in an ultrasonic bath for 60 min. A particular attention was given to the proper dissolution of ciprofloxacine, which needs more time than the other FQs to dissolve. The content of the flask was then adjusted to 100 ml with methanol. The stock solutions were kept at +4 °C and were stable for 3 months.

Then 1 $\mu g\,ml^{-1}$ single FQ intermediate solutions were prepared by diluting the respective stock solutions with diluted formic acid (pH 2.5; 0,14%, v/v). Single internal standard intermediate solutions were also prepared in the same way. These solutions were stable for 2 weeks at +4 °C.

Six final solutions were prepared by diluting FQs intermediate solutions and internal standard intermediate solutions with diluted formic acid (pH 2.5). The final solution concentrations were in the range MRL/4 to MRL × 2 (which corresponds to 32.5–300 $ng\,ml^{-1}$ for most of the FQs, see Table 1), with 300 $ng\,ml^{-1}$ internal standard. Kept at +4 °C, they were stable for 2 weeks.

2.3. Instrumentation

The solid-phase extraction (SPE) of the samples was performed using SDB-RPS disposable extraction disk cartridges (10 mm, 6 ml) from 3M Empore (St Paul, MN, USA) containing mixed C_8 and cation exchange phases.

The LC system consisted of a Waters Alliance 2690 quarternary solvent delivery system (Waters Corporation, Milford, MA, USA). The chromatographic separation of the FQs was performed using a Symmetry Shield RP-8 (150 mm × 3.9 mm; 5 μm particle diameter) reversed phase analytical column from Waters. A guard column Symmetry Shield RP-8 was placed in front of the analytical column.

The ESI-MS/MS detection of the FQs was achieved using a Quattro LC triple stage quadrupole instrument from Micromass (Manchester, UK). The positive ionisation mode was used and the ions were monitored in the multiple reaction monitoring (MRM) mode.

2.4. Method

2.4.1. Preparation of the pig kidney samples

Fifty pig kidneys were minced and homogenised using a 1094 Homogenizer (Scientifica Panzeri) followed by a fine Turrax disruption. One gram of minced pig kidney was directly spiked with 1.0 ml of final solution of FQs at concentrations corresponding to MRL/4, MRL/3, MRL/2, MRL and MRL × 2 for the preparation of calibration curves. Ten millilitres of acetonitrile were added to the tube containing the sample. The tube was vortexed for 1 min and then agitated horizontally for 15 min. After agitation, the tube was centrifuged at 4000 rpm (2808 × *g*) for 10 min. Ten millilitres of the supernatant were evaporated to dryness at +37 °C under a stream of nitrogen. The residue was redissolved in 2.0 ml ammonium acetate buffer 5 mM pH 4.0. The dissolution was achieved using vortex and ultrasonic bath for 15 min.

The SDB-RPS extraction cartridge was conditioned using 2 × 1.0 ml of methanol, 2 × 1.0 ml of water and 2 × 1.0 ml of ammonium acetate buffer 5 mM pH 4.0. The cartridge was then loaded with the extract, drop by drop. Finally the compounds were eluted from the cartridge using 4 × 1.0 ml of a mixture of acetonitrile and ammonium hydroxide 1 M (75/25; v/v). The eluate was evaporated to dryness at +37 °C under a stream of nitrogen and redissolved in 300 μl of diluted formic acid pH 2.5. This solution was filtered on a 0.45 μm filter prior to LC–MS/MS analysis.

2.4.2. LC and ESI-MS/MS conditions

The LC separation of the FQs was achieved using a gradient elution. The initial mobile phase consisted of 90% A and 10% B, where A was diluted formic acid (pH 2.5) and B was acetonitrile containing 0.14% (v/v) of formic acid. From 0 to 10 min, the percentage of B increased from 10 to 62%. At 10.5 min, B percentage was set at 100% and was stable for 2 min. Finally at 13 min, B percentage was set at 10% and was stable for 2 min for reconditioning of the analytical column.

The flow-rate of the mobile phase was 1.0 $ml\,min^{-1}$. A T-piece splitter (4:1) was used between the LC column and the MS detector in order to introduce 200 $\mu l\,min^{-1}$ effluent into the ion source of the mass spectrometer. The column temperature was 25 °C. Fifty-microlitre aliquots of the extracts were injected in the LC–MS/MS system.

