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Multiresidue determination of (fluoro)quinolone antibiotics in swine kidney using liquid chromatography–tandem mass spectrometry

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Abstract

New antibiotics were recently developed, among which are the (fluoro)quinolones. This paper presents an analytical method which allows the determination of 11 (fluoro)quinolones in swine kidneys: norfloxacin, ofloxacin, cinoxacin, oxolinic acid, nalidixic acid, flumequine, enrofloxacin, enoxacin, ciprofloxacin, danofloxacin and marbofloxacin. The procedure involves a rapid and efficient pre-treatment by solid-phase extraction (recoveries 83–98%), followed by the sensitive and selective determination of all compounds in a single run using LC–ESI-MS–MS. Multiple reaction monitoring (MRM) was used for selective detection of each (fluoro)quinolone. Quinine was selected as internal standard. The accuracy of the method, expressed as recovery, was between 89 and 109%; the repeatability had a maximum RSD lower than 15%. The limits of detection (LOD) were much lower than the respective Maximum Residue Limits (MRL)/4. © 2002 Published by Elsevier Science B.V.

Keywords: Antibiotics; Fluoroquinolones; Quinolones

1. Introduction

Antibiotics are antibacterial agents derived from living organisms but some classes of synthetic products also have an antibacterial activity. Intensive use of antibiotics in human and veterinarian medicine as well as in industrial farming (food additives) [1–4] has lead to a significant increase in antimicrobial resistance, having therefore important consequences on public health [2,5].

To safeguard human health, the European Union (EU) has produced legislation (Council Regulation 2377/90/EEC [6]) establishing safe maximum residue limits (MRL) for residues of veterinary drugs in animal tissues and derived food stuffs entering the human food chain such as milk, meat and eggs [7]. Their monitoring is necessary to ensure that they are not present at levels that may pose health risks to the public (EU Council Directive 96/23/EC) [8].

From the analytical point of view, methodologies

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have already been successfully developed and established to identify and quantify some classes of antibiotics [9–12]. Recent reviews [9,10] show that measuring residual concentrations of many antibiotics, at or near the MRL, is still a difficult task.

The more recent class of (fluoro)quinolones has been less studied although they are widely used (Fig. 1). For these antibiotics, a number of LC methods using UV or fluorescence detection have been developed for applications in various tissues such as bovine, porcine, poultry or fish [13–18]. These procedures were however restricted to a limited number of quinolones. A recent paper [19] proposed a LC-fluorescence method for nine quinolones in chicken tissue, providing limits of detection far below the MRLs. However, this method does not allow the analysis in a single run, but requires three different runs, with different elution programmes.

To achieve multianalyte performance, another approach is then necessary. Liquid chromatography hyphenated to electrospray ionisation-tandem mass spectrometry (LC–ESI-MS–MS) combines high specificity, sensitivity and allows rapid and multiresidue determinations in complex matrices, together with structural information [20–25]. For instance, Doerge and Bajic [21] proposed a method for oxolinic acid, nalidixic acid, flumequine and piromidic acid in catfish muscle, while D'Agostino et al. [23] determined norfloxacin, enoxacin, ciprofloxacin and ofloxacin. Delepine et al. [24] developed a procedure for six quinolones in pig muscle and Volmer et al. [25] published procedures for the determination of seven quinolones in milk, four in salmon tissues and six in human urine, using liquid–liquid extraction. However, all these studies only addressed a few compounds. In the present work, we have therefore developed an analytical methodology allowing the detection of 11 (fluoro)quinolones, within the same run, in swine kidney samples. This involved first the development of a rapid, efficient and multiresidue sample pre-treatment using solid-phase extraction (SPE).

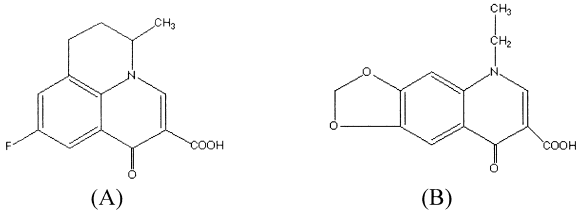

Fig. 1. Structure of two (fluoro)quinolones: (A) flumequine; (B) oxolinic acid.

2. Experimental

2.1. Instrumentation

Solid phase extraction was carried out on a Chromabond vacuum manifold for 24 columns (Macherey-Nagel, Duren, Germany) connected to a membrane pump (Barnant, Barrington, IL, USA), while tissue disruption was realised using a T25 Ultra-Turrax from IKA Labortechnik (Darmstadt, Germany).

