Residue depletion of colistin in swine after intramuscular administration

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ABSTRACT

A newly formulated colistin sulphate solution was prepared in a previous study as a potential agent for intramuscular injection and its effectiveness, toxicity and pharmacokinetics were investigated. In order to provide more information to establish scientific guidance for safe use of this preparation, its residue depletion in swine tissues following intramuscular administration was investigated in this experiment. Fifty healthy cross-bred piglets (13.3 ± 0.9 kg) were used in this study. Five animals were kept as untreated controls and the other 45 animals were intramuscularly injected with the colistin preparation at a dose of 2.5 mg/kg of body weight. From the treated piglets, 5 animals were randomly selected and sacrificed at different withdrawal times. Liver, kidney and muscle tissues were sampled to examine the colistin residue levels by microbiological assay. The results showed that the colistin residue in liver and muscle decreased quickly and could not be detected at 1 day after the final dosing. However, the residue depletion in the kidneys was much slower than that in other tissues and even a small quantity of drug could be detected at 14 days after withdrawal. Using the method recommended by the Committee for Veterinary Medical Products (CVMP), a withdrawal time of 10 days was established for the safe use of the newly formulated colistin sulphate solution.

Key words: colistin, intramuscular administration, microbiological assay, residue depletion, swine, withdrawal time.

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INTRODUCTION

Colistin (polymyxin E) is one of the main polymyxins and it has been used frequently as a veterinary medicine for the promotion of growth and prevention and control of diseases in modern animal husbandry[2,4]. Through binding to the outer membrane of bacterial lipopolysaccharides and endotoxins of Gram-negative bacteria, colistin can effectively kill bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Bacillus* spp., *Salmonella* spp., and *Haemophilus* spp.[14,19]. In human medicine, colistin has also provided an effective treatment for critically ill patients with meningitis, cystic fibrosis and sepsis caused by multi-drug resistant Gram-negative bacilli[1,16,21].

In recent years, while many bacterial strains have developed strong resistance to multiple antibiotics, the reported potential to generate resistance to colistin has been low[6,7]. Under such circumstances, colistin provides an effective alternative treatment to combat Gram-negative bacteria that are sensitive to this antibiotic. However, colistin's application is limited in veterinary medicine in many countries, such as China, where only premix and oral granules are available. These preparations do not effectively meet the growing needs of animal production in China in treating general infections. It would therefore be of great interest to develop an injectable colistin sulphate solution to provide a new therapy for a wide range of drug-resistant Gram-negative infections.

In our previous study, a newly formulated colistin sulphate solution[15] was prepared as a potential agent for intramuscular injection, and its effectiveness, toxicity and pharmacokinetics were investigated. In order to provide more information to establish scientifically-based guidance for the safe use of this preparation, its residue depletion in swine tissues following intramuscular administration is investigated in this study.

MATERIALS AND METHODS

Reagents and materials

Colistin sulphate standard (749 μg base activity per mg) and raw colistin sulphate (643.5 μg base activity per mg) were purchased from Meiji Seika Kaisha Ltd. Beijing Branch (Beijing, China). The colistin sulphate standard was used to analyse colistin levels in swine tissues and raw colistin sulphate was used to prepare the newly formulated colistin sulphate solution for animal treatment as described previously[15]. The indicator organism, *Bordetella bronchiseptica* (ATCC 4617), for microbiological assay was obtained from the China Institute of Veterinary Drug Control (Beijing, China). Difco antibiotic media and tryptic soy broth were purchased from Beijing Seaskybio Technology Co. Ltd. (Beijing, China). Other reagents were of analytical grade and were obtained from Beijing Chemical Reagents Company (Beijing, China).

