Fluoroquinolone Residues in Raw Cow's Milk

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Abstract

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The aim of the study was to monitore the levels of fluoroquinolone residues in bulk samples of raw cow's milk. The bulk samples of raw cow's milk (n = 150) were obtained from 58 different milk suppliers in the South Moravian and Vysočina Regions. The samples were analysed by reverse phase high-performance liquid chromatography method with fluorescence detection and gradient elution. 87.3% of the raw milk samples were positive for the fluoroquinolones residues. Flumequine was present in none of the samples. The levels of other fluoroquinolones investigated were below the recommended maximum residue limit. The results of the study indicate that fluoroquinolones are frequently administered to the dairy cows in spite of the recommendations of CVMP. The most frequently determined was enrofloxacin and its indicator residue, i.e. ciprofloxacin. An efficient control of the veterinary drugs residues in milk is very important to ensure the safety of the milk and milk products.

Keywords: veterinaty drug; RP HPCL; food safety; MRL; LOD; LOQ

The monitoring of the veterinary drugs residues is an important part of the food safety control in raw materials and foods of animal origin. To ensure the safety of food of animal origin for consumers, maximum residue limits (MRL) of veterinary drugs used with the food animals have been set for raw materials of animal origin (Botsoglou & Fletouris 2001; Commission Regulation 37/2010).

Since 1976, when the first monofluoroquinolone flumequine was developed, very many fluoroquinolone representatives have been synthetised and described. Compared with the classic 1st generation quinolones that were characterised by a narrow spectrum of action and poor tissue penetration ability, fluoroquinolones feature many attributes of ideal antimicrobials (Sarkőzy 2001; Emmerson & Jones 2003). Fluoroquinolones are used for the treatment of infections caused by various bacterial agents in both human and veterinary medicine. Fluoroquinolones are effective for the therapy of serious diseases, e.g. septicaemia, gastroenteritis, and respiratory diseases caused by Gram-negative bacteria. They are also used for the treatment of infections of the urinary tract and skin, and of infections of soft tissues caused by Gram-negative and certain Gram-positive aerobic bacteria. They are effective in the therapy for mycoplasma infections and infections caused by atypical bacteria. Incidence of side adverse effects of fluoroquinolone treatment, particularly in human medicine, has been reported (Flomenbaum *et al*. 2006; CVMP 2007).

In veterinary medicine, they are useful especially in the therapy for gastrointestinal and respiration infections (Botsoglou & Fletouris 2001). Fluoroquinolones are used in intramammary prepara-

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tions for the treatment of mastitis in lactating dairy cows, for dairy cows during dry periods, and also for mastitis prevention (Gruet *et al*. 2001).

Although fluoroquinolones found a wide application in primary agriculture, their administration to food animals has been discussed in recent years in connection with the increased incidence of resistance among human pathogenic micro-organisms. From the onset of the bacterial resistance point of view, fluoroquinolones belong to the high-risk groups of antimicrobial drugs (WHO 1998; CVMP 2007). The World Health Organisation (WHO) and Word Organisation of Animal Health (OIE) have defined quinolones as "critically important antimicrobials" for human and animal health, respectively (FAO 2007).

Due to extra-label use of veterinary drugs or noncompliance withdrawal periods, much higher residue levels might appear in the edible animal products (Botsoglou & Fletouris 2001). The published data indicated that violative residues were found in raw or heat treated milk samples in countries all over the world (Botsoglou & Fletouris 2001; Tolentino *et al*. 2005; Fonseca *et al*. 2009; Kaya & Filazi 2010; Addo *et al*. 2011). Quality control of the purchased raw cow's milk implies that 0.16% samples showed violative concentrations of residues (Kopunetz 2010).

Within the EU, each state is obliged to monitor foodstuffs for the residues of veterinary drugs and to present a National Residue Monitoring Plan that takes into account the specific situation in its country (Botsoglou & Fletouris 2001). In the Czech Republic, milk is monitored for the presence of veterinary drugs residues in accredited laboratories as part of their food chain monitoring for exogenous substances, and also during quality checks of the raw milk. Screening tests for veterinary drugs residues in milk rely mainly on rapid tests and microbial inhibition methods (plate diffusion method) (Botsoglou & Fletouris 2001; Czech National Reference Laboratory 2008). In targeted testing for fluoroquinolone residues, modern analytical methods such as the high-performance liquid chromatography (HPLC) analysis and mass spectrometry are used (Hernández-Arteseros *et al*. 2002; Marazuela & Moreno-Bondi 2004).