Different SPE disposable cartridges were tested: SCX (sulfonic cation-exchange) from IST-Isolute, C_{18} and BEC (Bond Elute Certified, mixed C_{18} and cation-exchange phases) from JT Baker (Phillipsburg, NJ, USA), Sep-Pack cartridges (anion-exchange phase) from Waters (Milford, MA, USA) and SDB-RPS and MPC-SD disk cartridges (both mixed of C_8 and cation-exchange phases) from 3 M Empore (St Paul, MN, USA).

LC equipment was a Waters Alliance 2690 quaternary solvent delivery system (Waters, Milford, MA, USA). ESI-MS–MS was carried out using a Quattro LC triple stage quadrupole instrument from Micromass (Manchester, UK). Direct infusion experiments were done through a 250-μl Hamilton (Reno, NV, USA) gas-tight syringe and a Harvard Apparatus (South Natick, MA, USA) model 11 syringe pump. A Valco zero dead volume T-piece splitter (9:1) from Micromass was used between the LC column and the ESI interface in order to reduce the flow-rate of the chromatographic effluent down to 100 μl min^{-1} before entering the MS detector.

2.2. Reagents and standards

Ultra pure water was obtained from a Millipore Mill-RO 10 Plus deionisation system followed by a Milli-Quarter system (18 MΩ cm resistivity) and a sub-boiling quartz distillation unit (Quartex s.a., Paris, France). LC grade acetonitrile and methanol,

acetic acid 96%, formic acid, anhydrous Na_2SO_4 and ammonia were from Merck (Darmstadt, Germany). Nitrogen (99.999%) (desolvation and nebuliser gas) and argon N_{50} (MS–MS collision gas) were from Air Liquide (Liège, Belgium).

The (fluoro)quinolone standards norfloxacin, ofloxacin, cinoxacin, oxolinic acid, nalidixic acid and flumequine were obtained from Sigma–Aldrich (St Louis, MO, USA) while enrofloxacin, enoxacin and ciprofloxacin were from Bayer (Leverkusen, Germany), danofloxacin from Pfizer (Groton, CT, USA), and marbofloxacin from Vetoquinol (Aartselaar, Belgium). The compounds tested for possible internal standards for MS–MS quantifications, quinine and tinidazole, were obtained from Sigma–Aldrich.

Swine kidney samples were provided by the ''Institut d'expertise vétérinaire'' (IEV, Ministry of Health, Brussels, Belgium). The samples were proved to be free from agents inhibiting the bacterial growth since they reacted negatively to the official Belgium renal test.

2.3. Standard solutions preparation

A standard stock solution (100 mg l^{-1}) was prepared for each antibiotic by dissolving 2 mg of standard in 200 μl NaOH 1 *M* and adjusting to 20 ml with methanol. Two different internal standard stock solutions were also prepared by dissolving either quinine or tinidazole (100 mg l^{-1}) in MeOH. Secondary standard solutions were prepared by dilution in MeOH, one for each internal standard at a concentration of 150 μg l^{-1}, one for flumequine at 6000 μg l^{-1} and one containing a mixture of all the other (fluoro)quinolones at 600 μg l^{-1} each. Two sets of ternary solutions containing 100 μl of flumequine solution and 100 μl of the mix solution were finally prepared in order to obtain a suitable range of working solutions from MRL/4 to four times the MRL defined in the EU Regulation [6] (Table 1). Each set of solutions was finally spiked with 100 μl of one of the two internal standard solutions.

2.4. Solid phase extraction of the (fluoro)quinolones from swine kidney

SPE has become the dominant mode of extraction of the various classes of antibiotics, including quinolones, from biological matrices [26,27]. In the present procedure, 1 g of swine kidney was minced and spiked with 150 ng of quinine (selected internal standard) before turrax disruption. For linearity and quantification tests, the samples were also fortified with the different (fluoro)quinolones, at concentrations corresponding to MRL/4, MRL/2, MRL, MRL×2 and MRL×4. A concentration of 150 μg kg^{-1} was used as ''indicative MRL'' for the target antibiotics the MRLs of which are not fixed yet (cf. Table 1).

