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Quantification of fluoroquinolone (enrofloxacin, norfloxacin and ciprofloxacin) residues in cow milk

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Abstract

The current study was designed to observe the withdrawal period of Fluoroquinolone antibiotics (enrofloxacin (ENRO), ciprofloxacin (CIPR) and norfloxacin (NOR) in cow milk. After simple deproteination of milk sample with 15% (w/v) trichloro acetic acid (TCA), the supernatant was subjected to analysis by spectrophotometer at λmax 272.5 nm for enrofloxacin, 276 nm for ciprofloxacin and 438 nm for norfloxacin. The mean ± SE value of enrofloxacin excreted in milk was 23.64 ± 1.41 µg/mL ranging from 1.85 to 52.03 µg/mL, average concentration of norfloxacin excreted in milk was 12.42 ± 0.51 µg/mL and for Ciprofloxacin it was 13.83 ± 0.67µg/mL. The washout period of enrofloxacin observed in this study was 6 days in cow milk and for norfloxacin and ciprofloxacin the washout period were 5 days and 6 days, respectively. Therefore, milk should be marked after completing the withdrawal period of these drugs in order to protect the human from its harmful effects.

Key words: Milk, Enrofloxacin, Norfloxacin, Ciprofloxacin, Fluoroquinolones

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1. Introduction

The repetitious use of veterinary antibiotics to control diseases in farm animals may result in the incidence of residues in foodstuffs of animal origin that can cause pathogen resistance to antibiotics used in human medicine [1-2]. Therefore, the protection of public health against probable harmful effects of these veterinary drugs has been a main concern for the drug regularity authorities [3]. In the past few years, new types of antibiotics have been developed to replace those which were losing their antibacterial activity. However, majority of the antibiotics developed nowadays, are related to those already in use, so the need to preserve their effectiveness is even more urgent. Fluoroquinolones (FQs) are a highly effective group of antimicrobial drugs used both, in veterinary and human medicine worldwide. Ignorance to observe label directions for these antibiotics or unintentional adulteration of feed for cow could cause disrupt residues in milk for human consumption. Exploitation of these pharmaceutical drugs may give rise to public health (e.g. antibiotic resistance, allergic reactions, etc.), industrial and environmental (e.g. cheese or yoghurt production etc.) problems and the countries must screen the presence of these drugs and other veterinary residues in live animals and animal products [4].

An important measure of averting is the withdrawal time of milk after a cow has been treated with antibiotics, either systemically or intra-mammary. Each drug has a specific withdrawal period, however, under practical situations the excretion period of drug residues may differ due to different conditions from the withdrawal period indicated. This being the case with conformist milking systems with more or less regular milking intervals, there is a complete lack of information on the competence of the indicated withdrawal periods in a situation with more recurrent milking and with irregular intervals as in automatic milking [5].

Fluoroquinolone antibiotic (Enrofloxacin, Norfloxacin and Ciprofloxacin) has been permitted for use in food origin animals and is effective against organisms resistant to antibacterial substances usually used in veterinary medicine, such as aminoglycosides, β-lactam antibiotics, macrolides and tetracyclines [6-7]. The use of these drugs in lactating/breeding animals may leave the residues in meat and milk. Therefore, animal food may be a probable threat for the consumers, cause allergic and also lead to the emergence of drug-resistant bacteria. To protect consumer's health European Union set maximum residue limits (MRLs) for many drugs regarding meat, milk and others foods (Regulation no. 2377/90). Particularly the MRL

for enrofloxacin and its metabolite ciprofloxacin in milk was set as 100 mg/kg for all animal species [8-9]. The current study was designed to study the elimination or withdrawal period of fluoroquinolone residues in cow milk.

2. Materials and Methods

Animals: Twenty-four healthy female cows were selected for this study. The animals were maintained in animal shed, Department of Livestock Management, University of Agriculture Faisalabad. In the month of July and August 2012, all the animals were kept under similar conditions.

