Detection of residues of quinolones in milk

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Abstract

For the detection of enro- and ciprofloxacin (EFX, CFX) residues in milk on the level of an expected MRL of <10 µg/kg as well screening (microbial inhibitor test with E.coli, ELISA) as well as confirmation (HPLC) methods were developed and applied to the examination of milk of 3 cows treated with Baytril®. The detection of further quinolones by these methods was tested in orientating experiments. HPLC analysis is performed after solid phase extraction with oxalic acid/triethylamine/acetonitrile as an eluent and fluorometric detection (295 nm/495 nm); limit of was ≤2.5 µg/kg for CFX, EFX, marbofloxacin and danofloxacin. In the milk of the treated cows CFX, EFX, CFX metabolite M1 and two unidentified substances were detected. No false negative results of the screening methods with respect to HPLC values ≥3.0 µg/kg were observed.

Introduction

Enrofloxacin (EFX) belonging to the group of quinolones is widely used in the treatment of bacterial infections in veterinary medicine, but it is not (yet) licensed for the treatment of lactating cows. A biologically active metabolite ciprofloxacin (CFX), which has been restricted to use in human medicine, is excreted with milk after EFX treatment. A maximum acceptable daily intake (ADI) value of 18.75 µg EFX + CFX/person was derived. According to EU Regulation 2901/93 a maximum residue limit (MRL) of 30 µg/kg is fixed for muscle, liver and kidney. Based on a daily food intake of 500 g of combined tissue and 1500 ml milk following the ADI-concept a MRL of about 2.5 µg EFX + CFX/kg for milk has to be expected. The MRL of marbofloxacin (MFX) in milk is 75 µg/kg (EU Regulation 2017/96).

Methods for an integrated detection system (1) which comprise a microbiological inhibitor test for screening, an antibody-capture immunoassay (ELISA) for preliminary confirmation and a HPLC method for confirmation were developed and put to test with milk samples of EFX-treated cows. The detection of further quinolones by these methods was tested in orientating experiments

Materials and methods

Quinolones:

EFX, CFX and metabolites M1-M4 of CFX were donated by Bayer, Wuppertal (DE), danofloxacin (DFX) by Pfizer, Karlsruhe (DE), MFX by Laboratoire Pharmaceutique Véterinaire, Lure Cedex (F) and Sarafloxacin (SFX) by Abbott Laboratories, Chicago (US) and flumequin (FQ) was purchased at Sigma (DE). For further dilutions 1 million µg/kg stock solutions in alkaline methanol were prepared.

Treatment trial:

3 healthy cows (somatic cell counts <50 000/ml, milk yield 19-23 kg/day) of the experimental herd of the Federal Dairy Research Centre were treated i.v. with 20 ml of Baytril® (Bayer, DE); samples were taken before treatment, 2, 4, 6, 8, 10 hours after treatment and afterwards during normal milking time (14/10 hours), stored at -18°C and analyzed within at 3 weeks at maximum.

Microbial inhibitor test:

Agar diffusion test at pH 0.5 with *Escherichia* coli ATCC 11303 (2) as test microorganism and bromcresol purple as indicator in microtitre plates. Milk samples are heated (10 min at 80°C) before analysis. On each test plate negative and positive controls (10 µg EFX/kg) have to be analyzed.

Incubation:

1 h at 6°C and 18 h at 37°C. Test results are evaluated visually (purple = positive, yellow = negative) or by photometric measurement by an ELISA reader (3). The following detection limits (µg/kg) were determined: CFX 4, DFX 8, EFX 10, FQ 500, MFX 10 and SFX 20 (4).

ELISA:

Antibody-capture immunoassay for EFX with 100% cross reaction with CFX and limit of detection (LOD) of 1.56 µg EFX equivalents/kg (5).

HPLC:

The vacuum manifold was an Adsorbex solid-phase extraction unit (Merck). The SPE columns contain 500 mg Chromabond C18ec (Macherey-Nagel). A model L-6200 gradient pump, F-1080 fluorescence detector, AS-2000 autosampler, D-6000 interface, LC-organizer and L-5025 column thermostat were supplied by Merck. The analytical cartridges were EcoCART 125 mm/3 mm (Merck, DE) with Superspher 60RP 8ec.

