

# HPLC METHOD WITH FLUORESCENCE DETECTION FOR THE IDENTIFICATION AND QUANTITATIVE DETERMINATION OF FLUOROQUINOLONES IN MILK

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**Keywords:**  
flouroquinolone,  
HPLC, milk

**Abstract:** The aim of this study was to develop a rapid HPLC method with fluorescence detection for the separation of five fluoroquinolones in milk: norfloxacin (NFX), ofloxacin (OFL), moxifloxacin (MOX), ciprofloxacin (CFX), enrofloxacin (EFX) and the validation of a HPLC method for the determination of NFX and CFX in milk. The separation was performed on a Phenomenex Luna C 18 column, 150x4, 60 mm, 3 mm, having as mobile phase a mixture of: A = 20 mmol KH<sub>2</sub>PO<sub>4</sub> tetra methyl ammonium hydroxide 10% brought to pH 3 with H<sub>3</sub>PO<sub>4</sub> 85% and B = methanol in linear gradient of concentration. Mobile phase flow rates were variable from 0.7 to 0.8 ml/min. For the determination of NFX and CFX flow rate was set at 0.8 ml/min. For determination, a fluorescence detector was used (excitation at 278 nm and emission at 455 nm). Analysis of milk samples was done after protein precipitation with 20% perchloric acid. In these conditions, the chromatographic retention times of the 5 fluoroquinolones were: MOX (rT = 4.31 min), EFX (rT = 6.14 min), OFL (rT = 8.05 min), CFX (rT = 9.24 min) and NFX (rT = 10.73 min). The method is linear and specific for the concentration range of 10-1000 ng/ml milk. The method developed shows good separation of the five fluoroquinolones and allows a rapid screening of their content in milk. A simple method was validated for the determination of ciprofloxacin and norfloxacin in milk, the analytical performance of the method was verified through the most important validation parameters.

**Cuvinte cheie:**  
fluoroquinolone,  
HPLC, lapte

**Rezumat:** Scopul acestei lucrări a fost elaborarea unei metode rapide HPLC cu detecție de fluorescență pentru separarea a cinci fluorochinolone din lapte: norfloxacină (NFX), ofloxacină (OFL), moxifloxacină (MOX), ciprofloxacină (CFX), enrofloxacină (EFX) precum și validarea unei metode HPLC de dozare a CFX și NFX din lapte. Separarea s-a făcut pe o coloană Luna Phenomenex C 18, 150x4,60 mm; 3 μm având ca și fază mobilă un amestec de A= KH<sub>2</sub>PO<sub>4</sub> 20 mmol cu hidroxid de tetra metil amoniu 10% adus la pH=3 cu H<sub>3</sub>PO<sub>4</sub> 85% și B= metanol în gradient liniar de concentrație. Debitul fazei mobile a fost variabil 0,7- 0,8 ml/min. Pentru dozarea CFX și NFX debitul a fost fixat la 0.8 ml/min. În determinări s-a folosit un detector de fluorescență (excitare la 278 nm și emisie la 455 nm). Analiza probelor de lapte s-a făcut după precipitarea proteinelor cu acid percloric 20%. În aceste condiții cromatografice, timpii de retenție au fost: MOX (tR= 4.31 min), EFX (tR= 6.14 min), OFL (tR= 8.05 min), CFX (tR = 9.24 min,) și NFX (tR=10,73 min). Metoda este liniară și specifică pe domeniul de concentrație 10-1000 ng/ml lapte. Metoda elaborată prezintă o bună separare a celor cinci fluorochinolone și permite un screening rapid al conținutului lor în lapte. A fost validată o metodă simplă de determinare a ciprofloxacinei și a norfloxacinei din lapte, fiind verificate performanțele analitice ale metodei prin cei mai importanți parametri de validare.