The objective of the present study was to give the information on the presence of fluoroquinolone residues for which maximum residue limits were set in raw cow's milk. These imply the residues of enrofloxacin (ENRO) and its indicator residue = sum of enrofloxacin and ciprofloxacin (CIPRO), and the residues of marbofloxacin (MARBO), danofloxacin (DANO) and flumequine (FLU), and to determine their concentrations.

MATERIAL AND METHODS

Milk samples. The bulk samples of raw cow's milk (n = 150) were obtained from 58 different milk suppliers in the South Moravian and Vysočina Regions. The samples of bulk milk were collected from the milk collection route of the dairy plant. The milk samples were kept at temperatures not exceeding 4°C until the testing time.

Chemicals and materials. The standards of fluoroquinolones, i.e. of enrofloxacin (17849), flumequine (F7016), marbofloxacin (34039), danofloxacin (33700), ciprofloxacin (17850), and norfloxacin (N 9890) were purchased from Sigma Aldrich (St. Louis, USA). The chemicals for HPLC analysis, i.e. acetonitril, methanol and dichloromethane (HPLC grade) were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid and phosphoric acid (suprapure grade) were from Fluka Chemie AG (Buchs, Switzerland) and Sigma Aldrich (St. Louis, USA). NaOH (analytical grade), was purchased from Penta (Prague, Czech Republic). The hardware included an analytical balance (Kern & Sohn GmbH, Balingen, Germany), a cooling centrifuge (Mechanika Precyzyjna, Bytom, Poland), a rotary vacuum evaporator (Bűchi, Postfach, Switzerland), and a vortex (Merck, Darmstadt, Germany).

Preparation of standard fluoroquinolone solutions. The stock solutions at active substance concentration of c = 1 g/l were first prepared by dissolving an adequate amount of the chemotherapeutic in 1 ml of 0.1 mol/l NaOH and adding deionised water to a total volume of 25 ml. The stock solutions of chemotherapeutics were then further diluted with deionised water to obtain working solutions with a concentration in the 0.1–1.0 mg/l range that were then used to prepare fortified milk samples for the method validation.

Preparation of samples for HPLC analysis. Quinolones were extracted from the milk samples by the liquid/liquid extraction method. Concentrated phosphoric acid solution (0.5 ml) was added to 5 ml of milk and vortexed for 5 seconds. Acetonitrile (10 ml) was pipetted into the mixture

for extraction by vortexing for 1 minute. The resulting mixture was then centrifuged at 4000 rpm for 10 min at 5°C. The supernatant was collected (10 ml) and acetonitrile was evaporated. Two ml of dichloromethane were added to the residue, and the mixture was again vortexed for 10 s and centrifuged at 4000 rpm for 10 min at 5°C. The 1 ml sample collected from the upper aqueous layer was diluted with the mobile A phase at a ratio of 1:1 for HPLC analysis.

HPLC conditions. The HPLC assay was performed on an Alliance 2996 liquid chromatograph with a 2475 fluorescence detector (Waters, Milford, USA). The separation was performed on an Atlantis T3 chromatographic column 4.6 × 250 mm with the particle size of 5 µm (Waters, Milford, USA). The mobile A phase consisted of 0.2% trifluoroacetic acid, the mobile B phase of a methanol + acetonitrile mixture (1:5), the gradient was linear from 25% B phase to 80% B phase, and the flow rate was 0.7 ml/min. The injection volume was 20 µl and the column temperature was 30°C. The wavelengths selected for the fluorescence detection were $\lambda_{ex}/\lambda_{em}$ = 280/450 nm for MARBO, CIPRO, DANO, and ENRO, and $\lambda_{ex}/\lambda_{em}$ = 312/366 nm for FLU.

Method validation. An external standard method was used for both qualitative and quantitative evaluations. Each sample was measured in two parallel assays, and at the same time, a blank sample per series was also measured. Norfloxacin was used as a surrogate standard. The milk samples not containing any of the analytes monitored were analysed to test for the method selectivity.

The calibration curves of the matrix samples were generated in the range of 3.5–140.0 µg/l for marbofloxacin, 10.0–400.0 µg/l for norfloxacin, 6.5–260 µg/l for ciprofloxacin, 2.0–80.0 µg/l for danofloxacin, 6.2–250.0 µg/l for enrofloxacin, and 1.2–48.0 µg/l for flumequine.

