SIMULTANEOUS DETERMINATION OF FLUOROQUINOLONES IN FEED BY LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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Abstract

An HPLC method with fluorescence detection (HPLC-FLD) was developed for the simultaneous quantitative determination of enrofloxacin, ciprofloxacin, sarafloxacin, and difloxacin in feed. The samples were extracted with 0.25% formic acid in ultrasonic bath and purified by solid phase extraction (SPE) on Strata-X cartridges. The samples were analysed on Luna C8 liquid chromatography column with gradient programme by acetonitrile and 0.025 M phosphoric acid with 0.0025 M sodium 1-heptanesulfonate monohydrate. The method was successfully validated according to the requirements of the European Decision 2002/657/EC. Recoveries of the components from spiked feed samples ranged from 55%–70%. Repeatability was <7%. The method presented here proved to be efficient, rapid, and a selective approach for determination of fluoroquinolones in feed.

Key words: feed, fluoroquinolones, residues, liquid chromatography.

Fluoroquinolone (ciprofloxacin (CIP), enrofloxacin (ENR), sarafloxacin (SAR), and difloxacin (DIF)) antibiotics derived from nalidixic acid and belong to the second generation of quinolones. They constitute a very important group of synthetic antimicrobials, which are still widely used in human and veterinary medicine (4, 5). The antibiotics are broad-spectrum effective antibacterial agents. Their action is based on DNA gyrase, an essential bacterial enzyme that maintains superhelical twists in DNA (12). The general structure contains carboxylic group, which makes all these compounds acidic. The *pK_a* values of fluoroquinolones in water obtained by capillary electrophoresis method are as follows: CIP–5.86, ENR–5.88, SAR–5.62, and DIF–5.66 (5, 6, 11). The molecular structure of the fluoroquinolones is shown in Fig. 1. In the veterinary medicine, fluoroquinolones are used for the treatment of a high number of infections and added to feeds, which allow to keep the animals healthy. In contrast, the illegal and excessive use of antibiotics in feed may cause some problems in animal farms and also spread to the environment (5). More significant is the fact that this kind of situation can expose humans and animals to antibiotic resistant microbes. Several methods for the determination of fluoroquinolones in tissues, biological fluids, and food were reported (1, 3, 7, 9, 10). Only a few methods with HPLC-UV, HPLC-FLD, and HPLC-MS/MS detection have been described for the analyses of fluoroquinolones in feed (4, 5, 8).

The purpose of this study was to develop a rapid and sensitive method for determination of fluoroquinolone residues in feed. The chromatography conditions of the presented method were based on previously described analytical study for the determination of fluoroquinolones in different matrices. The sample preparation was modified by using different extraction solvent and SPE to the clean up step (1, 3, 4, 7, 9, 10).

Material and Methods

Reagents. All reagents used were of analytical grade and analytically pure. Phosphoric acid (CAS: 7664-38-2) was from Sigma-Aldrich (USA), sodium 1-heptanesulfonate monohydrate (CAS: 207300-90-1) and 98% formic acid (CAS: 64-18-6) were from Fluka (USA), sodium hydroxide (CAS: 1310-73-2) was from POCH (Poland), acetonitrile (CAS: 75-05-8) and methanol (CAS: 67-56-1) were obtained from J.T. Baker (the Netherlands). Water was deionised (>18 MΩcm⁻¹) by the Milli-Q water-purification system (USA).

Analytical standard and standard solutions. ENR (CAS: 33699), CIP (CAS: 33434), SAR (CAS: 33497), and DIF (CAS: 33984) were from Sigma-Aldrich (USA). All the fluoroquinolones were of a minimum 97% degree of purity. Strata-X (polymeric sorbent-surface modified styrene divinylbenzene, 200 mg, 3 ml) cartridges were obtained from Phenomenex (USA), syringe filters 0.22 μm PVDF (polyvinylidene fluoride) were from Restek (USA).

Fig. 1. Molecular structure of the antibiotics.