## INTRODUCTION

Quinolones are synthetic antibiotics that act bactericidal by inhibiting DNA gyrase and topoisomerase IV, essential enzymes for the transcription and DNA replication. Fluoroquinolones, fluorinated derivatives of quinolones, have broader spectrum of activity than these, including *Pseudomonas aeruginosa* and gram-positive and gram-negative bacteria. Quinolone-resistant bacteria (for example, nalidixic acid, oxolinic acid), may be susceptible to fluoroquinolones, but fluoroquinolone-resistant bacteria generally have a cross-resistant to other fluoroquinolones.(1)

Initially, fluoroquinolones had a good activity in vitro for the *Campylobacter* species, but since 1991, *Campylobacter jejuni* and *Campylobacter coli* resistance in humans has been reported. This resistance coincided with the introduction of fluoroquinolones in veterinary medicine.(2,3,4)

The use of fluoroquinolones in veterinary practice is quite common. Consumed by pregnant women, milk contaminated with fluoroquinolones may cause the accumulation of fluoroquinolones in the growth cartilage of long bones and produce irreversible arthropathy with functional impairment in the fetus. Prescribing fluoroquinolones in children during growth is reserved for severe cases, when alternative treatments cannot be used or proved ineffective. Ciprofloxacin is the cheapest fluoroquinolon and is often prescribed for the treatment of animals. Norfloxacin has also an increased incidence in veterinary medicine. FDA (Food and Drug Administration) includes ciprofloxacin in category C of risk in pregnancy.

Although the European Union has established the number and frequency of analyzes for the antibiotics residues through the EU Directive 96/23, the analytical methods

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Article received on 5.11.2013 and accepted for publication on 20.01.2014  
ACTA MEDICA TRANSILVANICA March 2014;2(1):151-153

## CLINICAL ASPECTS

available until now are insufficient to achieve these requirements. Worldwide, it is necessary to develop methods for the simultaneous determination of several types of antibiotics which would increase food safety.(5,6)

### PURPOSE

The aim of the study was to develop a rapid HPLC method with fluorescence detection for the separation of five fluoroquinolone (FQ) in milk: norfloxacin (NFX), ofloxacin (OFL), moxifloxacin (MOX), ciprofloxacin (CFX), enrofloxacin (EFX) and the validation of a method for the determination of NFX and CFX.

### METHODS

#### 1. Reagents and standards

All solvents were ultrapure, HPLC quality, and were purchased from Merck: acetonitrile, phosphoric acid, tetramethyl ammonium hydroxide, perchloric acid. Potassium dihydrogen phosphate was purchased also from Merck. Ultrapure water was obtained with a Millipore water purification system. For the analysis, we used commercially milk, from different companies, with fat content of 1.5% and 0.1%.

Norfloxacin, ofloxacin, moxifloxacin, ciprofloxacin and enrofloxacin standards were purchased from Sigma Aldrich.

#### 2. Equipment and chromatographic system

Measurements were performed on a Merck Hitachi chromatographic system consisting of: L-7100 binary pump with degasser L-7612, L-7200 automatic injector with thermostat L-7360, fluorescence L-7480 detector.

Equipment used: AB54S balance (Mettler-Toledo), pH meter MP225 (Mettler-Toledo), centrifuge 2-15 (Sigma), mixer 10 (Falc Instruments), water purification device Direct Q (Millipore), ultrasonic bath Transsonic T700H (Elma).

Column. Separation was performed on a Kromasil 100-RP8 chromatographic column, 150 mm x 4.6 mm, 5  $\mu$ m with Kromasil RP 8 precolumn at a temperature of 25°C. The temperature of the column was 35°C.

Detection: excitation at 278 nm and emission at 455 nm, injected sample volume was 10  $\mu$ l, analysis time was 20 minutes with a 5 minute period for the rebalancing of the column.

#### 3. Mobile phase

The mobile phase for the separation of the five FQ (norfloxacin, ofloxacin, moxifloxacin, ciprofloxacin and enrofloxacin) was a mobile phase mixture of A: 20 mmol  $\text{KH}_2\text{PO}_4$ , tetra methyl ammonium hydroxide 10% brought to pH 3 with 85%  $\text{H}_3\text{PO}_4$  and B: methanol in gradient profile 0-7.5 min 80% a and 79% A and between 7.5 and 25 minutes 79% - 75% A. Mobile phase flow rate was variable from 0.7 to 0.8 ml/min.