Animal treatment

The 50 healthy cross bred piglets (Large white × Landrace × Pietrain) used in this experiment with an average body weight of 13.3 ± 0.9 kg were supplied by Beijing Laboratory Animal Research Center (Beijing, China). The animals were housed in individual metabolic cages in the animal facility of China Agricultural University, with continuous ventilation and heating systems. All procedures used in the experiment were approved by China Agricultural University Animal Care and Use Committee. Animals were fed a drug-free balanced diet and had access to water *ad libitum* during adaptation (3 weeks) and the subsequent treatment period. Five animals were kept as untreated controls and the other 45 animals were weighed and injected intramuscularly into the neck with colistin at 2.5 mg/kg of body weight for 3 consecutive days. Five treated animals were randomly selected and sacrificed by an intravenous overdose of sodium pentobarbital at 0.5, 1, 2, 3, 5, 7, 10, 14 and 21 days after the last injection. The untreated animals were slaughtered using the same method to obtain control tissues after the adaptation period. The liver, kidney, and muscle (thigh and injection sites) tissues were sampled and stored at –80 °C until the colistin content was analysed.

Extraction of colistin from tissues

Extraction of the drug from swine tissues was achieved by the method described previously[11] with some modifications.

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Five grams of tissue was homogenised in 10 mℓ of 0.1 M hydrochloric acid and then in an additional volume of acetonitrile (10 mℓ for muscle samples, 20 mℓ for liver and kidney samples). The supernatant was collected and the precipitate was homogenised again in 20 mℓ acetonitrile for 5 minutes. After centrifugation at 6500 *g* for 10 minutes at –4 °C, the total supernatant was filtered through a 0.22 μm filter and evaporated until dry under vacuum at 50 °C. The dried residue was dissolved in 3 mℓ of 1 M phosphate buffer solution (PBS, pH 6.6) and centrifuged at 1000 g for 5 minutes and 280 μℓ supernatant was used for the microbiological assay.

Analysis of drug content

The concentration of colistin in tissues was tested by its inhibitory effect against *Bordetella bronchiseptica* (ATCC 4617) according to the method described in United States Pharmacopeia[20] with some improvements. Fifteen millilitres of autoclaved Difco antibiotic medium 9 was distributed into test plates (diameter: 100 mm) and left to solidify into a smooth base layer of uniform depth. The stock suspension of the indicator organism (ATCC 4617) was diluted in tryptic soy broth so that its transmittance at 580 nm in a l-cm cell averaged 60 %. One millilitre of diluted stock suspension was mixed with 5 mℓ of autoclaved Difco antibiotic medium 10 that had been cooled to 45–50 °C. The medium was then distributed into the test plates containing base layer to obtain a seed layer inoculum with an even surface. After the medium was cooled to room temperature (RT), 280 μl of the extraction solution was poured into Oxford cups (inside diameter 6 mm; height 10 mm), which were placed directly on the surface of the seed layer. After 3-hour diffusion at RT and incubation for 18 hours at 37 °C, the diameter of inhibitory circles was measured with a vernier calliper.

Standard curve

The calibration curves were prepared on the basis of the inhibition zones and the colistin concentrations of a series of standard spiked samples. Drug free swine tissues obtained from the untreated animals were used to develop standard spiked samples. A series of standard spiked samples containing 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025 and 0.0125 μg/g of colistin were prepared by adding 0.25 mℓ of the working standard solution (128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg/mℓ) into 5 g tissues. The working standard solution was prepared by dissolving colistin standard in 1 M PBS (pH 6.6). After the standard spiked samples were left at RT for 30 minutes to let the drug diffuse into the tissues. They were analysed as described above.

Table 1: **Average diameter** (mm) **of inhibitory circles for the standard curves** (*n* = 7)**.**

Colistin concentration (μg/g)	Tissue		
	Muscle	**Liver**	**Kidney**
0.0125	ND	ND	ND
0.025	ND	10.96 ± 0.33	10.04 ± 1.37
0.05	11.94 ± 0.50	13.22 ± 0.10	12.52 ± 0.38
0.1	14.00 ± 0.52	14.67 ± 0.44	14.43 ± 0.43
0.2	16.22 ± 0.36	16.25 ± 0.88	16.19 ± 0.42
0.4	17.57 ± 0.32	17.55 ± 0.58	17.68 ± 0.44
0.8	18.86 ± 0.37	18.75 ± 0.65	18.95 ± 0.28
1.6	20.11 ± 0.32	19.99 ± 0.88	20.19 ± 0.50
3.2	21.72 ± 0.31	21.47 ± 0.86	21.70 ± 0.37
6.4	23.50 ± 0.35	23.09 ± 0.83	23.44 ± 0.34
Linear equation*	$y = 0.1806\ x - 3.4834$	$y = 0.2078\ x - 3.9985$	$y = 0.1874\ x - 3.6272$
Correlation coefficient	0.995	0.997	0.995

ND = not detectable.
*y log concentration (μg/g); x = diameter (mm).