Table 1
Validation of method

Parameter	Analyses			
	ENR	CIP	SAR	DIF
Analite name				
CC α ($\mu\text{g/kg}$)	231.00	219.00	224.00	115.00
CC β ($\mu\text{g/kg}$)	242.00	226.00	237.00	124.00
LOD ($\mu\text{g/kg}$)	22.00	24.00	24.00	12.00
LOQ ($\mu\text{g/kg}$)	51.00	45.00	54.00	26.00
Correlation coefficient, r	0.99	0.99	0.98	0.99
Linearity (working range) ($\mu\text{g/kg}$)	100-300	100-300	100-300	50-150
Recovery (%)	67.00	59.00	72.00	64.00
Repeatability (%)	6.96	1.70	0.05	1.79
Within-laboratory reproducibility (%)	5.13	2.19	2.73	3.94

a)

b)

c)

Fig. 2. a) Standard solution mixture of fluoroquinolones 200 ng/mL (CIP, ENR, SAR) and 100 ng/mL (DIF), b) Blank sample chromatogram, c) Spiked sample chromatogram 100 µg/kg (CIP, ENR, SAR) and 50 µg/kg (DIF).

Individual stock standard solutions (1 mg/mL) were prepared in methanol with 1 M sodium hydroxide. Working and mixed standard solutions were prepared in acetonitrile and 0.025 M phosphoric acid with 0.0025 M sodium 1-heptanesulfonate monohydrate. Stock standards solutions were made in amber volumetric flasks and stored at -18 °C whereas working mixed standard solutions were made in amber volumetric flasks and stored at 4°C.

Instrumentation. The liquid chromatography equipment (Shimadzu VP Series, Japan) with a binary pump (LC-10ATvp), gradient controller (SCL-10 Avp),

online degasser (DGU-14A), and column thermostat (CTO-10ASvp) was coupled to a FLD (RF-10 AXL). The column was Luna C8 (5µ, 150 mm×4.6 mm) (Phenomenex). CLASS-VP V6.13 SP2 workstation was used as the data acquisition system. MPW-6K15 centrifuge (MPW Med. Instruments, Poland), SPE-12G (J.T. Baker, the Netherlands), and VLM – EVA EC1/EC2L (VLM, Germany) sample evaporator were used in sample preparation.

Sample preparation and extraction. All blank feed samples were obtained from different regions of

Poland. The samples used for validation procedure were previously determined to be free of the analysed compounds. The samples of ground feed (1 g) was fortified and 30 ml of 0.25% formic acid was added then the samples were homogenised with a Vortex mixer for 2 min, put in a ultrasonic bath to mix well for 15 min, and then centrifuged at 4,500 rpm for 15 min at 5°C.

Clean up. Aliquots of supernatants were applied into Strata-X cartridges preconditioned with 5 ml of methanol and 5 ml of water. After the application, columns were washed with 5 ml of water and vacuumed-dried for 10 min. The analytes were eluted with 0.1% formic acid in acetonitrile (0.1:99.9, v/v), (twice 3 ml). The eluates were evaporated to dryness under a stream of nitrogen at 45°C and residues were dissolved in acetonitrile and 0.025 M phosphoric acid with 0.0025 M sodium 1-heptanesulfonate monohydrate (500 µl) and filtered through 0.22 µm PVDF syringe filters into amber vials.

Liquid chromatography-fluorescence detection. The mobile phase for HPLC analysis consisted of acetonitrile (A) and 0.025 M phosphoric acid with 0.0025 M sodium 1-heptanesulfonate monohydrate (B) in gradient elution. The flow rate of 1.2 ml/min and injection volume of 20 µl were used. A gradient elution programme was started with 20 of A, decreased to 19% of A for 5 min, and increased to 21 of A for 10 min, kept at 21 of A for 10 min, then returned to initial composition for 20 min and equilibrated for other 7 min before the next injection. Chromatographic separation was performed for 27 min maintained at 30°C in a column oven thermostat. The fluorescence detector was set at the excitation wavelength = 280 nm and emission wavelength = 465 nm.

Validation procedure. The samples of feed were spiked with a mixture of four fluoroquinolones on three levels: 100, 200, and 300 µg/kg (CIP, ENR, SAR) and 50, 100, and 150 µg/kg (DIF). The spiked samples were analysed using the developed method. The obtained results were evaluated according to the Commission Decision 2002/657/EC (11). A validation study of the analytical method was performed in terms of linearity, specificity, precision, repeatability, and within-laboratory reproducibility, recovery, decision limit (CC α), and detection capability (CC β). The limit of detection (LOD) and limit of quantitation (LOQ) were checked by analysing of blank samples and looking for interfering peaks (8).