The mobile phase for the quantitative determination of CFX and NFX: mobile phase A was 20 mmol  $\text{KH}_2\text{PO}_4$  tetra methyl ammonium hydroxide 10% brought to pH 3 with 85%  $\text{H}_3\text{PO}_4$  and B- linear gradient 80% methanol to 76% A for a period of 20 minutes. Mobile phase flow rate was 0.8 ml/min.

#### 4. Analytical method

Stock solutions. Stock solutions of FQ were prepared with concentration of 1 mg/ml in methanol acidified with HCl from which the working solution were prepared with concentrations of 2-10 mg/ml CFX respectively NFX. These solutions were used for spiking the milk samples with FQ.

Internal standard. As internal standard was used 5 $\mu$ g/ml NFX for determining CFX and 5 $\mu$ g/ml CFX for determining NFX.

Calibration curve. Calibration curves were in the range 20-100 ng/ml in milk and were prepared by spiking milk

samples with working solutions and respectively internal standard. After spiking milk samples with known concentrations of CFX or NFX and respectively internal standard, proteins were precipitated with 150 ml 20% perchloric acid. After vortexing for 30 seconds the samples were centrifuged for 10 minutes at 15,000 rpm. 10  $\mu$ l clear supernatant were injected into the chromatographic system.

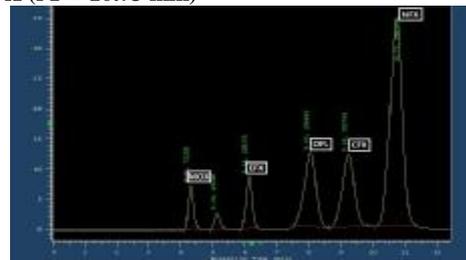
Preparation of control samples QCA, QCB, QCC. Control solutions were prepared with concentrations of 2 mg/ml, 5 mg/ml to 8 mg/ml CFX and respectively NFX.

### RESULTS

#### 1. Separating FQ mixed

After preparation and injection of milk samples marked with concentrations of 100 ng/ml fluoroquinolones, the five fluoroquinolones separated in less than 11 minutes, the order of separation is as follows: MOX (rT = 4.31 min), EFX (rT = 6.14 min), OFL (rT = 8.05 min), CFX (rT = 9.24 min) and NFX (rT = 10.73 min) (figure no. 1).

Figure no. 1. The chromatogram of a milk sample with concentration of 100 ng/ml FQ: MOX (rT = 4.31min), EFX (rT = 6.14 min), OFL (rT = 8.05 min), CFX (rT = 9.24 min) and NFX (rT = 10.73 min)



#### 2. Validation of a dosage method for CFX and NFX in milk

The specificity of the method. It was verified on six blank samples of milk taken individually. Under the experimental conditions described above at the retention time of CFX or NFX there are no matrix interfering (figure no. 2 and figure no. 3).

Figure no. 2. The chromatogram of a blank milk sample

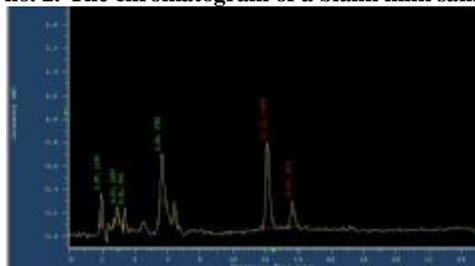
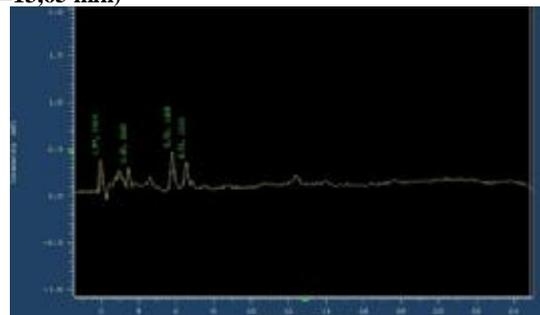