Table 2: **The coefficient of variation and recovery of microbiological assay** (*n* = 6)**.**

Tissue	Fortified level	Intra-day variability		Inter-day variability	
		Recovery	**C.V.**	**Recovery**	**C.V.**
	(μg/g)	(%)	(%)	(%)	(%)
Muscle	0.1	91.77 ± 7.36	8.0	84.73 ± 8.84	10.4
	0.8	100.04 ± 4.11	4.1	98.15 ± 8.75	8.9
	3.2	100.24 ± 4.94	5.0	99.37 ± 7.60	7.7
Liver	0.1	89.32 ± 4.00	5.0	84.95 ± 8.37	9.8
	0.8	93.84 ± 5.72	6.1	92.86 ± 5.50	5.9
	3.2	102.53 ± 5.09	5.0	96.82 ± 5.38	5.6
Kidney	0.1	90.12 ± 5.94	6.6	88.83 ± 9.24	10.4
	0.8	90.48 ± 3.91	4.3	91.24 ± 8.56	9.4
	3.2	99.58 ± 3.86	3.9	101.25 ± 5.36	5.3

Fortification

To ensure the feasibility of the detection method for colistin in swine tissues, a fortifying test was performed on the muscle, liver and kidney. The spiked samples at 3.2, 0.8 and 0.1 μg/g fortification levels were prepared by adding 0.25 mℓ of the working standard solution (64, 16 and 2 μg/g) into 5 g tissue, left at RT for 30 minutes to let the drug diffuse into the tissues and analysed as described above. The precision (inter-day and intra-day) of the method was assessed using 6 replicates of fortified samples at 3 fortification levels on 6 different days.

Statistical analysis

Student's *t*-tests were used to compare the differences between colistin concentrations in the samples and determine their statistical significance at a probability of <0.05. The withdrawal time was established by linear regression analysis of the log-transformed tissue concentrations and the time interval when the 1-sided 95% upper tolerance limit was below the European Union (EU) Maximum Residue Limit (MRL)[3].

RESULTS

The results of the linear equation, the correlation coefficient and the diameter of the inhibitory circle of the microbiological method are listed in Table 1. The linear range of the method was 0.05–6.4, 0.025–6.4 and 0.025–6.4 μg/ml for the muscle, liver and kidney, respectively. Colistin level cannot be detected at 0.0125 μg/g for liver and kidney tissues and 0.025 μg/g for muscle tissue, therefore the limits of detection (LODs) for the muscle, liver, and kidney tissues were 0.05, 0.025, and 0.025 μg/g, respectively. The recovery and precision of 3 spiked levels are summarised in Table 2. The intra-day mean recovery method was between 89.32 % and 102.53 %, with coefficients of variation (CVs) of 3.9– 8.0 %; the inter-day mean recovery method was in the range of 84.95 % to 101.25 % with CVs of 5.3–10.4 %.

The results for residual concentrations of colistin in swine tissues at various periods after administration are summarised in Table 3. At 12 hours post-treatment, the highest residue level ($P < 0.001$) was found in kidney tissue (16.794 ± 1.251 μg/g) and the lowest residue level was found in the liver (0.381 ± 0.012 μg/g) and thigh muscle (0.280 ± 0.011 μg/g). The colistin residue concentrations in liver and thigh muscle decreased so quickly that they could not be detected 1 day after the final dose. However, the residue depletion of colistin in kidney was much slower than that in other tissues, and a small quantity of drug could even be detected 14 days after the final dose in 3 piglets. The residue depletion curves of kidney and muscle at the injection sites are shown in Figs 1 and 2, which were plotted according to the method recommended by the Committee for Veterinary Medical Products (CVMP)[3]. The withdrawal time in kidney and muscle at the injection site were estimated to fall below the EU MRLs (95 % tolerance limit and 95 % confidence) at 9.29 and 5.42 days, respectively.