Results

Since feeds constitute a very complex matrix to analysis, the usage of acetonitrile as extraction solvent previously described (9) was replaced by water solution of formic acid. The components separation from matrix was achieved by liquid chromatography conditions modification. In our laboratory, the method was successfully validated according to new requirements of the European Decision 2002/657/EC. All parameters are shown in Table 1. The specificity of the method was checked by analysing a mixture of different types of

blank and spiked feed samples. The chromatograms of standard solution mixture and spiked and blank samples are shown in Fig. 2. No interfering peaks were detected in analyte retention time. The decision limit (CC α) and detection capability (CC β) of the method were determined on mixture of different feeds. The high correlation coefficients (0.98–0.99) indicated good correlations between analytes concentrations and peak areas from 100–300 µg/kg. The overview of the recovery and repeatability of the method on mixture of different types of feed samples were relatively high. The matrix compositions had not any impact on recovery and repeatability results. Stability of single stock standard solution of fluoroquinolones stored at -18°C retained for at least 6 months. A working mixture was made by adding each stock standard solution and diluting with solvent to the grade mark. The mixtures stored at 4 °C have been stable for at least 1 month. The stability of compounds in feed samples stored at 4 °C is at least 3 months but at room temperature, the stability of the analytes in matrix samples did not retain 1 month.

Discussion

Feed appears to be one of the most difficult matrix to isolate its compounds, especially when analysis is performed with classical detectors. The cause of these problems is large amounts of biological compounds: proteins, stains, lipids, vegetable oils, and lots of others components applied by producers. These compounds cause the interference; that is why it is necessary to develop analytical method, which will be efficient enough to eliminate interfering compounds. Therefore, optimisation of clean up step and isolation conditions are crucial. In this study, some experiments were conducted with different extraction solvents, extraction and clean up techniques, chromatography columns, and elution programmes.

Based on the structure of the molecules and their solubility properties, acidic solvents: formic acid, acetic acid, and citric acid in different percentages in water were compared. When formic and citric acids were used as the extraction solvents, all of the fluoroquinolones were extracted. With acetic acid, recovery was lower for CIP and ENR, only with 0.25% formic acid more than 55% recovery for all analytes was obtained. Most of the noted methods for the extraction of residue compounds from feed samples require a long time (more than 30 min) to the accomplished correctly extraction step. In this study the time for the extraction procedure was reduced to 15 min with an ultrasonic treatment.

Two different extraction techniques were implemented in this study: MSPD (matrix solid phase dispense extraction) with C18 sorbent and SPE (solid phase extraction). MSPD extraction was found inappropriate to separate analytes from impurities in feed samples. During the experiments, SPE extraction coupled with C18, Strata-X and Oasis HLB columns have been tried to clean up the extract. It was not

possible to separate fluoroquinolones from impurities with C18 column usage. Oasis HLB reduced interference of matrix but not enough and recoveries of compounds were very low. When the Strata-X cartridge coupled with syringe filters PVDF 0.22 µm was used the best and satisfactory result was obtained.

The separation of analytes from feeds is usually performed using C18 and C5 chromatography column (3-5). In this study, three different columns (C5, C8, and C18) were tested. The best results were obtained using the C8 column for fluoroquinolone determination. This column permits a visible separation and shapes of all peaks in the single run. The resolution was calculated for components and all values were higher than 1.5 what was the measure of the ability to separate the analytes. The isocratic and gradient programme was evaluated for the separation all of the fluoroquinolones. It was very difficult to achieve a desirable result. The presented study is the first one, which describes the usage of mobile phase containing pair agent – 0.025 M phosphoric acid with 0.0025 M sodium 1-heptanesulfonate monohydrate and acetonitrile for the determination fluoroquinolones in feeds. The isocratic separation system did not allow isolating compounds from impurities in matrix, especially CIP and ENR. It was necessary to apply the gradient separation system. Different organic solvents: ethanol, methanol, and acetonitrile were checked. The positive results were obtained with all of them but ethanol caused too high pressure on column. The best peak shapes and separations were achieved with acetonitrile as the organic component (4, 5).

The above results show that a new HPLC- FLD method developed for simultaneous determination of ENR, CIP, SAR, and DIF in feed has satisfactory accuracy and precision and is sensitive and reliable. This method can be used to detect four fluoroquinolones in different kinds of feed in routine analysis.

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