The ESI-MS/MS conditions were the followings: +3.2 kV capillary voltage, source block and desolvation temperatures at 130 and 400 °C, respectively. Desolvation and nebuliser gas (N_2) flows were 650 and 80 $l\,h^{-1}$, respectively. Argon pressure in the collision cell was 2.5×10^{-3} mbar. The cone voltage and collision energy for MRM acquisitions are presented in Table 1. The dwell time was 100 ms/transition. Two transitions were followed for identification but only one was used for quantitation (in bold in Table 1).

3. Results and discussion

The experiments were divided in two parts: optimisation of the method for amphoteric and acidic FQs and validation.

3.1. Optimisation

The method previously developed for the quantification of the seven amphoteric FQs (norfloxacine, ofloxacine,

enoxacine, enrofloxacine, ciprofloxacine, marbofloxacine and danofloxacine) could not give satisfactory repeatability results for the analysis of the acidic FQs (cinoxacine, flumequine, nalidixic acid and oxolinic acid) [27]. In order to develop a method targeting both chemical groups different parameters were studied.

3.1.1. The preparation of the FQs standard solutions

Both amphoteric and acidic FQs showed a poor dissolution capacity in diluted formic acid pH 2.5. The addition of 20–30% methanol could not improve their dissolution. Stock solutions, which concentration was not higher than 100 $\mu g\,ml^{-1}$, were finally prepared by diluting the FQs in 2 ml of ammonium hydroxide 2 M followed by sonication in an ultrasonic bath. Sixty minutes of sonication were necessary in order to obtain the proper dissolution of the FQs, especially for cinoxacin. Then the volume of the flasks was adjusted to 100 ml with methanol. The homogeneity of these solutions was successfully tested using repeated injections in LC–MS/MS.

3.1.2. The LC separation

First of all, in order to achieve a good repeatability of the results, the MS ionisation conditions have to be stable during the analytical run. Therefore, the composition of the mobile phase was set in order to keep the formic acid concentration constant during the whole elution gradient. For this purpose, the formic acid concentration in solvent A (diluted formic acid pH 2.5) was determined (0.14%; v/v) and the same formic acid concentration was used in solvent B (acetonitrile).

Second, the chromatographic profile, especially for the acidic FQs, was optimised. An endcapped analytical column was selected reducing the interactions between the free silanol groups and the nitrogen moiety of the FQs and thus reducing peak tailing. Moreover, a C-8 stationary phase was chosen in order to reduce the retention time of the acidic FQs compared with a C-18 stationary phase and to further improve the symmetry of the chromatographic peaks.

Third, some memory effects were observed after repeated injections of pure standard solutions of acidic FQs. This might be again related to the higher affinity of acidic FQs for the LC column at acidic pH (higher retention times) compared to amphoteric FQs and to their incomplete elution from the LC system. Indeed the use of 2 min elution with 100% solvent B (acetonitrile/0.14% formic acid) at the end of the elution gradient further improved the repeatability of the results.

3.1.3. The MS detection

The MS analysis of individual FQ standard solution showed some MRM interferences between FQs in case of coelution. These interferences could not be avoided by using different fragmentation pathways. Therefore, the slope of the elution gradient was modified in order to allow the separate elution of interfering FQs (i.e. flumequine/oxolinic acid, cinoxacine, oxolinic acid) but a non linear gradient was used in order to shorten the analytical run. As can be seen on Fig. 2, the 11 FQs could be separated within 15 min.

Besides, the repeatability of the results for the acidic FQs could further be improved by selecting a second internal standard, chemically similar to the acidic FQs. This standard should be used not only as an internal standard for the sample preparation step but also as internal standard for the MS ionisation process. 2-Phenyl-4-quinoline carboxylic acid (cincophen) was selected as acidic internal standard for the quantification of the acidic FQs.

Finally, coefficient of variations lower than 5% could be obtained for the four acidic and the seven amphoteric FQs in pure solution, what shows a very good repeatability of the results in LC–MS/MS.

3.1.4. The sample preparation of the pig kidney samples

The different steps of the sample preparation of the pig kidney samples were found to be critical for the repeatability of the procedure. As higher recoveries often lead to better repeatability results, several parameters were optimised in order to achieve high recoveries. During this optimisation stage, the recoveries were calculated in terms of “real recoveries” without taking into account any internal standard. By this way, any loss of sample during the extraction could be tracked.

