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Residue Depletion of Nitrovin in Chicken after Oral Administration

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ABSTRACT: In this study, the residue depletion of nitrovin in chicken was studied after feeding the birds with dietary feeds containing 10 mg/kg of nitrovin for 7 consecutive days. Tissues (muscle, fat, kidney, and liver) and plasma were collected at different withdrawal periods and determined by a high-performance liquid chromatography−ultraviolet (HPLC−UV) method. The limit of detection for nitrovin in tissue and plasma samples was 0.1 ng/(g or mL), and the inter- and intrarecoveries from the blank fortified samples were in the range of 71.1−85.7%. At the withdrawal period of 0 days, the residue concentration of nitrovin in plasma was the highest (average of 84.98 ng/mL) compared to those in muscle, fat, liver, and kidney (average of 21.04, 61.18, 24.04, and 68.28 ng/g, respectively). At the withdrawal period of 28 days, the residue levels of nitrovin in muscle, fat, liver, and plasma were all higher than 1.0 ng/(g or mL) and the highest concentration was in liver (average of 5.8 ng/g). These data are in support of the ban of nitrovin as a feed additive in food-producing animals.

KEYWORDS: Nitrovin, residue depletion, chicken, tissue, plasma, HPLC

INTRODUCTION

Nitrofurans are a class of broad-spectrum antibacterial drugs that all contain the characteristic molecular structure of a 5-nitrofuran ring. The common nitrofurans are shown in Figure 1. They have been widely used as feed additives in livestock and aquaculture to treat certain bacterial infections for many years.[1] However, furazolidone, nitrofurantoin, nitrofurazone, furaltadone, and nifursol and their metabolites have been proven to show potential carcinogenic and mutagenic effects.[2] Therefore, the use of the five drugs in food-producing animals has been prohibited in most countries.

Nitrovin (NTV) is also a synthesized nitrofuran drug (Figure 1). Because of no confirmatory result available showing its harmful effects similar to its banned homologues, it is still licensed as a feed additive in some regions at a level of 10−25 mg of NTV/kg of feedstuff.[3] However, some researchers have proven that NTV is mutagenic in *Salmonella typhimurium*.[4] Therefore, the Ministry of Agriculture of China has prohibited the use of NTV in food-producing animals in consideration of its potential similar harmful effects similar to the five banned nitrofurans.[5]

The ban of the five drugs is mostly because of their metabolites. Researchers have proven that their parent drugs were metabolized rapidly *in vivo*, that the parent drugs could not be detected even within a few hours after cessation of treatment, but that their metabolites as tissue-bound residues were stable for longer periods.[6−10] These protein-bound metabolites can be released in the stomachs of consumers to show harmful effects.[11] Therefore, these metabolites are usually regarded as the marker residues of their respective parent drugs, although the scientific panel of the European Food Safety Authority (EFSA) has stated that the issue of carcinogenicity is not of concern for human health when a nitrofurazone metabolite is present in food at low concentrations,[12] and a new biomarker of nitrofurazone (cyano metabolite) has been reported.[13] By now, there have been many papers reported for the determination of these metabolites in various animal-derived products.[14−23]

However, there has been no paper reported for the study of the metabolism of NTV in animals. Some papers have been reported for the determination of NTV in feeds,[24−29] and only one paper has been reported for the determination of NTV residue in animal tissues.[30]

The chemical structure of NTV is different from the five banned nitrofurans. In the molecules of the five drugs, there is a C═N bond in the side chain of furan ring and their metabolites are generated as a result of the breakage of the C═N bond (Figure 1). However, there is a C═C bond at the same position in the molecule of NTV, and the C = C bond is more stable than the C═N bond and unlikely to be broken at this position to generate the NTV metabolite. Although there are also two C═N bonds in the molecule of NTV, the two bonds are unlikely to be broken. Then, it is speculated that NTV is not metabolized *in vivo*, and it is excreted and accumulated in animal tissues as the parent drug. Therefore, the parent NTV is defined as the target analyte in animal-derived products in the official method of China (unpromulgated). The objective of this study was to research the residue depletion of NTV in broiler chicken after feeding the animals with NTV-contained dietary feeds.

