Residue of Rifaximin in Milk of Lactating Dairy Cows Following Administration of Intrauterine Infusion

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Abstract. In this article, a new intrauterine infusion containing rifaximin (RIF) was prepared, a sensitive UPLC-MS/MS method for the determination of RIF in milk was developed, and the residue of drug in milk of dairy cows was carried out. Twelve healthy dairy cows were selected by random and treated by RIF intrauterine infusion at a dosage of 25 g/head (contain RIF187.5 mg). Milk samples were collected before dosing (0 h) and at different time intervals (6, 9, 12, 18, 24, 36, 42, 48, 60, 72, and 96 h) after treatment. The results showed that the limit of detection (LOD) and limit of quantitation (LOQ) of UPLC-MS/MS were 0.5 ng/mL and 1.0 ng/mL, respectively. The residues of RIF in the milk samples from 6 h to 96 h after administration were not detected, which were lower than LOQ (1.0 ng/mL) and lower than maximum residue limits (MRLs) (60 ng/mL in milk). This study indicated that administration of RIF by uterine routine has a minimal distribution rate into the milk, and the RIF intrauterine infusion could be used in lactating cows with zero milk-withdrawal period.

1. Introduction
Endometritis in dairy cows is one of the important postpartum diseases (Bovine Endometritis and the Inflammatory Peripheral Cholinergic System) which lead to lower fertility, less milk production, and serious economic loss [1]. The administration of antimicrobial drugs by the uterine route offers a convenient and efficient option for the treatment of bovine endometritis, because of the high drug concentrations at the affected parts.

Rifaximin(RIF) is a semisynthetic antibiotic, belonging to the family of rifamycins. It was widely used at treatment of various diseases in human, such as acute diarrhoeal enteritis [2], small intestine bacterial overgrowth [3-5], travelers’ diarrhea [6-8], irritable bowel syndrome [9-11], ulcerative colitis [12], clostridium difficile [13-15] and hepatic encephalopathy [16-18].

Due to the characteristics of low-aqueous solubility, P-glycoprotein efflux, and low-permeability, Rifaximin is considered as a non-absorbed oral antibiotic (<0.4%), a best choice for topical preparation. Recently, in veterinary medicine, RIF has been attracted widespread attention, which has been used in the treatment and prevention of animal diseases, such as bovine mastitis and endometritis, dermatological diseases, hepatic encephalopathy in pets. It is published that RIF has shown excellent clinical effects on the therapy of hepatic encephalopathy in dogs and cats [19]. Bertocchi et al. demonstrated the high therapeutic efficacy of RIF combined with cephacetrile for the treatment of bovine mastitis caused by S. aureus [20] Furthermore, Polat et al. published the results on comparison of intrauterine ozone and rifaximin treatment in cows with subclinical endometritis [21].
More than 96% of RIF is excreted in the faeces as unchanged drug. Previous related studies demonstrated the residual depletion of RIF in milk after using in cattle cow. Sun Lei et al. conducted a study on the elimination of RIF residues in milk in dairy cows that were administered rifaximin during the dry period, and found that only a small number of samples contained drug residues, which were all lower than the MRL (60 ng/ml). The drug residue in milk after administering RIF uterine infusion different from intramammary infusion. Moreover, to our knowledge, there are no reports about the residual depletion in milk of RIF in dairy cows after administration of intrauterine infusion.

In the present work, we prepared a new RIF intrauterine infusion, described and validated a highly efficient UPLC–MS/MS method for the determination of RIF in milk. Then, the residue of RIF in milk of dairy cows treated was evaluated, and the withdrawal time was proposed.

2. Materials and Methods

2.1. Preparation of RIF Intrauterine Infusion

A 25 g scale of 0.75% RIF suspension for cure of endometritis in dairy cows was prepared, as described below and illustrated in Fig.S2. Suspending agent (Aladdin Biochemical Technology, Shanghai, China) completely dissolved in appropriate amounts of oily dispersive vehicle (RCT basic IKAMAG® safety control, IKA®, Staufen, Germany) under continuous stirring using a thermostatic magnetic stirrer (C-MAC HS 4, IKA®, Staufen, Germany) at 60-80 °C for 30 minutes. After cooling to 40 °C, surfactants (Aladdin Biochemical Technology, Shanghai, China), RIF and the rest of oily dispersive vehicle were added, and RIF intrauterine infusion was finally obtained through mixing well.

2.2. Animals

Twelve apparently healthy, endometritis-free lactating dairy cows, which didn’t receive any antibiotic administration in the past one month, were obtained from a large-scale dairy farm (Beijing, China). And in order to avoid unnecessary errors in the whole process of the experiment, the selected test cows weigh 400kg~550kg and are 2~4 years old. During the whole experiment, the average daily milk output of dairy cows was about 31.0 kg before and after administration. Cows were housed and fed as usual. All study protocols were approved by the Ethics Committee of the Feed Research Institute from CAAS.