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the analysis of the matrix samples fortified with fluoroquinolone standards, and evaluated as 3 signal to noise ratio (S/N) and 10 S/N in µg/kg. The repeatability and recovery were determined from 12 parallel analyses of the milk samples fortified with the standards solutions of known concentrations, 0.5, 1.0, and 1.5 MRL. To determine the analyte recovery for the individual fluoroquinolones, the analytical results obtained with the fortified samples were compared with an externally generated calibration curve of the standards. Their mean values are summarised in Table 1 together with the coefficients of variation for repeatability. The decision limit CCα and the detection capability CCβ as defined by the Commission Decision 657 (2002) were determined by analysing the matrix milk samples at the concentration levels of 0.5, 1.0, and 1.5 MRL of each analyte.

Statistical evaluation. Basic statistical evaluation of the results was performed using Statistica 7 statistical software (StatSoft, Prague, Czech Republic).

RESULTS AND DISCUSSION

Food safety is an important issue in the EU. Foodstuffs that contain violative antibiotic residues constitute public health hazards including toxicological (direct toxic effect), microbiological

Table 1. Validation results of HPLC with fluorescence detection: recovery, repeatability, LOD, LOQ, R^2, CCα and CCβ values

Parameter	Marbofloxacin	Ciprofloxacin	Danofloxacin	Flumequine	Enrofloxacin
Recovery (%)	89.1	97.1	91.6	82.6	103.0
RSD (%)	5.4	10.1	3.4	3.7	7.1
R^2	0.98	0.99	0.99	0.99	0.98
LOD (µg/kg)	7.0	6.0	4.0	3.0	6.0
LOQ (µg/kg)	21.0	18.0	12.0	10.0	18.0
CCα (µg/kg)	79	108	31	51	104
CCβ (µg/kg)	83	116	32	52	108

RSD = relative standard deviation; LOD – the limit of detection; LOQ – the limit of quantification; *n* – number of measurements; R^2 – linearity; CCα - decision limit; CCβ – detection capability

(transmittance of antibiotic resistance, adverse effects on the ecology of human intestinal microflora), and immunopathological (hypersensitivity reactions) hazards (Roeder & Roeder 2000). The incidence of some side adverse effects of fluoroquinolone treatment has been described. The most important side effects of fluoroquinolones include renal impairment, ocular disorders, and damage to articular cartilage. Articular cartilage damage is irreversible and for that reason the preparations containing fluoroquinolones must not be administered to adolescents and pregnant women. Other adverse effects include, e.g., frequent gastrointestinal disturbances (diminished appetite, nausea, abdominal pain, vomiting, diarrhoea), headache, insomnia, tension, or skin allergy manifestations (Flomenbaum *et al*. 2006).

The Committee for Medicinal Products for Veterinary Use (CVMP) classifies quinolone preparations into critically important antimicrobials that should be administered in accordance with prudent use principles. Prudent use of quinolones is defined as the practices that maximise therapeutic effect while minimising the emergence of resistance (CVMP 2007; WHO 1998). At present, injection preparations containing danofloxacin, enrofloxacin, difloxacin, and marbofloxacin are registered in the Czech Republic for use in lactating dairy cows. Raw cow's milk, irrespective of whether it is to be processed or sold directly to consumers, must meet the quality standards set by EU legislation. Neither raw cow's milk nor milk of other animal species may be placed on the market if it contains the residues of pharmacologically active substances in quantities in excess of the set MRLs, or if it contains substances whose use in food animals is prohibited (Commission Regulation 1662/2006). MRL levels set by EU legislation for fluoroquinolones in milk differ widely between the individual compounds, from 0.03 mg/kg for danofloxacin and 0.05 mg/kg for flumequine to 0.075 mg/kg for marbofloxacin and 0.1 mg/kg for the sum of enrofloxacin and ciprofloxacin residues (Commission Regulation 37/2010).

For the detection of the selected fluoroquinolones in raw cow's milk, HPLC analysis with fluorescence detection was validated (Table 1). In the process of the method validation, CCα and CCβ were determined in compliance with the legislation (Commission Decision 657/2002) (Table 1).