MATERIALS AND METHODS

Reagents and Chemicals. NTV standard was purchased from the China Institute of Veterinary Drug Control, Beijing, China. The crude NTV was obtained from the Nanjing Pharma Chemical Company (Nanjing, China). Liquid-chromatographic-grade acetonitrile was purchased from Merck KGaA (Darmstadt, Germany). Liquid-chromatographic-grade methanol was from Dikma (Lake Forest, CA). Other chemical reagents were analytical-grade from the Beijing Chemical Company (Beijing, China). Standard stock solution of NTV (1 mg/mL) and the working solutions (0.5, 1, 10, 20, 50, and 100 ng/mL) were

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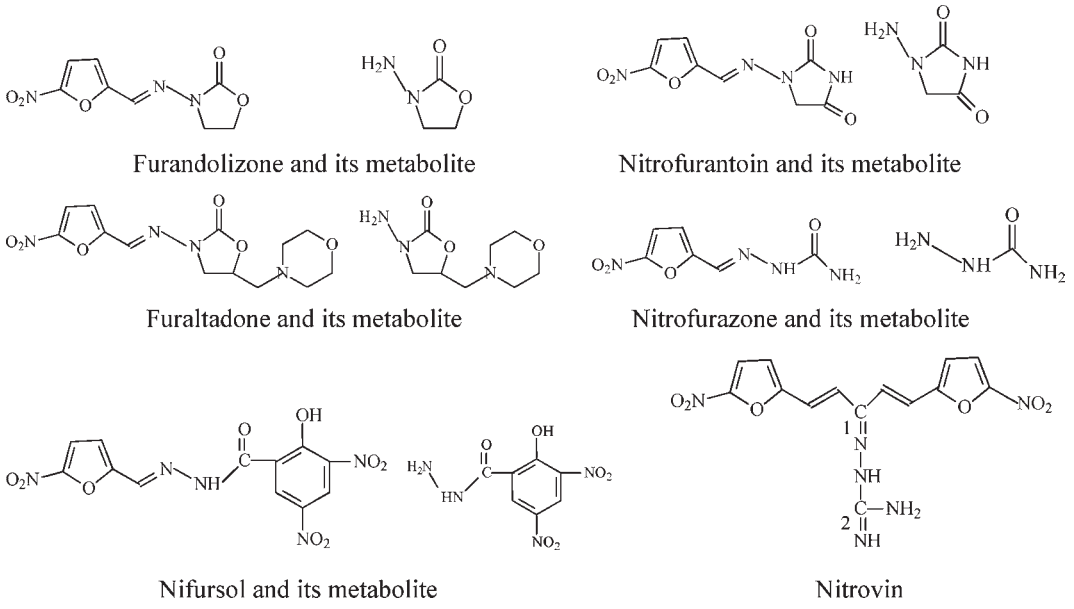

Figure 1. Chemical structures of nitrovin, some nitrofurans, and their metabolites.

prepared with acetonitrile/methanol (1:1, v/v) and stored at 4 °C to be stable for 4 weeks. Sodium acetate buffer was prepared by dissolving 0.6797 g of $CH_3COONa \cdot 3H_2O$ in 700 mL of deionized water, and the pH was adjusted to 5.5 using 10% acetic acid. Then, the mixture was diluted to 1000 mL with deionized water.

Chromatographic Conditions. The high-performance liquid chromatography (HPLC) system consisted of Shimadzu LC-6AD liquid chromatography, a SPD-20A UV detector (Shimadzu, Japan), and a reversed-phase Diamonsil C_{18} column (150 × 4.6 mm, 5 μm) (Dikma, Lake Forest, CA). The mobile phase consisted of acetonitrile (A) and sodium acetate buffer (B) with a binary gradient elution mode. The gradient elution started with 30% A, linearly increased to 80% A in 8 min, was maintained for 7.5 min, was then brought back to 30% A in 0.5 min, and was maintained at these conditions for 4 min, with the total running time of 20 min. The other HPLC conditions were as follows: injection volume, 20 μL; flow rate, 0.4 mL/min; and detection wavelength, 380 nm. Qualitative analysis was obtained by comparing the retention time of the chromatogram peak of the samples with that of the NTV standard. Quantification was calculated according the chromatogram peak area of the NTV standard.