2.3. Drug Administration and Sample Collection

The genital was thoroughly disinfected with potassium permanganate solution (Sinopharm Chemical Reagent Co., Ltd, China) and the uterus was flushed with a moderate amount of physiological saline three times, then intrauterine infusion occurred. About 187.5 mg RIF (25 g drug preparation) was slowly pushed into the cow's uterus through the injection catheter, and then the catheter was pull out after administrate for 5 minutes.

The milking procedures must be observed before milking, it includes wiping each teat with antiseptic wipes, dipping the teats with povidone iodine solution for about 30 s, and wiping the surface of teats with clean cloth towel. Then milk samples were collected into 50-mL centrifuge tube (Corning Life Sciences, USA) before dosing (0 h) and at 6, 9, 12, 18, 24, 36, 42, 48, 60, 72 and 96 h after drug administration, the final samples were mixed samples from four udder quarters about 40 mL.

2.4. Milk Sample Preparation

Twenty milliliter acetonitrile (Fisher Scientific, Fair Lawn, USA) and 5 g anhydrous sodium sulfate (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) was added to the well-mixed milk samples (5 g), the former is to precipitate protein and extract RIF, and the latter is to remove moisture, after mixing it with a vortex for 1min and centrifuged for 5 min at 2583×g (Sorvall™ ST40, Thermo Fisher Scientific, Fair Lawn, US), supernatant was transferred into a 50-mL centrifuge tube. Over the next step, underlying precipitate was crushed and extracted again with 10mL acetonitrile under the same condition, supernatant was transferred to the previous tube, then well-mixed. The presence of 10 mL
N-hexane (Beijing Chemical Works, Beijing, China) saturated with acetonitrile would remove lipid substances. After 100 mL heart-shaped bottle with all the lower extract and 10mL isopropanol (Beijing Chemical Works, Beijing, China) was dried using rotary evaporator (RV 8 V, IKA®, Staufen, Germany) at 40 °C, the residues were fully redissolved with 3 mL acetonitrile, and then it was used to separate and purify RIF via solide phase extraction (Oasis® HLB, Waters, Milford Massachusetts, USA). The eluate was collected and dried again under nitrogen at 40°C. Finally, before detection by UPLC-MS/MS, the residue was mixed with 1mL mobile phase and filtered using a syringe filter (0.22 μm, Waters, Milford Massachusetts, USA).

2.5. Chromatographic Conditions
The method establishment and validation about the concentration of RIF in milk utilized ACQUITY UPLC™ system equipped with Xevo™ TQ Mass Detector (Waters, USA). And a Acquity UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm) (Waters, Milford Massachusetts, USA) was used to separate and analysis, the temperature was kept at 40°C and the injection volume was 2 μL. The flow rate was set at 0.3 mL/min with the mobile phases 0.1% formic acid solution (Fisher Scientific, Fair Lawn, US) (A) and acetonitrile (B). Moreover, the gradient elution condition was carried out: initial, 30% A; 3 min, 80% A; 4.5 min, 30% A. MS was performed in electrospray ionization mode which was used in the positive ion mode (ESI+). And the parameters were determined by automatic instrument tuning in order to obtain the perfect analytical conditions for detecting the parent and the daughter ion mass as shown below: the desolvation gas flow and temperature were 800 L/h and 400 °C respectively, cone voltage and capillary voltage were set at 3V and 3 kV . Data was finally acquired with MassLynx 6.1 under multiple reactions monitoring (MRM) mode.

2.6. Method Validation
For assessing the specificity, the mobile phase (initial proportion 30% formic acid solution and 70% acetonitrile), blank milk, spiked milk with RIF (60 ng/mL) and blank milk with RIF were determined to eliminate the interference. Matrix-matched calibration curves were conducted using extracted blank milk spiked with RIF at 1, 10, 30, 60, 120, and 200 ng/mL. The limit of detection (LOD) and the limits of quantification (LOQ) of RIF in milk were determined at the lowest concentrations, which gave signal to noise ratio (S/N) of more than 3 and 10, respectively. Furthermore, to indicate analyte losses in the process of sample analysis, recovery rates was performed with the same analytes as above, and samples with same concentration was determined five times. Ultimately the precision and recovery rate were evaluated by calculating the %RSD. For the determination of the intra-day precision, the analytes that QC sample with the addition of standard of three levels of different concentrations (120 ng/mL, 60 ng/mL, 1 ng/mL) were analyzed five times within one day, while the inter-day precision was proven with repetition of the above-mentioned step five days.Several stability test were assessed as part of validation under different conditions, including 25°C, 4°C, and -20°C by testing the spiked milk samples. During the stability tests, three replicate samples were analyzed using the proposed UPLC-MS/MS method. All samples were kept away from light. GraphPad (Version 5, La Jolla, CA, USA) software package was used to analyze the data of method validation.