First, higher recoveries could be obtained for the FQs by increasing the time of contact between the FQs and the organic solvent during the liquid extraction with acetonitrile. This was achieved by using a 15 min agitation step in acetonitrile and 10 min centrifugation of the samples. The addition of pure acetonitrile to the spiked samples for liquid extraction was preferred to the addition of acidic or basic mixtures of acetonitrile and aqueous buffer. Indeed, using acetonitrile:acetic acid (4:1; v:v) and acetonitrile:ammonia (6:1; v:v), “real recoveries” in the range 20–51% and 26–62% were achieved, respectively. Whereas, using pure acetonitrile addition, “real recoveries” were higher than 50% for each of the 11 FQs, including the acidic FQs.

Second, before loading on the SPE cartridge, the acetonitrile extract was evaporated to dryness and the residue was redissolved in an aqueous buffer in order to reduce the elution power of the sample solution. No washing of the SPE cartridge with aqueous buffer was used after loading as it dramatically reduced the recoveries of the FQs. However, no interference from the matrix was observed in the chromatogram. The retention of the FQs on the SPE cartridge was evaluated by collecting fractions during the sample loading and analysing the fractions in LC–MS/MS. Ammonium acetate 5 mM pH 4.0 buffer was selected as giving the best recoveries for the FQs. On the other hand, the loading of the sample drop by drop was demonstrated to be a critical parameter in order to maximise the retention of the FQs on the SPE cartridge. Finally, a mixture of acetoni-