Sample Preparation. An amount of 2.00 g of homogenized tissue sample or plasma and 10 mL of methanol/acetonitrile (1:1, v/v) were added to a polypropylene centrifuge tube. The tube was stirred vigorously in a water bath (70 °C) for 20 min. After centrifugation at 4000 revolutions/min for 10 min, the supernatant was collected and the residue was extracted again with another 10 mL of methanol/acetonitrile (1:1, v/v). The extracts were combined and evaporated to dryness on an evaporator at 40 °C. Then, the dry residue was dissolved in 1 mL of acetonitrile/water (1:1, v/v) under vortex. About 2 mL of *n*-hexane was added, and the mixture was shaken vigorously for 2 min. After centrifugation at 5000 revolutions/min for 5 min, the supernatant was discarded. The lower phase was analyzed with the HPLC−ultraviolet (UV) method before filtration through a 0.22 μm Millipore filter.

Animal Experiment. The animal experiment was carried out at the Animal Experiment Center of the College of Animal Science and Technology, Agricultural University of Hebei, Hebei, China. A total of 36 7 day broiler chickens from Baoding Xun Yuan Poultry Industry Co., Ltd. were randomly divided into two groups. Group 1 consisted of 30 birds as experimental animals, and group 2 consisted of 6 birds as control animals. All of the animals were fed with a standard feed containing no antibiotics for 7 days, and the general health conditions were monitored by a veterinarian. Broilers were raised in whole 4-tier multi-step cages. The animal unit was equipped with a continuous ventilation and heating system, and the temperature was adjusted according to the age of the animals.

After the acclimation period, the birds of group 1 were fed with the medicated feeds containing 10 mg of NTV/kg of feed for 7 consecutive days. The birds of group 2 were given the nonmedicated standard feed. All of the animals had free access to feed and water. During the medication period, the total feed consumption of group 1 was 20 kg; therefore, the average NTV dosage was 6.67 mg/animal. After the NTV medication period, the birds of the two groups were all provided with nonmedicated feeds. At days 0, 3, 7, 14, 21, and 28 after withdrawal of NTV, 5 birds from group 1 were selected randomly and slaughtered. Muscle, fat, kidney, liver, and plasma from each animal were collected separately and placed in plastic bags marked with the code number. All of the samples were transported to the laboratory as soon as possible and stored at −20 °C until analysis. The samples of muscle, fat, kidney, liver, and plasma from the birds of group 2 were also collected as blank samples to evaluate the proposed HPLC method.

RESULTS AND DISCUSSION

HPLC Conditions. For determination of the residue depletion of NTV with the HPLC−UV method, the first step is to select the appropriate mobile phase and detector wavelength. Some researchers have reported the use of acetonitrile/0.1% formic acid[27] or acetonitrile/water[28,29] as the mobile phase, and those researchers have shown NTV could only be eluted from the chromatography column with high ratio of acetonitrile. In this study, the gradient acetonitrile/sodium acetate buffer was used to elute NTV and a symmetric and acute peak was obtained (Figure 2A). NTV was eluted at 15.95 min when the acetonitrile ratio was about 30%, which is different from the reported methods.

There are two furan rings in the molecule of NTV; therefore, it has UV absorbency in the range of 220−260, 360−380, and 470−500 nm. Therefore, NTV has been detected with a diode array detector or UV detector at wavelengths of 254, 365, 375, 378, and 495 nm in the reported papers.[24−29] After comparison

