ANALYTICAL PROCEDURE FOR THE DETERMINATION OF FLUOROQUINOLONES IN ANIMAL MUSCLE

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Abstract

A liquid chromatography–fluorescence detection method for the determination of five fluoroquinolones in muscle tissue was proposed. The method is selective and robust enough for the required purposes. The whole procedure was validated in accordance with the Commission Decision 657/2002/EC. Detection capabilities were from 132.8 to 421.0 µg/kg, and recoveries ranged from 51.7% to 80.91%, depending on the analyte.

Key words: muscle, fluoroquinolones, residues, chromatography, validation.

Fluoroquinolones (FQs) are a very important class of antibacterial compounds widely used in veterinary practice for therapeutic purposes. They are highly active against a wide range of Gram-negative and Gram-positive bacteria, including those resistant to β-lactam antibiotics and sulphonamides. FQs have a satisfactory effect in the treatment of severe intestinal and respiratory infections in food-producing animals, such as poultry, pigs, calves, as well as in fish raised in aquaculture (5, 8).

Residues of FQs may occur in animal tissues if the adequate withdrawal times are not observed or if the FQ compounds are improperly administered. In order to protect consumer health, the European Union (EU) established maximum residue levels (MRLs) for sum of ciprofloxacin and enrofloxacin at the level of 100 µg/kg in muscles of all kinds of food producing animals, sarafloxacin – 200 µg/kg and difloxacin – 400 µg/kg for bovine muscles, and danofloxacin – 200 µg/kg for poultry muscles (6). The wide range use of FQs in veterinary medicine and possibility of the occurrence of the residues in food of animal origin has prompted the development of a control programme (2) that necessitated robust and reliable analytical methods. For the determination of FQs in various biological matrices several analytical procedures have been described in the literature and the majority of them bases on liquid chromatography (LC) coupled with ultraviolet (UV), fluorescence (FLD) or mass spectrometric (MS) detection (7).

The present work reports the development of a selective and sensitive LC-FLD method for the determination of ciprofloxacin (CIP), enrofloxacin (ENR), sarafloxacin (SAR), danofloxacin (DAN), and difloxacin (DIF) in muscle samples. The method uses the “dispersive solid-phase extraction” for the cleanup of the muscle extract (10). The whole procedure was validated according to the quality criteria of Commission Decision 2002/657/EC (1) and the obtained results showed that applied sample processing allows for a successful detection of FQs in muscle samples.

Material and Methods

Materials. Ammonium hydroxide (25%), acetonitrile, methanol, acetone, and acetic acid (HPLC grade) were from J.T. Baker (The Netherlands). Baker also supplied a non-polar sorbent Bakerbond octadecyl (C₁₈) 40 µm (Cat. No. 7025-00). The sorbent was pre-washed twice with hexane and dried at 50°C. Sodium acetate, sodium hydroxide, sodium chloride were obtained from Sigma-Aldrich (Germany). Phosphoric acid and hexane-1-sulphonic acid sodium salt were purchased from Merck (Germany). Water was purified trough a Mili-Q Plus system from Millipore (USA).

Standards and standard solutions. Analytical standards of ciprofloxacin, enrofloxacin, sarafloxacin, danofloxacin and difloxacin were obtained from Riedel-de Haën (Germany). The FQs individual standard solutions at the level of 1 mg/mL were prepared separately in 1 M solution of sodium hydroxide in methanol and stored in dark at <~16°C for no longer than six months. The FQs mixed solution at the level of 100 ng/mL was prepared by dilution of individual standard solutions in methanol and stored in dark at <6°C, for no longer than one month. The FQs working standard solutions were prepared by serial dilution of the FQs mixed solution with methanol (stable for at least one month). The FQs working standard solutions were added to the muscle samples at appropriate microliter aliquots corresponding to 0.25xMRL, 0.5xMRL, 1.0xMRL, 1.5xMRL, 2.0xMRL, and 4.0xMRL.
**Extraction.** The muscle samples were obtained from healthy animals (cattle and poultry) that were not treated with any veterinary drugs. The samples were minced, mixed, and deep-frozen until analysis.