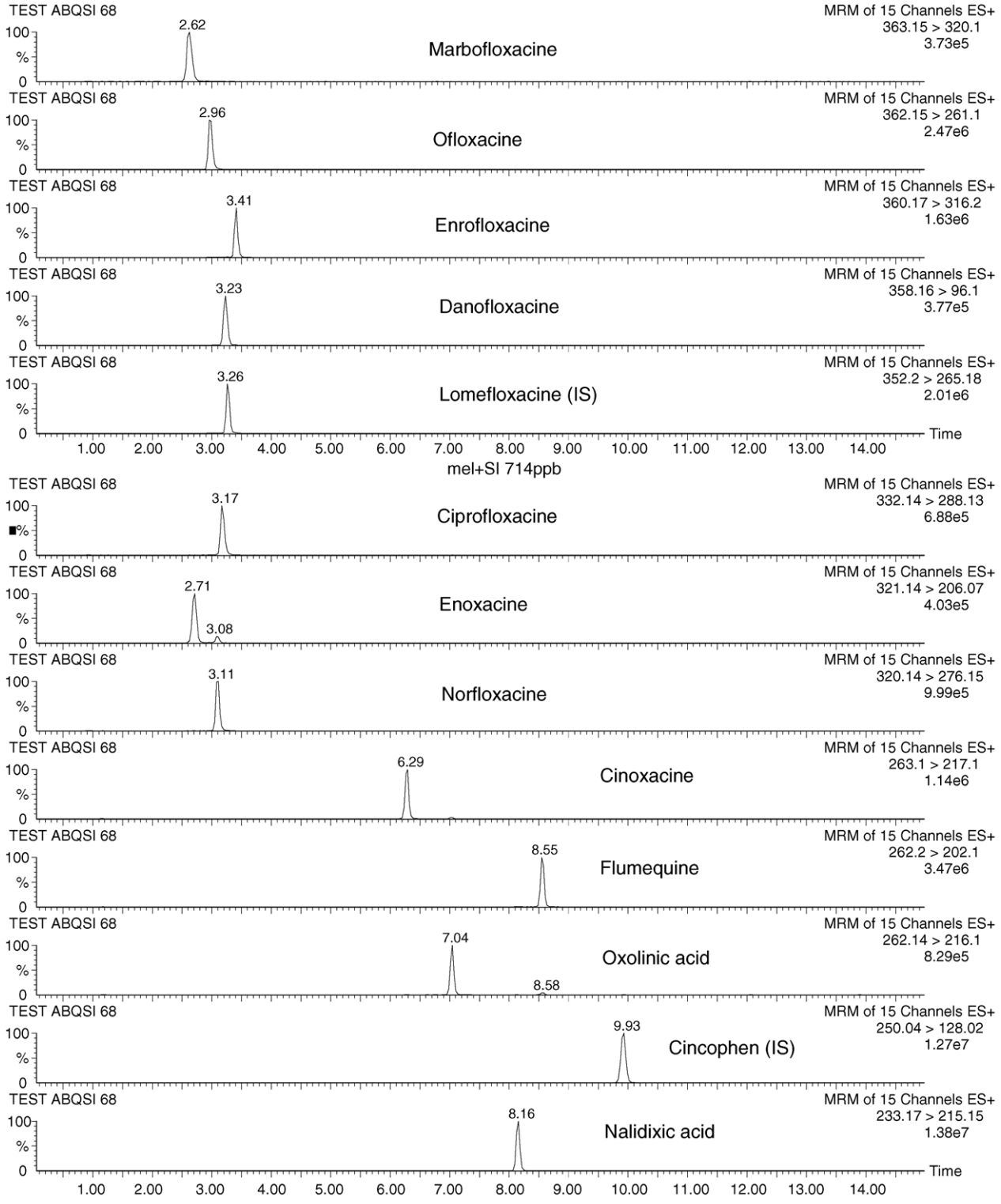

Fig. 2. Reconstituted ion chromatogram obtained after the SPE-LC–MS/MS analysis of a pig kidney sample spiked with 11 FQs and two internal standards at 700 μg kg^{-1}.

trile and ammonium hydroxide (75/25; v/v) showed a higher elution power than the same mixture of methanol and ammonium hydroxide in order to elute the FQs from the SPE cartridge.

After optimisation of the method, the "real recoveries" of the FQs, calculated without any internal standard, at three concentration levels (37.5, 150 and 300 μg kg^{-1}), were in the range 55.9–99.9%.

3.2. Validation

3.2.1. Selectivity

The selectivity of the method was investigated using "blank" pig kidney samples. These samples were called "blank" as they reacted negatively to microbiological test for quinolones at the University of Ghent. These samples were also analysed using the LC–MS/MS method for the detection of the 11 targeted FQs. Thanks to the high sensitivity of this technique (limit of quantification (LOQ) of 1 $\mu g\,kg^{-1}$ = 165 pg injected on column), the test showed the presence of some FQ residues at very low concentrations in the pig kidneys, residues which could not be detected by the previous microbiological testing. Flumequine, lomefloxacine and cincophen residues were observed at concentrations lower than the limit of quantification of 1 $\mu g\,kg^{-1}$ and much lower than the MRL (flumequine: 1500 $\mu g\,kg^{-1}$). Therefore they were not taken into account for the quantification of the FQs in the spiked samples. In particular, the presence of lomefloxacine and cincophen residues in the pig kidneys can be surprising. It could be due to a very slight cross-contamination of the analytical system with spiked samples, detectable in MS/MS.

3.2.2. Specificity

FQs being part of group B in substances of Annex 1, Council Directive 96/23/CE, three points of identification are required. Using LC–MS/MS, each precursor ion represents 1 point of identification, whereas each fragment ion represents 1.5 points. Therefore, in this method, two different transitions were followed for each FQ in the MRM mode. Concerning the quantification, only one fragmentation path was monitored (indicated in bold in Table 1).

3.2.3. Performance criteria

According to the performance criteria requested by the EU Decision No. 2002/657/CE for LC–MS methods, the relative retention time of the FQs was determined by calculating the ratio of the analyte retention time to the internal standard retention time. The relative retention time of each FQ in pig kidney samples corresponded to the ratio obtained in standard solutions, with a maximum variation of 2.5% as recommended (Decision No. 2002/657/CE). In addition, the retention time of each FQ was verified to be at least twice the retention time corresponding to the dead-volume of the analytical column. Concerning the MS detection, one ionic ratio was determined for each FQ. Relative ionic intensities (relative to the base peak) between 10% and 95% were determined. Maximum variations were 20% as recommended (Decision No. 2002/657/CE).

3.2.4. Response function

Three calibration curves (series) were successively analysed in pig kidney at six concentration levels (0–300 $\mu g\,kg^{-1}$). Each concentration level was prepared by duplicate and each sample was analysed twice. The responses were expressed as ratios between the FQ area and the internal standard area. Lomefloxacine and cincophen were used as internal standards for the amphoteric and the acidic FQs, respectively. Responses obtained for the three series were plotted as a function of the concentration. A weighted regression model using a weighting factor (W_j) of $1/X$ was applied instead of an ordinary least squares regression model in order to obtain a constant relation between variances and concentrations. The calibration curve obtained using this model was not forced through zero as this can introduce a bias in the method. Determination coefficients (r^2) between 0.9908 and 0.9998 could be obtained for the 11 FQs in the calibration range MRL/4–MRL × 2 (Table 2).