2.1 Drugs

For study, the commercial formulations of norfloxacin (DOCTORJIN, DAE Sung Microbiological Labs, Sedul, Korea), Enrofloxacin (VETY-ENROX10%, Leads pharma. (PVT) LTD. Islamabad, Pakistan) and Ciprofloxacin (CIPROSEL, Selemore Agencies (PVT) LTD. Lahore, Pakistan) were given to experimental animals.

2.2 Drug Administration

In the first phase of study, a single recommended dose of norfloxacin 5mg/kg was given intramuscularly to eight animals. Milk samples were collected at 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144 and 156 hours. In second phase of study Enrofloxacin @, 2.5mg/kg was given intramuscularly to eight animals. Milk samples were collected after 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156 and 168 hours. Similarly Ciprofloxacin @ 5mg/kg was given intramuscularly to eight animals and milk samples were collected at 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204, and 216 hours.

2.3 Collection of Milk Samples

A blank or control sample was collected prior to drug administration from each animal. Milk samples were collected at predetermined time and were stored at –20°C in deep freezer for further analysis.

Standard solutions of Fluoroquinolones

Standard stock solution of drugs (enrofloxacin, ciprofloxacin, norfloxacin) was prepared by dissolving each drug in water to obtain a concentration of about 100 µg/mL. These solutions were kept at −40ºC and were stable for at least 3 months. Calibration curve for each analyte was prepared by dilution of appropriate stock standard solutions.

2.4 Extraction of Milk Samples

Milk samples were homogenized on a high-speed vortex mixer. Samples (2 mL) were taken in glass tube and added into 4mL of 15% TCA to precipitate the protein. The mixtures were allowed to stand in dark for another 15 min. Then samples were centrifuged for 15 minutes at a rate of 10,000 rpm and the supernatants were filtered through 0.45µ syringe filter (Alltech) into 2-mL vials for analysis.

2.5 Method Validation

Method was evaluated with following performance indices: specificity, accuracy, precision, linearity and stability of analyte during sample storage.

2.6 Specificity

Specificity was checked by analyzing different milk products (pasteurized milk, UHT milk and flavored milk. Besides, known amounts of sulphonamides, and tetracyclines were spiked into blank milk samples to evaluate possible interferences encountered in the method.

2.7 Accuracy

Three sets (n = 6), of blank milk samples were fortified with 0.5, 1 and 1.5 times the permitted limit (i.e. MRL) of enrofloxacin and ciprofloxacin (100 µg/L), respectively and analyzed.

2.8 Precision

Three sets, (n = 6) of blank milk samples were fortified with 0.5, 1 and 1.5 times the MRL of enrofloxacin, norfloxacin and ciprofloxacin respectively. The overall standard deviation and coefficient of variation (%) were calculated.

2.9 Linearity

A linear calibration curve for each analyte was established in every batch of analysis to evaluate the instrument robustness on different days. Milk samples (n = 3) fortified at 0, 25, 50, 75, 100, 125 and 150µg/L of FQs were analysed.

Table.1 Fluoroquinolone (enrofloxacin, norfloxacin and ciprofloxacin) residues excreted in cow milk