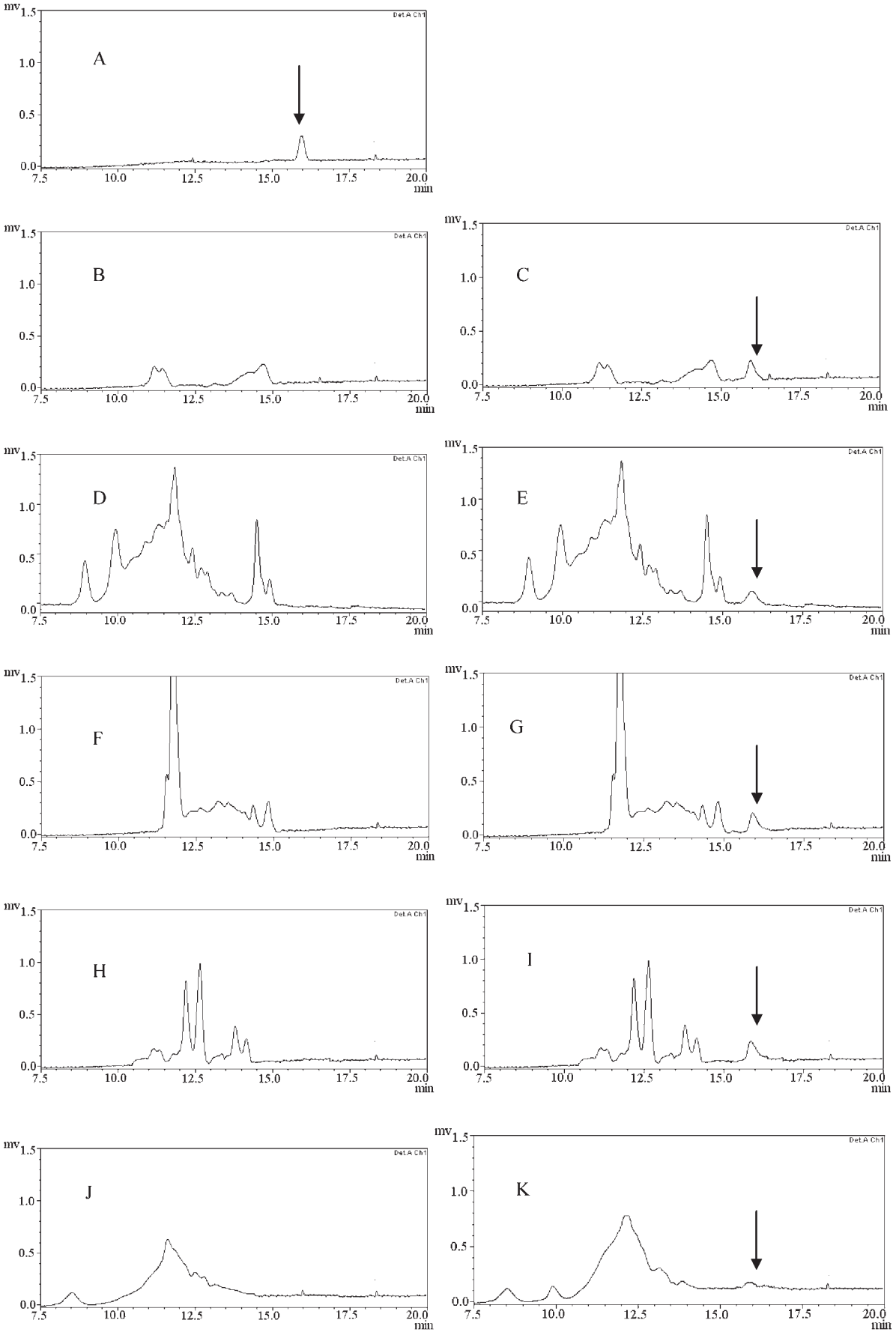

Figure 2. Continued

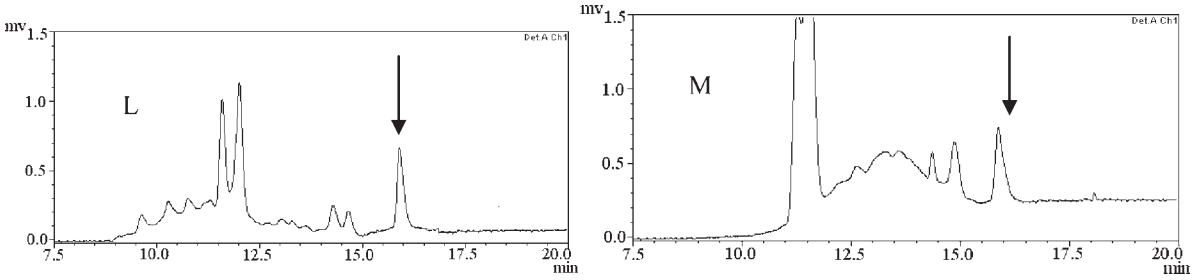

Figure 2. Chromatograms of (A) NTV standard (20 ng/mL), (B) blank muscle, (C) fortified muscle, (D) blank liver, (E) fortified liver, (F) blank kidney, (G) fortified kidney, (H) blank plasma, (I) fortified plasma, (J) blank fat, (K) fortified fat [0.5 ng/g, the fortification level in other sample was 10 ng/(g or mL)], (L) real plasma, and (M) real kidney.