Table 2
Calibration range, coefficient of determination, limits of detection (LOD) and quantification (LOQ) of the targeted (fluoro)quinolones

FQ	Injected calibration range ($\mu g\,kg^{-1}$)	R^2	LOD ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)
Amphoteric FQs				
Norfloxacine	37.50–300.00	0.9987	1.5	5
Ofloxacine	37.50–300.00	0.9908	1.2	4
Enoxacine	37.50–300.00	0.9990	0.9	3
Marbofloxacine	37.50–300.00	0.9980	2.1	7
Enrofloxacine	37.50–300.00	0.9994	0.9	3
Ciprofloxacine	37.50–300.00	0.9992	1.8	6
Danofloxacine	50.00–400.00	0.9998	0.9	3
Acidic FQs				
Cinoxacine	37.50–300.00	0.9982	0.6	2
Flumequine	375.00–3000.00	0.9996	0.3	1
Nalidixic acid	37.50–300.00	0.9989	0.3	1
Oxolinic acid	37.50–300.00	0.9987	0.6	2

3.2.5. Limits of detection/quantification and calibration range

The limit of quantification of each FQ was considered as the concentration giving a signal to noise ratio of 10. The limit of detection (LOD) was defined as 0.3 × LOQ. LODs and LOQs are presented in Table 2. LOQs much lower than MRL/4 could be obtained. The accuracy of the method was also verified at LOQ and in the whole calibration range by calculating the lowest and the highest quantifiable values (LQV and HQV [27]) between which the analyte recoveries and their confidence intervals are included in 80–120%. Lower and upper confidence limits of the mean percentage recovery at concentrations from MRL/4 to MRL × 2 were included in the acceptance interval of 80–120%.

3.2.6. Absolute recovery

During the validation, recoveries were determined for each FQ and were expressed in terms of "absolute recoveries". For this purpose, calibration curves including internal standard were performed for each FQ in aqueous solution and in spiked pig kidney samples. The FQs aqueous solutions were not submitted to the sample preparation procedure. The absolute recoveries of the analytes from pig kidney were evaluated by the ratio of the mean response obtained at each

Table 3
Mean absolute recoveries (%), accuracy (%) and precision results obtained for each (fluoro)quinolones (mean of results at MRL/4, MRL and 2MRL)

FQ	Absolute recovery ($k=3$)		Accuracy ($n=6$, $k=1$)		Precision ($k=3$)	
	Mean (%)	RSD (%)	Mean (%)	Confidence interval (%)	Mean r ($n=6$)	Mean R ($n=18$)
Amphoteric FQs						
Norfloxacine	101.1	4.9	99.4	6.5	8.3	13.7
Ofloxacine	99.4	6.7	104.6	9.8	11.4	13.5
Enoxacine	99.8	5.9	103.4	12.4	9.1	12.2
Marbofloxacine	100.1	6.4	105.2	12.5	11.0	15.4
Enrofloxacine	98.9	3.7	102.4	5.9	5.5	8.3
Ciprofloxacine	100.2	3.5	104.3	11.0	9.3	13.6
Danofloxacine	98.7	4.9	104.2	8.7	9.3	12.2
Acidic FQs						
Cinoxacine	102.6	5.9	107.1	6.8	5.1	12.5
Flumequine	100.8	1.3	105.4	3.4	4.0	9.8
Nalidixic acid	98.9	5.8	103.9	4.6	3.5	9.8
Oxolinic acid	104.2	6.7	104.1	5.4	5.1	9.1

r: repeatability coefficient, R: reproductibility coefficient, n: number of repetitions, k: number of days of analysis.

concentration level in pig kidney to the mean corresponding response in aqueous solution. Mean absolute recoveries between 98.7% and 104.2% could be obtained for all (fluoro)quinolones (Table 3). Absolute recoveries very close to 100% indicate that the behaviour of the internal standard during the sample preparation step is very similar to that of the analytes as expected. In addition, mean coefficients of variation (CV%) between 1.3% and 6.7% were determined, indicating a very good reproducibility of the extraction procedure throughout the whole calibration range (Table 3). It is interesting to note that the CVs obtained for the acidic FQs are lower than 6.7% and not higher than the CVs obtained with the amphoteric FQs, on the contrary of what could be achieved in previous experiments [27].

3.2.7. Accuracy

Accuracy was determined in pig kidney using six determinations ($n=6$) at three concentration levels (MRL/4, MRL, 2MRL), on the 3 days of validation ($k=3$) Table 3 shows the results obtained on day 2. Satisfactory accuracy results were achieved with recoveries included in the interval 80–120% and confidence intervals CI (%) lower than 15% at MRL and 2MRL (lower than 20% at the lowest concentration level MRL/4). Again, the accuracy results obtained for the acidic FQs were comparable to the results of the amphoteric FQs.