Day	Time (hr)	Enrofloxacin		Norfloxacin		Ciprofloxacin	
		Conc. (µg/mL)	Percent cumulative amount	Conc. (µg/mL)	Percent cumulative amount	Conc. (µg/mL)	Percent cumulative amount
1	12	19.85 ± 1.05	6.33 ± 0.67	47.75 ± 2.16	7.61 ± 0.88	13.06 ± 0.49	2.20 ± 0.20
	24	31.17± 0.73	16.26 ± 1.58	52.03 ± 2.76	15.97 ± 1.95	32.67 ± 1.87	7.73 ± 0.70
2	36	21.37± 0.61	23.04 ± 2.18	43.64 ± 2.58	22.95 ± 2.79	25.77 ± 1.24	12.07 ± 1.08
	48	16.70± 0.64	28.34 ± 2.69	35.29 ± 2.77	28.71 ± 3.05	21.24 ± 0.94	15.65 ± 1.40
3	60	14.38 ± 0.52	32.89 ± 3.10	26.02 ± 2.51	32.98 ± 4.34	18.80 ± 0.81	18.82 ± 1.68
	72	11.19 ± 0.55	36.47 ± 3.50	14.38 ± 0.52	35.26 ± 4.56	16.16 ± 0.75	21.55 ± 1.94
4	84	8.52 ± 0.26	39.16 ± 3.73	8.52 ± 0.26	36.60 ± 4.66	14.03 ± 0.74	23.91 ± 2.16
	96	6.22± 0.34	41.11 ± 3.88	4.78 ± 0.39	37.37 ± 4.75	11.90 ± 0.44	25.92 ± 2.33
5	108	3.57± 0.39	42.24 ± 3.98	2.15 ± 0.25	37.71 ± 4.79	9.49 ± 0.35	27.52 ± 2.46
	120	2.02 ± 0.46	40.88 ± 3.25	1.85 ± 0.01	56.67 ± 0.01	7.37 ± 0.42	28.78 ± 2.60
6	132	1.61± 0.14	48.59 ± 4.25	-------	-------	5.25 ± 0.33	29.67 ± 2.69
	144	-------	-------	-------	-------	3.30 ± 0.22	30.22 ± 2.74