An accurately weighed 1 g sample was placed in 25 ml centrifuge tube and vortexed for 1 min with 4 ml of acetonitrile, then the mixture was shaken for 10 min and centrifuged at 4 000 rpm for 10 min. After that, acetonitrile extract was separated, another 4 ml of acetonitrile were added to the matrix, and the isolation procedure was repeated in the same way. The acetonitrile extracts were coupled and 1.0 g of sodium chloride was added. The tube content was vortexed and centrifuged for 10 min.

To an aliquot of 4 ml of acetonitrile phase 0.1 ml of 10% ammonia solution was added and the whole solution was transferred to 5 ml centrifuge vial containing 100 mg of octadecyl sorbent. The vial was tightly capped and vortexed for 30 s. After the centrifugation for 5 min at 4 000 rpm, the solution was separated from the solids and transferred to the test tube. The solution was accurately evaporated to dryness under nitrogen stream at 40°C, the residue was dissolved in 400 µl of mobile phase.

**Liquid chromatography - fluorescence detection.** The chromatographic system consisted of a Shimadzu Class VP Series high performance liquid chromatograph (Germany) equipped with a degasser and a mixer of mobile phase. A fluorescence detector FR-10AXL with excitation wavelength of 280 nm and emission wavelength of 465 nm was used to analyse the tested solutions. LC control, data acquisition, and peak integration were performed by system controller SCL-10A utilising the RS-232C interface for communication with the CLASS-VP 6.10 chromatography workstation. The chromatographic separation was performed with isocratic elution on a Luna C18 (150 x 4.6 mm, 3 µ) analytical column (Phenomenex, Germany). The mobile phase for LC analyses consisted of acetonitrile and phosphoric acid (25 mM) with hexane-1-sulphonic acid (2.5 mM) solution (20:80, v/v). Typical chromatograms obtained from the standard solution of FQs at 100 ng/mL, the extract of blank bovine muscle, and the extract of muscle spiked with FQs at the level corresponding to 1 x MRL are shown in Fig. 1. The validation parameters obtained for FQ determinations in spiked muscle samples are listed in Table 1.

The FQs individual solution prepared in 1 M solution of sodium hydroxide in methanol and stored at -20°C were stable for 6 months. The stability of the FQs mixed solution stored at 4°C was 3 months, and the working solution was stable for 1 month. The muscle samples spiked at level of 100 µg/kg were examined every week, and the stability of the analysed samples was estimated for at least 6 weeks.

**Results**

In the prepared procedure, we used the isocratic elution on the column Luna C18 (150 x 4.6 mm, 3 µ) with the mobile consisted of acetonitrile and phosphoric acid (25 mM) with hexane-1-sulphonic acid (2.5 mM) solution (20:80, v/v). Typical chromatograms obtained from the standard solution of FQs at 100 ng/mL, the extract of blank bovine muscle, and the extract of muscle spiked with FQs at the level corresponding to 1 x MRL are shown in Fig. 1. The validation parameters obtained for FQ determinations in spiked muscle samples are listed in Table 1.

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**Table 1** Validation report

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>CIP</th>
<th>ENR</th>
<th>SAR</th>
<th>DAN</th>
<th>DIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear regression equation, (y=mx+b)</td>
<td>0.448x+0.564</td>
<td>0.244x+0.655</td>
<td>0.596x+1.341</td>
<td>0.476x+0.614</td>
<td>0.337x+1.032</td>
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<td>Correlation coefficient</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Decision limit, (µg/kg)</td>
<td>120.8</td>
<td>116.5</td>
<td>216.0</td>
<td>231.7</td>
<td>356.8</td>
</tr>
<tr>
<td>Detection capability, (µg/kg)</td>
<td>145.5</td>
<td>132.8</td>
<td>240.0</td>
<td>276.0</td>
<td>421.0</td>
</tr>
<tr>
<td>Recovery, (%)</td>
<td>51.7</td>
<td>64.5</td>
<td>71.2</td>
<td>78.9</td>
<td>80.9</td>
</tr>
<tr>
<td>Repeatability, (%)</td>
<td>4.4</td>
<td>3.0</td>
<td>3.5</td>
<td>7.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Within-laboratory reproducibility, (%)</td>
<td>10.9</td>
<td>7.8</td>
<td>11.7</td>
<td>14.5</td>
<td>9.2</td>
</tr>
<tr>
<td>Uncertainty, (µg/kg)</td>
<td>14.2</td>
<td>12.8</td>
<td>25.5</td>
<td>32.9</td>
<td>46.5</td>
</tr>
</tbody>
</table>