Table 3: **Residual concentrations** (µg/g) **of colistin in swine tissues at different intervals after the last intramuscular administration** ($n = 5$).

Interval time (d)	Mean residual concentrations of colistin			
	Kidney	Liver	Muscle	
			Thigh	Injection site
0.5	16.794 ± 1.251[a]	0.381 ± 0.012[c]	0.280 ± 0.011[c]	4.714 ± 0.272[b]
1	13.758 ± 0.701[a]	ND	ND	1.794 ± 0.112[b]
2	5.404 ± 0.767[a]	1.421 ± 0.063[b]		
3	1.866 ± 0.175[a]	0.699 ± 0.026[b]		
5	0.281 ± 0.014[a]	0.088 ± 0.007[b]		
7	0.107 ± 0.099	ND		
10	0.072 ± 0.008			
14	0.049 ± 0.009 (3/5[A])			
21	ND			

ND = not detectable.
[a,b,c]Denote significant ($P < 0.05$) difference in rows ($n = 5$).
[A]Number of individual animals with colistin concentration above the LOD.

DISCUSSION

Colistin is a complex mixture of more than 30 components with colistin A (polymyxin E_1) and colistin B (polymyxin E_2) being the 2 major components[8,18]. Although colistin A and B have a similar structure (only an additional CH_2 in the fatty acid of colistin A)[8,18], they have shown substantial differences in their chromatographic behaviour[12,17].

Although high performance liquid chromatography (HPLC) assay methods to measure colistin levels in biological material have been reported[5,12,17], some problems occurred with accurate analysis and quantification owing to the differences in chromatographic behaviour. In addition, colistin's weak ultraviolet absorption and lack of innate fluorescence made it difficult to detect by the HPLC method[5]. An immunological method has been described for measuring colistin in fish tissues[10], but the preparation of antigen was complicated. Therefore, a microbiological assay with some improvements was selected to measure the colistin level in swine tissues in the present study.

To the best of our knowledge, there is no information available about colistin residues in swine tissues after its intramuscular administration. In calf tissues, it was reported[11] that no colistin residue could be detected in liver and thigh muscle after intramuscular injection and the residue level of colistin was highest in kidney where it was eliminated slowly. In this

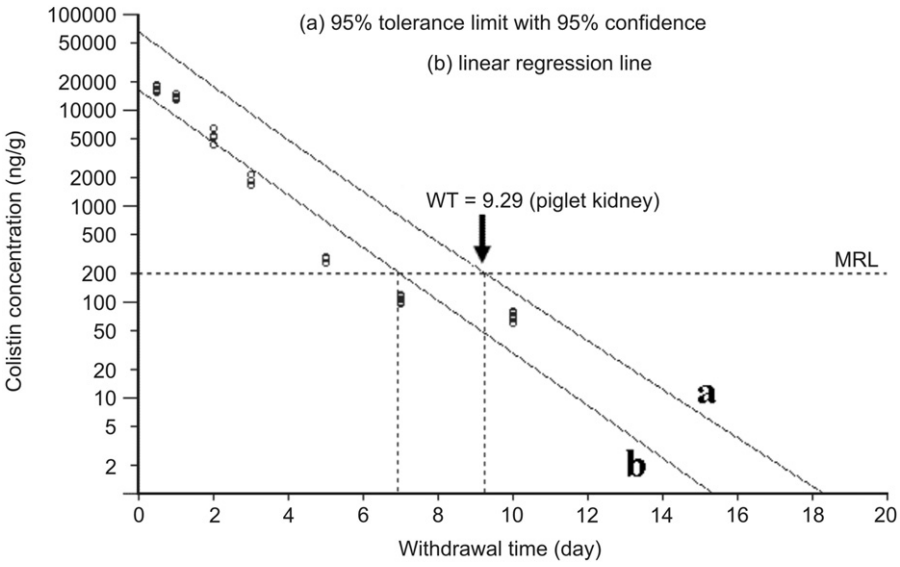

Fig. 1: **Plot of withdrawal time calculated for swine kidney when the 1-sided 95 % upper tolerance limit was below the EU MRL of 200 ng/g**.