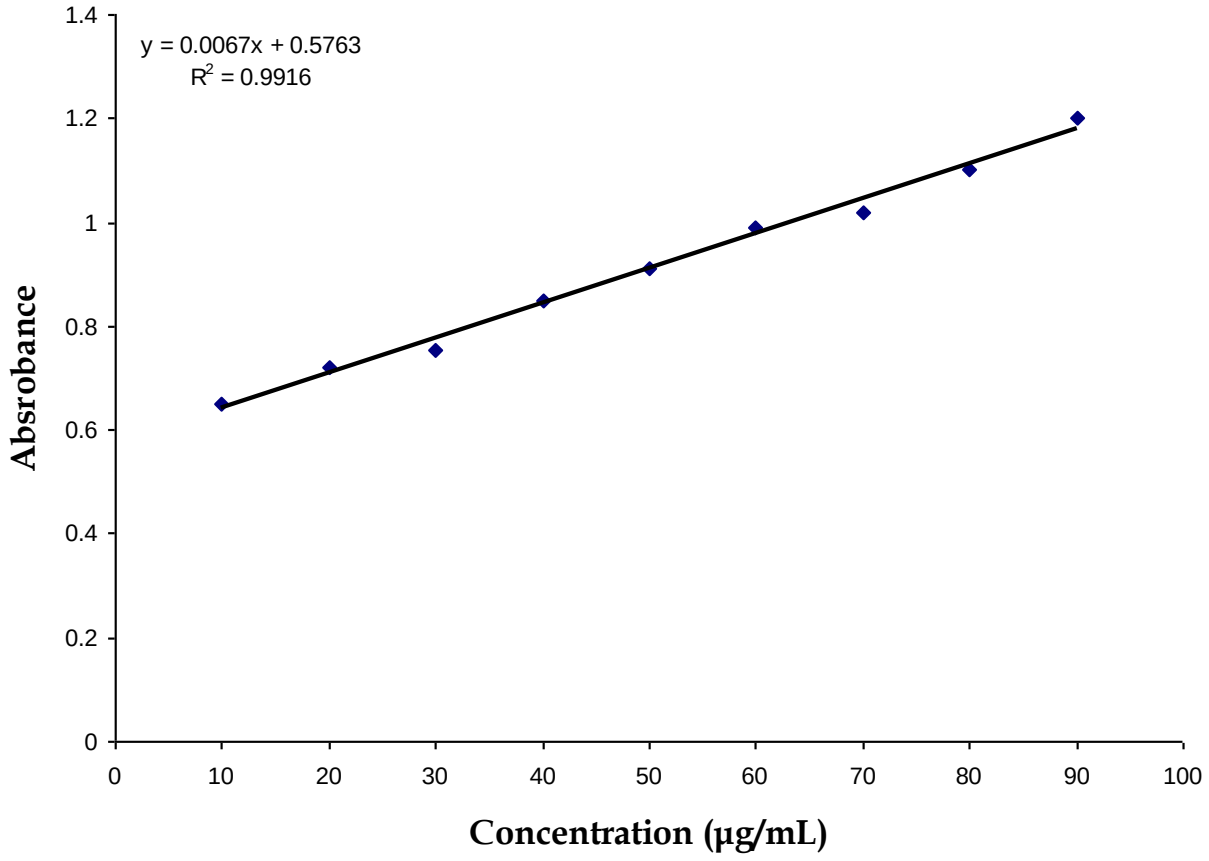

Fig.1 Standard Curve between Absorbance and Concentration (µg/mL) of Norfloxacin at λ_{max} 276 nm in Milk

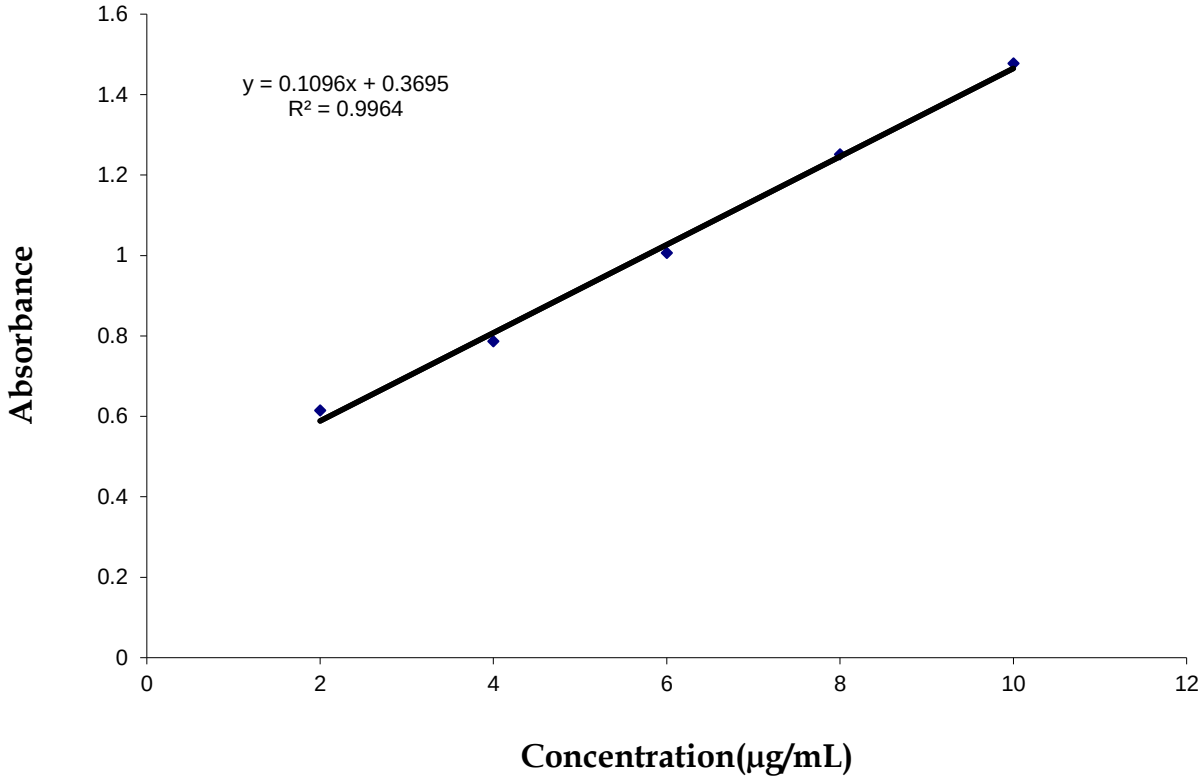

Fig.2 Standard Curve between Absorbance and Concentration (µg/mL) of Enrofloxacin at λ_{max} 272.5 nm in Milk