CIP - ciprofloxacin, ENR - enrofloxacin, SAR - sarafloxacin, DAN - danofloxacin, DIF - difloxacin.
Discussion

Traditional sample preparation strategies for the determination of residues of veterinary drugs in animal tissues involve isolation with an organic solvent (e.g. acetonitrile, methanol or ethyl acetate) followed by solid phase-extraction (SPE) or matrix solid-phase extraction (MSPD) (4, 9, 11-15). However, the cleanup involving SPE with polar, non-polar or ion-exchange sorbent materials can be reproduced with varying degrees of success that are strongly influenced by the manufacturer/batch of SPE cartridge used.

Dispersive SPE is similar in some respects to MSPD, but the sorbents are added to an aliquot of the extract rather than to the original sample as in MSPD. The high cost of the sorbent limits the sample size that can be used in MSPD. This leads to concern about sample representation and homogeneity, but dispersive SPE relies on the extraction process to provide a homogenous aliquot from the original of any size and only a small amount of sorbent is used. The dispersive SPE was successfully applied for the determination of sulphonamides in animal tissues (10).

In these studies, the extraction steps were performed with acetonitrile and co-extraction of endogenous compounds from bovine and poultry muscle was very small. The parameters evaluated for the optimisation of dispersive SPE procedure were as follows: pH of the sample extract, amount of the octadecl solvent and addition of sodium chloride. The extraction with acetonitrile gave the recoveries of tested FQs from 35% to 50%. The addition of 0.1 ml of 10% ammonia improved the recovery to 45%-60% and the water removing by the addition of sodium chloride.
stabilised pH value and improved the recovery from 50% to 80%. The use of 100 mg of the octadecyl sorbent was enough to remove the most of the endogenous matrix compounds and did not have an adverse effect on the recovery of target FQs from biological matrix.

Since the piperazine-fluoroquinolones are fluorescent, LC-FLD is mainly used as determination method for routine residue analysis. Fluorescence strongly depends on the pH of the medium. The highest fluorescence is obtained at a pH value from 2.5 to 4.5 (9, 11). Usually, the separation of FQ compounds is performed by LC using reversed-phase C18 or C8 silica columns with a mixture of low-pH environment – acetonitrile or/and methanol as mobile phase (14, 15). In this paper, the separation of the five FQs from endogenous compounds was performed by the Luna C18 analytical column and the mobile phase contained acetonitrile and phosphoric acid (25 mM) with hexane-1-sulphonic acid (2.5 mM) solution (pH=3.5). The experiments indicated that tested FQs could be fully separated from matrix compounds after application of isocratic elution. No interfering peaks from endogenous compounds as well as from other antibacterial agents were found in the retention time of the target FQs.

The validation preformed with the recommendations of the Commission Decision 2002/657/EC indicated linearity in the range from 0.25×MRL to 4.0×MRL for the five FQs. The obtained results were repeatable and reproducible, the repeatability RSDs for the five FQs were lower than 10% at all fortification levels and within-laboratory reproducibility were lower than 15%. The mean recovery 51.7% was obtained for CIP and 64.5% for ENR at all fortification levels. SAR was extracted with 71.2% efficiency from bovine muscle. DAN was extracted with 78.9 % and DIF with 81.00% recovery. The above results may suggest the influence of chemical structure on an isolation efficiency from the biological matrix. All tested FQs have amphoteric character; however, ENR and its metabolite CIP have slightly different structural formula than the other FQs compounds.

The developed procedure is simple, rapid, inexpensive, and the obtained validation results indicate that it can be used for residue control purposes. The whole procedure was verified in the proficiency test prepared in our laboratory for the local veterinary laboratories. In those studies, the muscle tissues were spiked with ENR at the level of 100 µg/kg. The obtained for all laboratories values of z-score varied from -0.77 to +0.11 and they indicated that the prepared procedure allow to analyse FQs at repeatable levels by different laboratories.

References