SFX (50 µg/kg milk) was added to the milk sample as an internal standard. A mixture of 5 ml milk, 3 ml acetonitrile (ACN), 2 ml trichloracetic acid and 5 ml water was centrifuged and filtered. To 12 ml filtrate 0.5 ml sodium acetate buffer and 10 ml water were added. SPE-column conditioning was carried out with 5 ml ACN and 5 ml water. Sample elution was effected with 2 ml buffer (oxalic acid, triethylamine, pH 2.9)/ACN (30:20); the eluate was concentrated by vacuum concentration; the volume was made up to 2 ml with eluent (final concentration 2:1). The mobile phase was a gradient of ACN and water or eluent respectively at a flow rate of 0.5 ml/min. The column was thermostated at 30°C, the injection volume was 10 µl. The detector was operated at excitation and emission wavelengths of 295 nm and 495 nm, respectively. If also DFX, MFX and FQ have to be detected the wavelength have to be changed to 325 nm/365 nm after 21 min and the analytical time is lengthened to 30 min. For evaluation, peak areas were included into linear regression equations derived from the analysis of aqueous standard solutions. The price of consumables per sample were calculated to be <9 DEM.

Fig. 1 shows a chromatogram of aqueous standard solution (2.5 µg/kg). Analyzing CFX 4 peaks appear; one could be identified as CFX metabolite M1, whereas the two others had different retention times than the tested CFX metabolites M1-M4. **Tab. 1** comprises the data of the examination of blind and spiked samples and the derived recovery values.

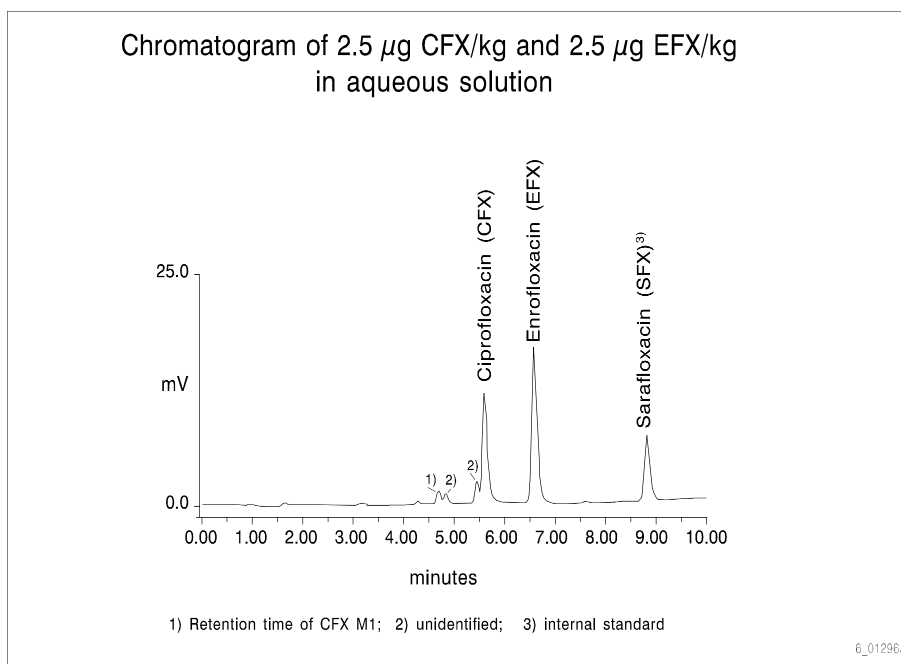

Figure 1

Table 1: Characteristics of the HPLC method for the detection of quinolones in milk

	Retention time (min)	LOD[1)] µg/kg	LOQ[1)] µg/kg	Recovery %	Recovery µg/kg	Repeatability s_r	Repeatability µg/kg
MFX	9.7	≤1	≤2.5	108[2)]	75.0	0.3	2.5
CFX	10.2	≤1	≤2.5	68	2.5	0.3	2.5
DFX	10.8	≤2	≤2.5	101	2.5	0.3	2.5
EFX	12.6	≤1.5	≤2.5	72	2.5	0.1	2.5
SFX	19.0		<50[3)]	76	50.0	–	–
FQ	26.2		<50[3)]	–	–	–	–

[1)] X_Q of negative milk + 3/6 s respectively; [2)] without SPE, [3)] preliminary

Treatment trial

Fig. 2 shows a chromatogram of a milk sample from the anamnestic phase and of a milk sample collected 24 hours *post applicationem*. For this experiment the analytical conditions were adjusted to the detection of CFX, EFX and SFX. It becomes obvious that in the milk sample of the treated cow several peaks appeared. One showed the same retention time as CFX metabolite M1 whereas the others could not be identified.