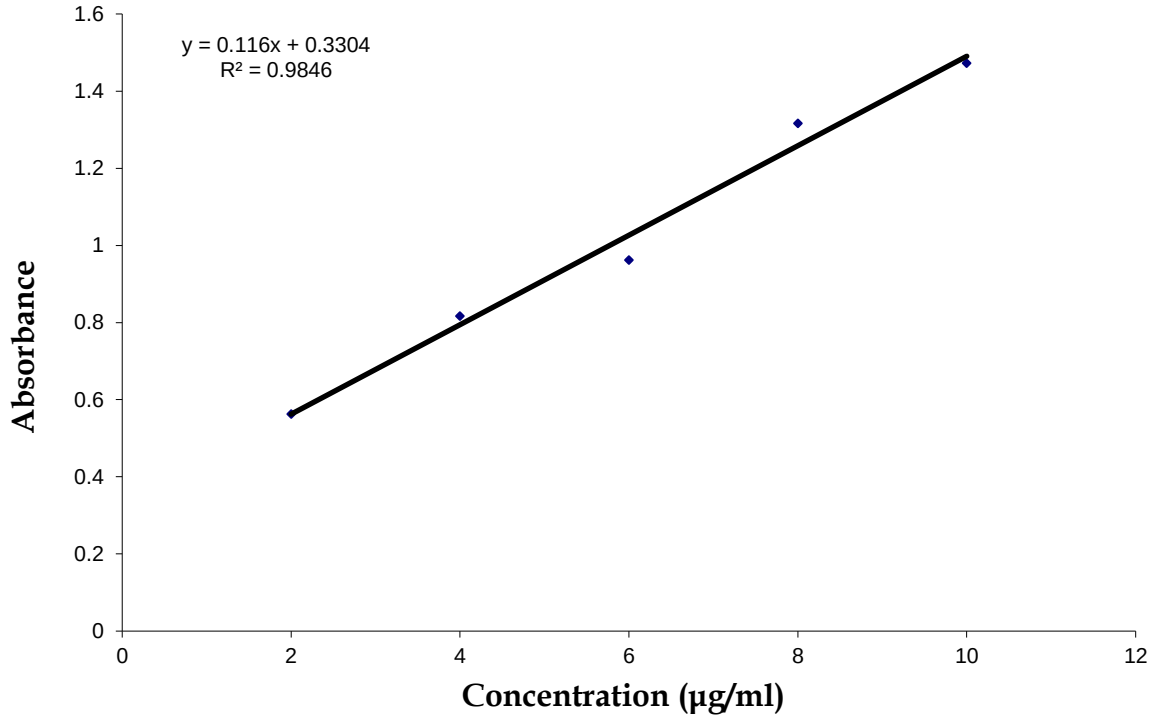

Fig.3 Standard Curve between Absorbance and Concentration (µg/mL) of Ciprofloxacin at λ_{max} 276 nm in Milk

Short-term stability in bovine milk during storage

Concentration, type of milk and storage temperature was tested for their significance toward analytes stability during storage. The average recovery in each experiment was determined.

2.10 Extraction Procedure

In a glass tube 2mL of milk standard solution containing drug, 4 ml of 15% TCA was added to precipitate the protein. The samples were centrifuged for 15 minutes at a rate of 4000 rpm. Into 2mL of filtrate, 8mL distilled water was added. Absorbance of the solution was measured at λmax = 272.5 nm against a reagent blank prepared as described above except that 1 mL milk was taken instead of standard and sample solution.