3. Results and Discussion
3.1. Method Validation
The result was showed in figure 1. There was no peaks of RIF are present in the mobile phase and blank milk sample at the retention time (about 2.21 min), and other representative chromatograms also indicate the inexistence of analyte-interfering, thus the developed detection method has high specificity.
Figure 1. Typical chromatograms of the blank milk extract (A) and of a milk sample spiked with RIF (60 ng/mL) (B) and the blank milk extract with RIF (60 ng/mL) (C).

The weighted regression calibration curve of the peak-area ratios versus concentrations in milk was plotted over the concentration range of 1 ~200 ng/mL. The results showed that the calibration curves demonstrated good linearity for milk. The mean equation of linearity was $y = x + (0.0181 \pm 0.658178)$ (n=5), and all the correlation coefficients ($r$) were higher than 0.9988. From the table 1, it was showed that the deviation between back-calculated amounts and nominal spiked amounts was within 2%.

The LOD was assumed as the concentration giving a peak signal, and the value of LOD was found to be 0.5 ng/mL(n=6) for RIF in milk. The limit of quantification (LOQ) for RIF in milk was estimated, which found at the level of 1.0 ng/mL. The LOQ for RIF were also used as the lowest standard concentration in calibration curves and recovery test.

The results showed that the recoveries of QC samples in the three levels of 1, 60, 120 ng/mL were $89.60\% \pm 5.92\%$, $92.60\% \pm 6.12\%$ and $92.44\% \pm 5.47\%$ respectively. The intra-day precisions ranged from 1.24 to 7.9%, while inter-day precisions ranged from 5.92 to 6.66%, which indicated that the method has good precision and accuracy (table 1).
Stability tests had been conducted with different storage conditions by assessing milk samples at three levels of RIF. The results founded that no significant degradation of RIF was found in milk samples. It is demonstrated that all samples are stable under the different conditions.

### 3.2. Daily Milk Production

During the entire experiment, the clinical symptoms of the dairy cows in each test did not change significantly. During the administration and after the drug was stopped, the dairy cows' uterine local symptoms, vaginal secretions, and mental appetite status had no significant changes compared to before the administration. The statistical results of average daily milk production before and after the administration were shown in table 2. There was no significant change in the daily milk production of cows after drug withdrawal (P>0.05).

#### 3.3. Residue of Rifaximin in Milk

The residue of RIF in milk of lactating dairy cow was studied after administered by uterine injection. The milk samples collected at different intervals of time were analyzed by the proposed UPLC-MS/MS method. None of the samples from 6 h to 96 h after administration were detected with RIF in it, that is to say, the concentration of RIF in milk sample was less than 1 ng/mL. Thus we propose that RIF intrauterine infusion is friendly to farmers with the advantage of no residue in milk, the withdrawal period could be set as 0 d. The comparison of RIF with other approved intrauterine infusion used for dairy cattle is summarized in table 3.
Table 3. The comparison of RIF with other approved intrauterine infusion used for dairy cattle.

| intrauterine infusion | specifications | dosage | Withdrawal time | Approval countries |
|-----------------------|---------------|--------|-----------------|-------------------|
| Rifaximin             | 25g:187.5mg   | once   | 0d              | In our study      |
| Cephapirin            | 19g:500mg     | once   | 0d              | Ireland           |
| tetracycline          | 2g            | once every 24 h, 1–3 continuously | 4d | United Kingdom |
| hydrochloride         |               |        |                 |                   |
| Florfenicol           | 25mL: 2g      | once every 72 h, 2–4 continuously | 7d | R.P.China      |
| Oxytetracycline       | 25g:250mL     | once every 48 h, 3–4 continuously | 3d | R.P.China      |
| Cefquinome sulfate    | 25g:0.9g      | once every 72 h, 1–2 continuously | 7d | R.P.China      |
| Doxycycline Hyclate   | 24g:2g        | once every 72 h, 1–4 continuously | 7d | R.P.China      |

4. Conclusion

In the present study, a fast, simple, sensitive UPLC-MS/MS method was developed and validated for determination of rifaximin residue behavior in milk following administration of intrauterine infusion. The study on residue of RIF in milk shows that none of RIF (less than 1.0 ng/mL) was found from 6h to 96 h after treatment, and the results shows that the drug residue in milk is very limited by using the RIF intrauterine infusion, and the RIF intrauterine infusion could be used in lactating cows with zero milk-withdrawal period.

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