Table 1
Maximum residue limits (MRL) of (fluoro)quinolones fixed by the European Union legislation [6] and approximate limits of detection (LOD) reached with the procedure developed in this work

Compound	MRLs for swine kidney (μg kg^{-1})	LOD (approx.) (μg kg^{-1})
Cinoxacin	ne	<20
Ciprofloxacin	[a]	<15
Danofloxacin	200	<10
Enoxacin	ne	<20
Enrofloxacin	[a]	<10
Flumequine	1500	<10
Marbofloxacin	150	<20
Nalixidic acid	ne	<10
Norfloxacin	ne	<10
Ofloxacin	ne	<10
Oxolinic acid	150	<15

ne, not existing.
[a] MRL for enrofloxacin + ciprofloxacin = 300 μg kg^{-1}.

Ten milliliters of acetonitrile and 2.5 g of Na_2SO_4 were added prior to homogenisation of the sample by turrax disruption for 2 min. The homogenate was centrifuged at 3000 *g* for 5 min. The supernatant was collected and filtered through 2.5 g of Na_2SO_4 on Whatman paper. The filtrate was acidified with 2.5 ml of acetic acid 96% before the solid-phase extraction step itself.

During the optimisation of the sample preparation method, SDB-RPS and MPC-SD (mixed phase) extraction disk cartridges (10 mm, 6 ml) were tested. They were conditioned with 2×1 ml of acetonitrile/acetic acid 96% (95/5, v/v), and the acidified filtrate was loaded on the disposable extraction cartridge. Then, the disk was dried under vacuum for 5 min, and the (fluoro)quinolones were eluted with 4×1 ml of a mixture of methanol and ammonia 1 *M* (75/25,

v/v). All the SPE steps were realised under a pressure lower than 20 kPa.

The eluate was evaporated to dryness at 35 °C under a nitrogen stream. The dry residue was dissolved in 300 μl of diluted formic acid solution (pH 2.5) and filtered (Chromafil 0.45 μm) prior to LC injection.

The extraction recovery experiments involving [^{14}C]Norfloxacin were carried out by addition of 4 ml of scintillation liquid (Ecoscint A National Diagnostics) to the final SPE extract and monitoring of the radioactivity using a Beckman scintillation counter.

2.5. LC and ESI-MS–MS conditions

LC separations were carried out using a Nucleosil 100-5 C_{18} (70×4 mm; 5 μm particle diameter) reversed-phase column (Macherey-Nagel). The linear elution gradient was from 2 to 70% of B in 5 min (70% of B for 1 min), where A was diluted formic acid (pH 2.5) and B was acetonitrile. The flow-rate of the mobile phase was 1 ml min^{-1} and 50-μl aliquots of the extracts were injected. Post-column LC split was used to reduce the flow-rate entering into the electrospray ionisation source down to 100 μl min^{-1} and the column and sample temperatures were set at 25 °C.

The ESI-MS–MS conditions were first optimised using direct infusion of each compound individually in a methanol/water/formic acid solution (50/50/0.1, v/v/v). The optimised ESI$^+$-MS–MS conditions were: 3.2 kV capillary voltage, source block and desolvation temperatures at 130 and 400 °C, respectively while the desolvation and nebuliser gas (N_2) flows were 650 and 75 l h^{-1}, respectively. Argon pressure in the collision cell was set at 2.5×10^{-3} mbar. The cone voltage and the collision energy parameters were optimised in the continuous flow mode for each particular antibiotic (described in Table 2). The Multiple Reaction Monitoring (MRM) mode of acquisition was used to enhance sensitivity

Table 2
LC retention times (min) and optimised MS–MS conditions and selected multiple reaction monitoring (MRM) transitions for the target (fluoro)quinolones

Compound	Ret. times (min)	Parent ions (*m*/*z*)	Cone voltage (V)	Coll. En. (eV)	Daughter ions[a] (*m*/*z*)
Norfloxacin	3.01	320	35	15	276
				25	*233*
Ofloxacin	3.01	362	25	20	318
				25	*261*
Cinoxacin	3.91	263	35	15	245
				20	*217*
Flumequine	4.88	262	35	20	244
				35	*202*
Enoxacin	2.97	321	35	20	257
				30	*206*
Oxolinic acid	4.18	262	40	20	244
				30	*216*
Nalidixic acid	4.78	233	40	15	*215*
				25	187
Marbofloxacin	2.97	363	30	20	345
				15	*320*
				50	122
Enrofloxacin	3.19	360	35	20	316
				30	*245*
Ciprofloxacin	3.06	332	30	15	288
				25	*245*
Danofloxacin	3.10	358	35	20	314
				25	*96*
Quinine (S.I.)	2.74	325	40	30	*160*

[a] The daughter ion used for quantification is shown in italics.

and selectivity, at the mass unit resolution (10% valley) with a dwell time of 100 ms/transition.