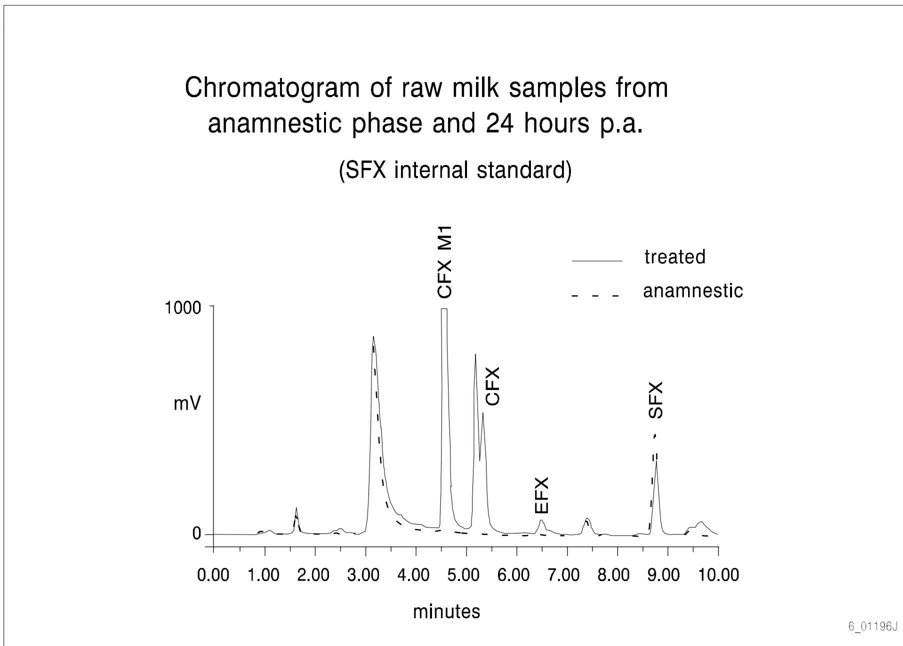

Figure 2

Tab. 2 summarizes the results of the treatment trial. It becomes evident that in the milk of cows treated with EFX its metabolite CFX can be detected in higher concentrations and for a longer period than EFX as also reported by Tyczkowska et al. (5). 82 hours p.a. CFX was <LOD and below the expected MRL of 2.5 µg/kg. The screening test with *E.coli* was positive in all samples with $\geq$3 µg CFX/kg and negative in samples $\leq$2 µg CFX/kg. The results of the ELISA were >LOD in all samples with at least 2 µg CFX/kg; 5 samples, in which no quinolones were detected by HPLC, showed values close to the LOD of the ELISA; in no case false negative results with respect to the HPLC values were observed.

Table 2: Excretion of enro- and ciprofloxacin (EFX, CFX) after i.v. treatment of 3 cows with Baytril®

Time after treatment (h)	EFX (µg/kg)[1)]	CFX (µg/kg)[1)]	ELISA (µg/kg)	*E.coli* test[2)]
-24	<LOD	<LOD	<LOD	0
-14	<LOD	<LOD	<LOD	0
0	<LOD	<LOD	<LOD	0
2	300	1863	>100	3
4	61.3	1232	>100	3
6	17.2	615	>100	3
8	9.8	273	>100	3
10	5.5	230	>100	3
24	<LOD	19.7	9.4	3
34	<LOD	11.0	6.7	3
48	<LOD	6.0	5.3	3
58	<LOD	4.6	4.5	3
72	<LOD	2.8	4.3	2
82	<LOD	<LOD	2.4[3)]	0
96	<LOD	<LOD	<LOD	0

[1)] Recovery rates considered; [2)] Number of positive results out of 3; [3)] n = 2, n = 1 <LOD

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