3.2.8. Precision

The precision of the method was evaluated at MRL/4, MRL and 2MRL by repeatability and reproducibility coefficients (%) (Table 3). Repeatability and reproducibility coefficients lower than 15% at MRL and 2MRL and lower than 20% at MRL/4 could be obtained for each FQ. Mean repeatability and reproducibility coefficients were 7.4% and 11.8%, respectively. In particular, repeatability and reproducibility coefficients lower than 5.1% and 12.5% respectively were obtained for cinoxacine, flumequine, oxolinic acid and nalidixic acid. Compared to previous results (mean repeatability and reproducibility coefficients of 33.2% and 37.3% [27]), this constitutes a major achievement of the method.

3.2.9. Stability study

The stability of the spiked pig kidney samples was tested at −20 °C (2, 4 and 7 weeks), +4 °C (1, 2 and 5 days) and +20 °C (1 day). As the samples are usually defrost just before analysis, their stability at +20 °C was not tested on several days. A reference sample was kept at −80 °C. Samples were prepared by spiking a blank pig kidney with the 11 FQs at MRL. The stability study design was an isochronous study that means that the samples were spiked on different days and analysed simultaneously at the end of the stability test period. On the day of analysis, the samples were defrost and spiked with 1 ml standard solution containing both internal standards at 2MRL. The results summarised in Fig. 3 show that both acidic and amphoteric FQs are stable in pig kidney

Temp. (°C)	Time	
	weeks	days
REF -80	7	
-20	7	
-20	4	
-20	2	
+ 4		5
+ 4		2
+ 4		1
+ 20		1

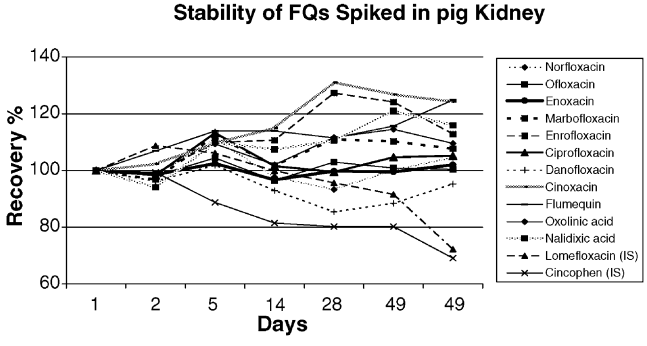

Fig. 3. Stability study of the spiked pig kidney samples at –20 °C, +4 °C and +20 °C (REF: reference sample stored at −80 °C).

samples stored for 7 weeks at −20 °C (recovery between 80% and 120%). At +4 °C, most FQs were stable for 5 days but three FQs (enrofloxacin, cinoxacin and flumequine) showed recoveries between 80% and 130%.

3.2.10. Homogeneity study

The homogeneity of the spiked pig kidney samples was evaluated at three concentration levels using aliquots of 1 g of minced pig kidney, spiked with the 11 FQs at MRL/4, MRL and 2MRL and with the two internal standards at 2MRL. As the samples were spiked after taking the aliquots, the homogeneity study was in this case testing the homogeneity of the blank matrix, the repeatability of the spiking process and the repeatability of the sample preparation procedure. Three independent sample preparations were performed at each concentration level ($n = 3$). Each sample was injected in duplicates. These analysis were repeated over 3 days ($k = 3$). Mean coefficients of variation between 3.2% and 10.0% could be obtained.

4. Conclusion

The multiresidue identification and quantification of 11 FQs in pig kidney could be successfully achieved using LC–MS/MS. The method presented in this paper was especially developed in order to allow the simultaneous analysis of amphoteric and acidic FQs which can usually hardly be determined using a single procedure. In this method, a common sample preparation, separation and detection procedure for all FQs was developed. It involves the liquid extraction of the FQs in acetonitrile and the solid-phase extraction of the liquid extract using SDB-RPS disposable extraction cartridges. The separation and detection of the FQs is then achieved using reversed phase LC with on-line ESI-MS/MS detection. The method was validated in-house for each FQ in pig kidney according to the Commission Decision No. 2002/657/CE. Satisfactory results could be obtained with respects to selectivity, linearity, accuracy and precision. Limits of quantification much lower than the maximum residue limits fixed by the European Commission could be obtained.

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