3. Results and discussion

3.1. Solid phase extraction of (fluoro)quinolones from swine kidney

Several SPE disposable systems and protocols were tested, using C_{18}, SCX (Sulfonic Cation Exchange), BEC (Bond Elute Certify), Sep-pack cartridges and SDB-RPS and MPC-SD disk cartridges. It has never been possible to extract efficiently all the different target (fluoro)quinolones within the same extraction run using C_{18} cartridges, due to their very different chemical properties (too wide pK_a range). When ion-exchange columns were used (cation-exchange for IST-SCX, anion-exchange for Waters Sep-Pack), higher extractions ranging from 40 to 75% for the different antibiotics could be achieved, but the extract appeared to be very dirty and the dry residues obtained after evaporation could not be redissolved completely in dilute formic acid (pH 2.5). It was therefore necessary to add a rinsing step with water and/or organic solvents within the extraction procedure but the recoveries for the target antibiotics decreased very rapidly. Mixed phase (C_{18} and cation-exchange) Bond Elute Certify columns produced very satisfactory results in terms of extraction efficiency (comparable to SDB-RPS) for all tested (fluoro)quinolones, but some minor interferences and baseline fluctuations could be observed on the ion reconstituted LC–MS–MS chromatograms.

Only two procedures appeared to meet all our requirements which include high recoveries for all the target (fluoro)quinolones, efficiency and rapidity [28]. They are based on the SDB-RPS and MPC-SD disks. Following the advice of manufacturer's experts, SDB-RPS (polymeric support) should be preferred for antibiotics extraction from solid matrices such as kidney (cartridges loaded with a very fluid acetonitrile liquid extract), while MPC-SD (more porous resin) should be used for milk or plasma samples. In practice, similar recoveries could be obtained for 10 of the (fluoro)quinolones using either cartridge, while flumequine was better extracted with SDB-RPS. This last solid phase was therefore selected for the extraction step. In order to achieve satisfactory recoveries for the 11 compounds in a single preparation process, no additional rinsing step was used after the sample loading, making of the sample preparation a fast and straightforward purification procedure.

Under these conditions, the estimated extraction recoveries from spiked kidney samples (at MRL/2 concentration) for the 11 target compounds reached very high values ranging from 83 to 98% with low relative standard deviations RSD (Table 3, first column). For example, the extraction recovery using

Table 3
First column: Extraction recoveries obtained by solid phase extraction (using SDB-RPS disk cartridges) of (fluoro)quinolones at MRL/2 concentration levels from swine kidney samples; second and third columns: Accuracy results of the whole procedure (SPE-LC–MS–MS), expressed as percent of recovery, for the quantification of (fluoro)quinolones in swine kidney samples spiked at two concentration levels

Residues	SP extraction recoveries (%)[a]	Recovery (%)[b] at 100 μg kg^{-1}	Recovery (%)[b] at 300 μg kg^{-1}
Cinoxacin	89	104	100
Ciprofloxacin	87	103	104
Danofloxacin	91	109	101
Enoxacin	87	107	108
Enrofloxacin	86	101	99
Flumequine	85	89	97
Marbofloxacin	83	103	103
Nalixidic acid	98	99	96
Norfloxacin	93	100	101
Ofloxacin	95	96	98
Oxolinic acid	97	95	105

[a] RSD ($n=6$) between 2 and 7%.
[b] RSD ($n=10$) between 4 and 8%.

[^{14}C]Norfloxacin spiked samples was 93±2% (n=6).

3.2. Hyphenated LC–ESI-MS–MS

The best compromise found to keep chromatographic run times as short as possible, but also to allow the analysis of crude kidney extracts (requiring a high column capacity) was a 70×4 mm C_{18} column. Coupled to a strong elution gradient from 2 to 70% acetonitrile in 5 min, at a flow-rate of 1 ml min^{-1}, retention times ranging from 2.5 to 5 min were obtained for the 11 antibiotics. The results are presented in Table 2. Other column dimensions were tested, such as 150×2 mm and 150×1 mm, but in these cases—what is probably due to the unique and simplified solid-phase extraction used for the 11 compounds together—a problem of saturation of the column was observed after the injection of kidney extracts, leading to peak tailing and retention time shifts. Moreover, the LC run time had to be increased to over 20 min.