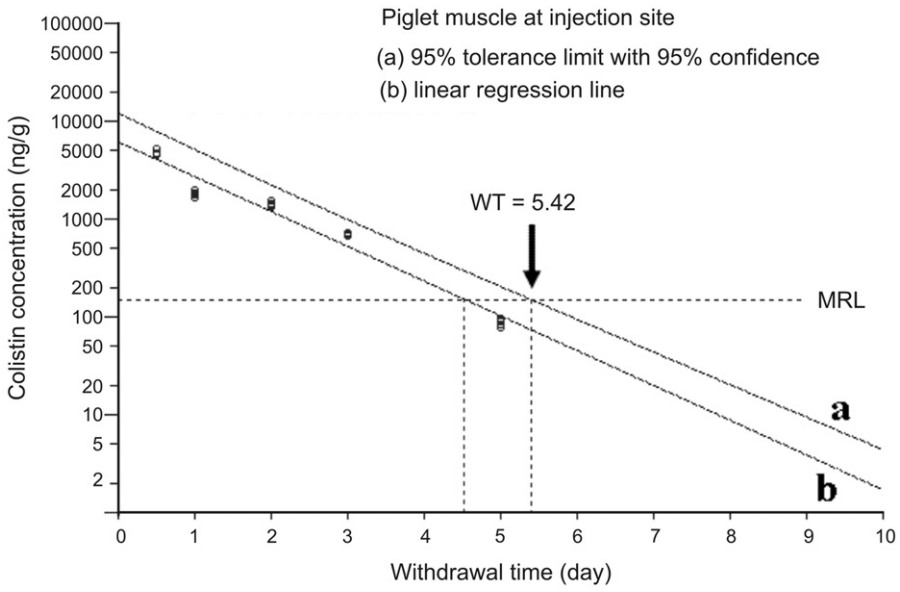

Fig. 2: **Plot of withdrawal time calculated for swine muscle at the injection site when the 1-sided 95 % upper tolerance limit was below the EU MRL of 150 ng/g**.

study in swine, similar results were obtained: the colistin residue levels in liver and thigh muscle could not be detected 24 hours after the last dosing, but the residue levels in the kidney could be detected up to 14 days after the last injection. Furthermore, the highest colistin concentration was measured in kidney tissue, indicating that the kidney is the main excretory organ for colistin in swine.

It was demonstrated that colistin was poorly absorbed and drug residue levels were usually undetectable in muscle, liver, and kidney tissues when administered at therapeutic dosages by the oral route[9]. The different results between intramuscular and the oral route could be explained by their different characteristics of absorption and elimination. When administered orally to animals, little colistin was absorbed and none of the drug was eliminated in the faeces *via* the bile. Conversely, when administered intramuscularly, most could be absorbed and be eliminated in the urine[13].

The CVMP of EU has established the MRLs[4] for colistin in swine: 150, 150 and 200 μg/g in muscle, liver and kidney, respectively. As shown in Table 3, colistin concentrations in liver and thigh muscle dropped below the accepted MRLs and were not detectable at the 2nd slaughter time point (24 hours). All of the colistin residue levels measured in the kidney and at the injection sites were below the accepted MRLs at 7 days post-treatment. However, due to the high inter-individual variability and the limited number of test animals, the withdrawal periods were estimated by the statistical method described previously to avoid potential hazards to human health[3]. The estimated withdrawal times in the kidney and at the injection site were 9.29 and 5.42 days, respectively (Figs 1, 2), falling below the MRLs (95 % tolerance limit and 95 % confidence). Therefore, the withdrawal time in kidney and the injection site should be 10 and 6 days, respectively. The longer withdrawal time of 10 days should be selected as the conclusive withdrawal time[3] for the safe use of this newly formulated colistin sulphate solution.

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