Figure no. 3. The chromatogram of a milk sample spiked with CFX 2 ng/ml (rT=12,12 min) and NFX 5 ng/ml (rT=13,65 min)



## CLINICAL ASPECTS

*The linearity of the method.* 5 calibration curves were injected with 6 levels of concentration. Linearity was assessed based on the linear regression coefficient between height and concentration. CFX average calibration curve was:  $c = 152.20 (\pm 12.10) \text{ ASC} + 21.62 (\pm 5.04) (R^2 > 0.998)$  and the average calibration curve for NFX was:  $c = 103.20 (\pm 8.23) \text{ ASC} + 35.25 (\pm 7.54) (R^2 > 0.998)$ .

*Precision and accuracy of the determination.* These were assessed by the percentage variation coefficient (CV%) and respectively the relative error (E%) for the determination made in the same day and on different days. The determination were under the limit of quantification of 20% and below 15% for the other levels of concentration (table no. 1 and table no. 2)

**Table no. 1. Precision and accuracy of the determination at 20 ng/ml, 40ng/ml, 80 ng/ml CFX for the determination made in the same day and on different days**

CFX ng/ml	C <sub>nominal</sub> ng/ml	Same day (n=5)			Different days (n=5)		
		C <sub>coverage found</sub> ng/ml ± SD	CV%	Rel. E%	C <sub>coverage found</sub> ng/ml ± SD	CV%	Rel. E%
QCA	20	18,05±1,29	7,16	-9,71	17,25±0,74	4,28	-13,71
QCB	40	45,48 ±2,90	6,37	13,71	43,46 ±3,78	8,71	8,66
QCC	80	85,57 ±3,38	3,95	6,97	81,73 ±2,59	3,17	2,16

**Table no. 2. Precision and accuracy of the determination at 20 ng/ml, 40ng/ml, 80 ng/ml NFX for the determination made in the same day and on different days**

NFX ng/ml	C <sub>nominal</sub> ng/ml	Same day (n=5)			Different days (n=5)		
		C <sub>coverage found</sub> ng/ml ± DS	CV%	E rel%	C <sub>coverage found</sub> ng/ml ± DS	CV%	E rel%
QCA	20	19,17 ± 2,89	15,11	-4,13	21,95 ±3,47	15,84	9,75
QCB	40	36,91±2,08	5,65	-7,71	37,33±3,50	9,38	-6,66
QCC	80	76,81±1,91	2,49	-3,98	78,95±5,48	6,95	-1,37

Fluoroquinolones present stability also stored at room temperature, freezing and thawing, stored in the injector and at long time periods at  $-20^{\circ}\text{C}$ , the relative percentage error was within the limits of  $\pm 15\%$ .

The accuracy for the dilution of samples was within the limits of  $\pm 11\%$  and the precision was less than 4%.

### DISCUSSIONS

In order to optimize the method, different mobile phases were tested and finally we stopped at the mobile phase composition previously mentioned. A concentrated solution of 100 ng/ml FQ was injected, the peaks were different from the interference due to various components of milk extracted together with these substances from milk. Among the most important, interference occurs at 5.15 minutes without affecting the specificity of the method (retention time is different from that of the studied fluoroquinolones).

In the chromatogram of a blank milk samples we can observe that at the retention times of the fluoroquinolones: ofloxacin, ciprofloxacin and norfloxacin there are no interference due to normal existing compounds in milk. Peaks that appear in the blank milk sample are physiological components of milk that appeared in all blank milk samples we have processed.

The method developed is rapid, sensitive, reproducible, does not require laborious samples processing and also is not very expensive like other methods in the literature

using more expensive and laborious techniques as capillary electrophoresis or MS techniques.(7,8,9)

#### **Acknowledgement:**

*The authors thank Assist. Dr. Ms. Aura Rusu for the fluoroquinolone standards provided.*

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