Chung *et al*. (2009) conducted a study in which they used HPLC analysis with fluorescence detection to determine ENRO and CIPRO residues. Compared with our study, their analysis validation recovery was higher for CIPRO (101.3%) but lower for ENRO (72.4%). The limits of detection were in their study 6.5 µg/kg and 1.1 µg/kg, and the limits of quantification 23.2 µg/kg, and 3.0 µg/kg for CIPRO and ENRO, respectively. Hermo *et al*. (2008) developed and applied various methods of liquid chromatography for the determination of quinolone residues in milk at MRL levels, and compared them with the mass spectrometry method. For all quinolones, a recovery in excess of 80% in the validation of LC methods was attained. The LC-FD method produced LOD in the 3–8 ng/g range. The limits of quantification (LOQ) were determined at levels below the MRL with the only exception of MARBO in LC-UV. In compliance with EU legislation, they determined CCα and CCβ values of the individual methods when performing the validation. Their LC-FD method validation of

Table 2. Concentrations of residues of fluoroquinolones determined in bulk samples of raw cow's milk

	Fluoroquinolone (µg/l)				
	enrofloxacin	ciprofloxacin	sum of enrofloxacin and ciprofloxacin	marbofloxacin	danofloxacin
n_1/n	115/150	122/150	131/150	22/150	33/150
$\bar{x}$ (µg/l)	10.24	8.23	16.70	5.42	3.41
Min (µg/l)	1.70	2.70	2.70	5.00	2.00
Max (µg/l)	18.60	17.40	27.40	6.00	10.20
SD (µg/l)	1.64	2.72	5.03	0.28	1.84
CV (%)	16.01	33.08	30.09	5.24	54.00

n_1 – number of positive samples; n – total number of examined samples; $\bar{x}$ - mean; min and max – minimum and maximum values; SD – standard deviation; *CV* – coefficient of variation

the fluoroquinolones analysed gave lower CCβ and CCα values that those in the present study (Table 1), with the exception of CCα value for MARBO, which is the same as in our study.

The residues of ENRO and of its main metabolite CIPRO were detected in 87.3% of the total number of the samples tested (Table 2). The presence of only ENRO was determined in 10 samples, and the presence of only CIPRO, its main metabolite, was determined in 13 samples. The maximum values of enrofloxacin and ciprofloxacin residues demonstrate that their levels in the samples were very low (Table 2). The sum of enrofloxacin residues and its indicator residues (= sum of enrofloxacin and ciprofloxacin) did not exceed the MLR value (0.1 mg/kg) in any of the bulk sample. In 13% of the samples, the total concentration of ENRO and CIPRO residues was less than 10 ± 5.03 μg/l, in 65.6% of the samples it was 10–20 ± 5.03 μg/l, and in 21.4% it was in the 20–25 ± 5.03 μg/l range. Only 4 samples (3%) revealed concentrations higher than 25 ± 5.03 μg/l. The highest ENRO and CIPRO concentration found was 27.4 ± 5.03 μg/l. The total sum of ENRO residues eliminated from the body is made up, besides the original substance, of two main metabolites, i.e. enrofloxacin amide and ciprofloxacin, and, to a lesser degree, other metabolites. If enrofloxacin is administered intravenously to dairy cows, ciprofloxacin may continue to be detected at relatively high concentrations over a longer period than enrofloxacin itself (Botsoglou & Fletouris 2001).

MARBO residues were detected in only 14.6% of the total of the samples examined (Table 2). MARBO is a fluoroquinolone that is used to treat bovine respiratory diseases. The studies monitoring the elimination of MARBO residues in livestock demonstrated that 73–89% of the total sum of residues in lactating dairy cows milk is made up of the original substance (Botsoglou & Fletouris 2001).

DANO residues were present in only 22% of samples. The residue concentrations were in the 2.7–27.4 ± 5.03 μg/l range (Table 2). DANO exhibits a wide spectrum of activity – it is effective against both Gram-negative and Gram-positive bacteria and mycoplasma, but it is less effective against anaerobic microorganisms (Aliabadi *et al.* 2003). The transfer of DANO from blood to milk was very rapid with maximum milk concentration having been reached only 8 h after the administration. On the basis of their study, Mestorino *et al.* (2009) recommend the use of 18% DANO administered subcutaneously for the treatment of mastitis and other infectious diseases in dairy cattle provided that a 3-day withdrawal period is observed.

The HPLC method failed to demonstrate the presence of flumequine in the bulk samples of raw cow's milk. The results are consistent with the fact that no flumequine-containing drug for use in lactating dairy cows is registered in the Czech Republic.

CONCLUSIONS

HPLC with fluorescence detection was used to detect the residues of the monitored fluoroquinolones (ENRO, CIPRO, MARBO, and DANO) in bulk samples of raw cow's milk. The results of the study indicate that fluoroquinolones are frequently administered to dairy cows in spite of the recommendations of CVMP. The residue levels determined did not exceed MRL in any of the samples. Flumequine was not present in any of the samples. The most frequently determined in the samples was enrofloxacin and its indicator residue, i.e. ciprofloxacin. An efficient control of the residues in milk is very important to ensure the safety of milk and milk products.

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