The use of atmospheric pressure chemical ionization (APCI) for antibiotic analysis has been reported by Delepine et al. [24]. The measurement of six fluoroquinolone residues in pig muscle was based on monitoring in the Selected Ion Monitoring (SIM) mode of the pseudo-molecular ion $[M+H]^+$ and the ion arising from the loss of one water molecule $[M+H-H_2O]^+$. In the present work, the analysis of the 11 (fluoro)quinolone residues extracted from swine kidney was also evaluated by APCI. Although it allowed the whole column eluent to enter the ion source (avoiding the 9:1 split necessary for electrospray ionization), no gain in sensitivity was observed. Moreover, the use of the 1 ml min^{-1} flow-rate caused rapid spoiling of the interface which needed to be cleaned after less than 30 injections of pig kidney extracts (the detection sensitivity decreased by 30% after 25 injections).

The ionization and fragmentation parameters were

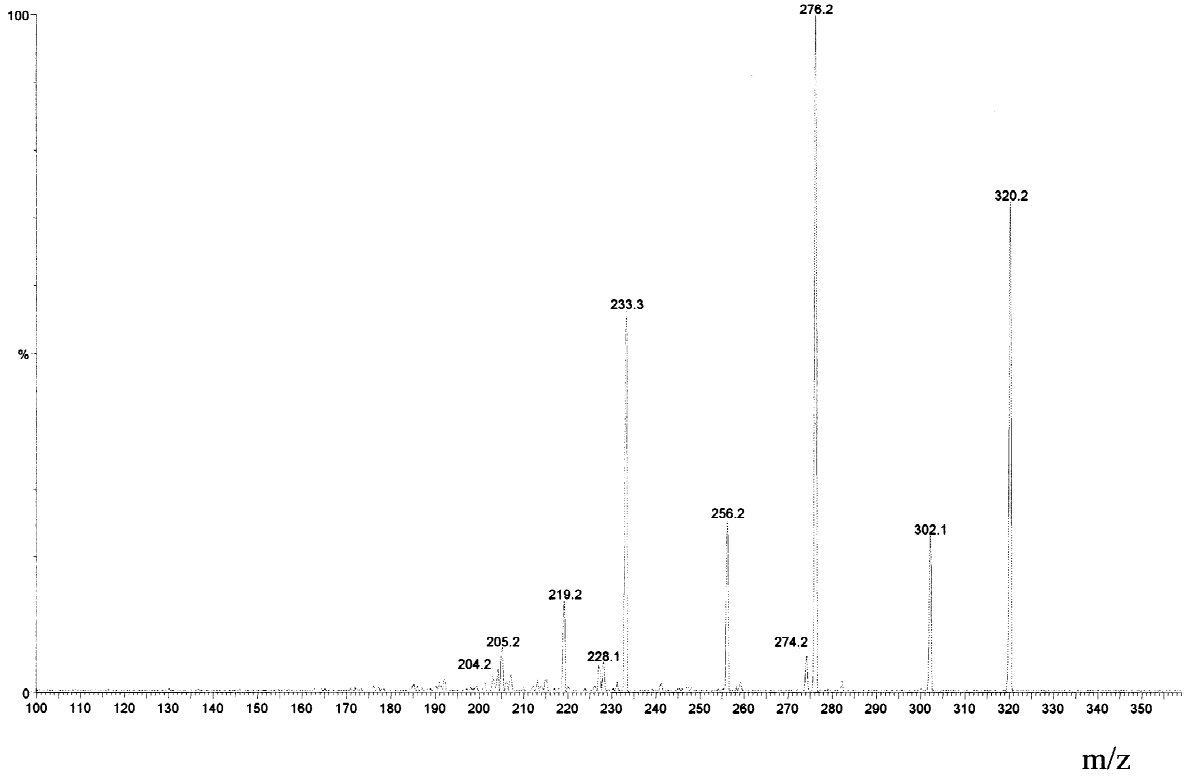

Fig. 2. Daughter ion spectrum obtained by direct infusion of norfloxacin and collision-induced dissociation (CID) (20 eV collision energy).