2.11 Statistical Analysis

After that the statistical analysis were applied and the results will be given as average ± SEM [13].

3. Results and Discussion

Percent Cumulative dose (%) of Enrofloxacin, Norfloxacin and Ciprofloxacin Excreted in whole Milk:

The mean percent cumulative amount excreted after intramuscular administration of Enrofloxacin, in Day-1 was 11.30 ± 1.13% ranging from 3.10% to 19.55%. During Day-5, after intramuscular administration of Enrofloxacin the mean percent cumulative dose ± SE value was 41.56 ± 3.62% ranging from 22.08% to 59.85% and after Day-6 two samples has the value 36.84% and 60.35% was detected in first twelve hours and after that no drug residues was detected in milk samples of cow.

The mean percent cumulative amount excreted after intramuscular administration of Norfloxacin, in Day-1 was 11.79 ± 1.41% ranging from 4.81% to 22.47%. During Day-5, after intramuscular administration of Norfloxacin the mean percent cumulative dose ± SE value was 47.19 ± 2.40% ranging from 21.23% to 56.67% and after no drug residues of Norfloxacin were detected in six-day milk samples of cow.

The mean percent cumulative amount excreted after intramuscular administration of Ciprofloxacin, in Day1 was 4.96 ± 0.45% ranging from 1.61% to 10.72%. During Day-5, after intramuscular administration of Ciprofloxacin the mean percent cumulative dose ± SE value was 28.15 ± 2.53% ranging from 17.37% to 40.88% and after Day-6 drug residues of Ciprofloxacin detected in cow milk samples was 29.94 ± 2.72% ranging from 18.58% to 43.09%.

The mean ± SE value of enrofloxacin excreted in milk was 23.64 ± 1.41 µg/mL ranging from 1.85 to 52.03 µg/mL, average concentration of norfloxacinexcretedin milk was 12.42 ± 0.51 µg/mL and for Ciprofloxacin it was 13.83 ± 0.67µg/mL. The concentration of Ciprofloxacin in milk was 105.3 µg/kg and for Enrofloxacin it was 105.5 µg/kg studied by Cinquina et al. (2003) [10]. Haritova et al (2003) [11] showed maximum milk concentration of Enrofloxacin was 2.38 µg/mL and area under the curve vs. time curve was 23.76 (2.21) µg h/mL.

Another study conducted by Ho, (2004) [12] showed the percent amount excreted for Enrofloxacin was in the range of 2.9-6.1% and for Ciprofloxacin it was 2.5-5.1% in cow milk. The percent cumulative amount (%) excreted in whole milk of seven lactating cows after intramuscular administration of Enrofloxacin (2.5 mg/Kg), norfloxacin (5 mg/Kg) and ciprofloxacin (5 mg/Kg) of its body weight is shown in Table 1.

3.1 Withdrawal period of Enrofloxacin, Norfloxacin and Ciprofloxacin in Cow milk:

Milk samples were collected after medication at regular interval till eight days. The mean amount of Enrofloxacin residues was 19.85 µg/mL to 1.61 µg/mL from Ist day to 6th day and no residues were detected after 6th day. So the wash out period for Enrofloxacin is 6 days. The mean amount of Norfloxacin residues was 47.75 µg/mL to 1.85 µg/mL from Ist day to 5th day and no residues were detected after 5th day therefore, the wash out period for Norfloxacin is 5 days. The mean amount of Ciprofloxacin residues was 13.06 µg/mL to 3.30 µg/mL from Ist day to 8th day and no residues were detected after 8th day therefore, the wash out period for Ciprofloxacin is 8 days.

4. Conclusions

Screening tests for milk and meat to detect the antibiotic residues before marketing these products should be compulsory to protect the human for its harmful effects.

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