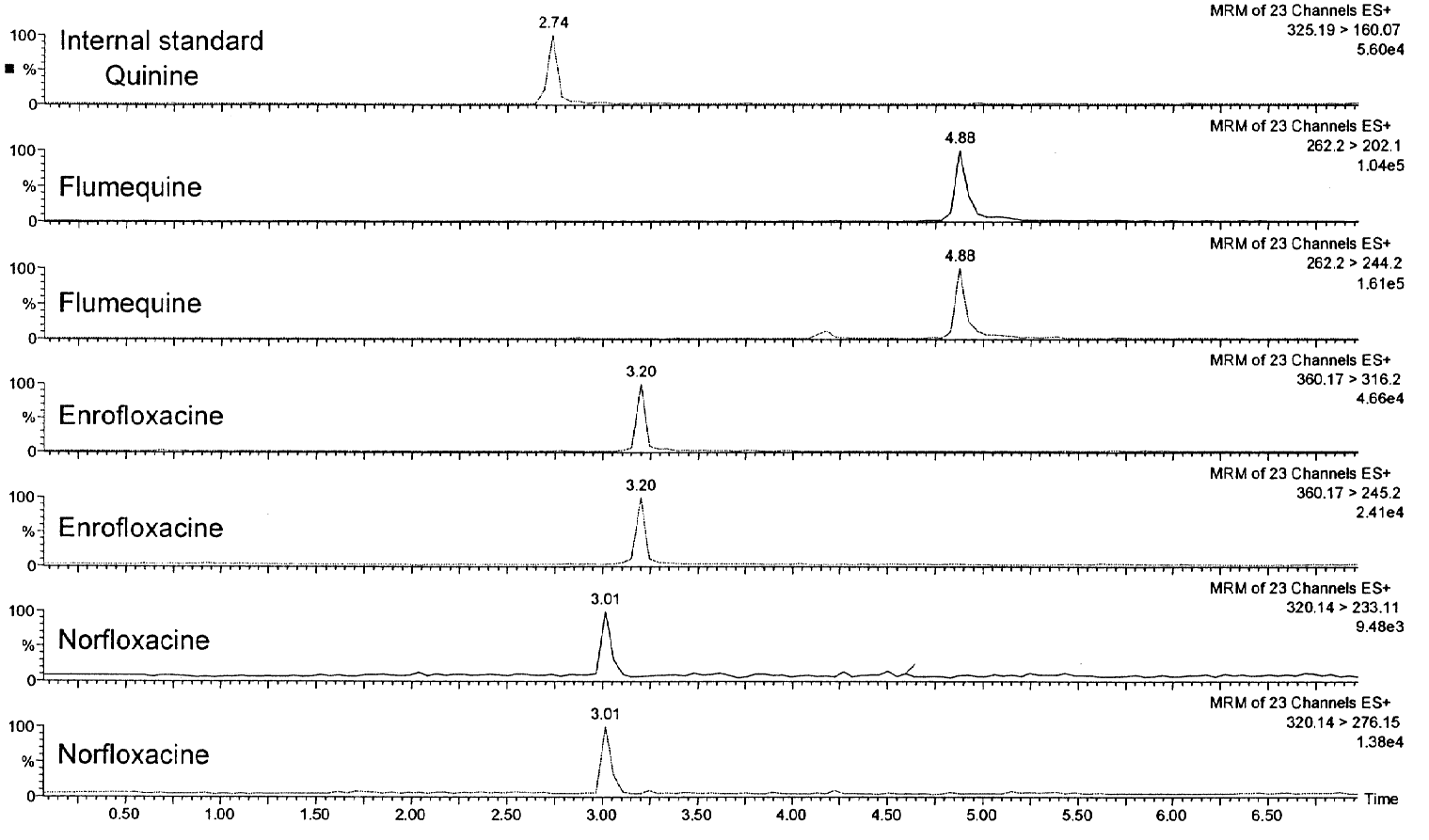

Fig. 3. Reconstituted ion chromatograms obtained by LC–ESI-MS–MS (MRM) analysis of a MRL/4 spiked swine kidney sample. The cases of enrofloxacin, norfloxacin and flumequine are depicted showing two MRM traces per compound. The chromatogram of the internal standard, quinine, is also included.

optimized for each compound individually. In MS–MS, the Multiple Reaction Monitoring mode (MRM) was used to enhance the sensitivity and selectivity of the determination. Consequently, for confirmatory identification, a minimum of two transitions was selected per antibiotic. An example (under the conditions described in Table 2) of daughter ions spectrum obtained by direct infusion and collision-induced dissociation (CID) of norfloxacin is presented in Fig. 2.

Examples of LC–ESI-MS–MS ion reconstituted chromatograms for a MRL/4 spiked kidney sample are represented in Fig. 3, which displays the cases of enrofloxacin, norfloxacin and flumequine. Two MRM traces per compound are given. The absence of interferences for these kidney extracts and the very high S/N ratios obtained can be observed. The chromatograms of blank samples (non fortified kidney material) showed no background due to the matrix.

The estimated limits of detection (LOD, given in Table 1) reached under these conditions allow the multiresidue monitoring of these 11 (fluoro)-quinolones in a unique 5.5-min chromatographic run, at concentrations much lower than the MRL/4 levels for all the residues.

3.3. Quantification tests

Preliminary quantitative tests using spiked kidney samples were carried out in order to prevalidate the SPE-LC–MS–MS procedure and to give an idea of the feasibility of a fully quantitative analysis of the 11 (fluoro)quinolones in a single run by LC–MS–MS. For each compound, the most specific transition (given in italics in the last column of Table 2) has been selected for quantification.

As no deuterated (fluoro)quinolone standards are available yet to enable MS quantifications by isotope dilution, different internal standards were tested for quantification, tinidazole and quinine. Tinidazole showed poor and variable solid-phase extraction recoveries (48–64%) thus hampering the access to accurate quantification. The best results, in terms of linearity and repeatability, were obtained using quinine. Moreover, the sensitivity of quinine (signal-to-noise ratio at 10 mg $ml^{-1} > 5$) is much higher than that of tinidazole (signal-to-noise ratio at 100 mg $ml^{-1} = 10$).

3.3.1. Linearity

The calibration curves, obtained for fortified swine kidney samples at increasing concentration of (fluoro)quinolones and prepared by SPE as described above, show that they are linear in concentration range MRL/4 to MRL×2 for the 11 compounds with all coefficients of correlation (R^2) being higher than 0.97. As an example, the equation for ciprofloxacin is $0.0071C_{CF} - 0.177$ ($n = 3$; $R^2 = 0.9853$) where C_{CF} is the concentration of the antibiotic in the fortified kidney.

3.3.2. Accuracy

The accuracy of the method was evaluated at two concentration levels: 100 and 300 μg kg^{-1} ($n = 10$). The results, expressed as recoveries, are summarised in Table 3 (second and third columns). For the 11 (fluoro)quinolones, the recoveries at both concentration levels are very satisfactory, being comprised between 89 and 109%, with relative standard deviations (RSD) on the mean values comprised between 4 and 8%. These results fulfil perfectly the requirements of the European legislation [29] which states that the accuracy of a confirmatory method should range from 80 to 110% for samples spiked with concentration levels above 10 μg kg^{-1}.

3.3.3. Repeatability

For each (fluoro)quinolone, the repeatability has been calculated at three concentration levels (MRL/2, MRL, MRL×2) from the concentration provided by the calibration curve. The relative standard deviation has been established for six measurements carried out within the same day. In all cases, the RSD value was lower than 15%. As examples, the RSD for flumequine were 14.9, 12.0 and 9.7% at MRL/2, MRL and MRL×2, respectively whereas for oxolinic acid, the respective values were 10.8, 8.7 and 7.7%.

4. Conclusion

In this work, a procedure has been developed which allows the extraction, identification and

quantification of 11 (fluoro)quinolone antibiotics in swine kidney samples. This method involves the extraction of the target residues by SPE on mixed phase extraction disk cartridges, followed by a very rapid, selective and sensitive determination using hyphenated LC–ESI-MS–MS in the MRM mode. Compared to other detection modes such as UV or fluorescence, the MS–MS mode shows its advantage of allowing multiresidue determination in complex matrices. The estimated detection limits reached are much lower than the Maximum Residue Limits fixed by the European legislation. The signal-to-noise (S/N) ratio obtained at the MRL/4 concentration level is very high and the absence of interference due to the matrix—leading to an unambiguous identification of each residue—on the chromatograms of the kidney extracts has been proved.

It will be of interest to extend this efficient and multiresidue procedure to other (fluoro)quinolones such as difloxacin, sarafloxacin, decoquinate or new compounds of this family.

On the whole, the procedure presented here appears therefore to be a promising candidate reference method for the quantitative analysis of (fluoro)-quinolones in foodstuffs from animal origin.

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