Table 1. Recoveries of NTV from Standard Fortified Samples

		intraday		interday	
	sample added [ng/(g or mL)]	recovery (%)	CV (%)	recovery (%)	CV (%)
muscle	0.5	80.4	7.9	77.0	8.4
	10	74.5	4.8	71.1	7.0
	50	72.8	5.2	78.2	5.8
fat	0.5	82.7	7.2	80.5	8.1
	10	76.8	6.0	79.5	8.3
	50	75.0	4.5	74.0	6.0
liver	0.5	75.3	8.0	78.4	9.2
	10	79.8	6.2	79.8	7.2
	50	80.2	7.3	76.3	8.8
kidney	0.5	83.1	7.4	85.7	7.9
	10	72.9	5.0	78.0	8.2
	50	78.0	5.4	72.5	6.0
plasma	0.5	73.1	6.9	80.6	7.5.
	10	79.5	6.1	78.4	7.5
	50	78.2	4.7	81.4	6.2

of the absorbencies at different wavelengths, the detection wavelength of 380 nm was selected in this study.

Linearity. NTV standards at concentrations of 0.5, 1, 10, 20, 50, and 100 ng/mL were injected into chromatography to develop the standard calibration curve by plotting the concentrations versus the peak areas. A good linearity over the range of 1−100 ng/mL was obtained. The standard curve equation was $y = 145.13x + 1059.1$, with the regression coefficient of 0.9993.

Sensitivity. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the peak/noise ratio (S/N) of 3:1 and 10:1, respectively, by measuring the peak height of the blank samples. The LOD and LOQ for NTV in tissues and plasma were 0.1 and 1.0 ng/(g or mL), respectively. The previous reported HPLC methods showed LODs of 0.05−0.2 mg/kg and LOQs of 0.2−2 mg/kg.[24−29] Therefore, the HPLC method is sensitive enough to detect the residue of NTV at low levels, although the sensitivity was lower than that of the recently reported liquid chromatography−tandem mass spectrometry (LC−MS/MS) method (0.09−0.26 ng/g).[30]

Accuracy and Precision. The accuracy and precision of the method (inter- and intraday) were determined using the NTV fortified blank samples at levels of 0.5, 10, and 50 ng/(g or mL). Intraday precision was assessed by performing six repetitive determinations at each level during a single day. Interday precision was assessed by performing duplicate analyses of each level per day over three different days. Inter- and intra-assay recoveries were in the range of 71.1−85.7%, with coefficients of variation (CVs) between 4.5 and 9.2% (Table 1).

Table 2. NTV Residues in Chicken Tissues and Plasma after a 7 Day Medication Period

sample	number	0 day	3 day	7 day	14 day	21 day	28 day
muscle (ng/g)	1	19.7	16.7	14.1	13.2	9.1	1.9
	2	21.7	17.2	13.8	11.9	10.0	3.2
	3	25.7	20.8	15.9	12.4	9.4	2.0
	4	18.6	14.7	12.7	8.2	2.6	ND[a]
	5	19.5	14.0	11.3	9.6	3.5	<1[b]
	average	21.04	16.68	13.56	11.06	6.92	2.02
fat (ng/g)	1	63.8	57.6	54.5	40.0	27.8	6.5
	2	65.9	58.0	49.8	41.2	23.5	3.9
	3	58.0	51.2	46.1	38.0	21.0	4.8
	4	61.5	54.9	50.8	37.5	19.2	2.7
	5	56.7	50.9	43.8	35.9	18.0	3.6
	average	61.18	54.52	49.00	38.52	21.9	4.30
liver (ng/g)	1	24.9	19.3	19.0	16.6	11.5	5.6
	2	21.7	17.3	16.2	14.8	10.9	5.2
	3	27.9	21.0	18.2	16.8	13.0	8.3
	4	20.0	15.4	11.9	9.1	5.9	3.1
	5	25.7	21.1	18.4	16.5	12.3	6.8
	average	24.04	18.82	16.74	14.76	10.72	5.80
kidney (ng/g)	1	69.0	56.5	36.9	19.5	2.9	ND
	2	76.2	59.0	40.3	22.8	6.8	ND
	3	65.0	49.0	32.2	15.4	<1	ND
	4	60.5	46.8	28.0	12.6	<1	ND
	5	70.7	52.1	38.8	19.6	4.7	ND
	average	68.28	52.68	35.24	17.98	3.28	
plasma (ng/mL)	1	87.5	67.1	41.5	28.9	16.3	1.8
	2	84.0	61.9	37.8	21.9	13.0	<1
	3	90.4	65.7	43.0	27.3	14.5	<1
	4	85.8	67.2	46.2	19.7	6.1	<1
	5	77.2	59.4	38.1	17.0	<1	ND
	average	84.98	64.26	41.32	22.96	10.18	1.20

[a] ND = not detected. [b] The concentration of <1 was calculated as 1 when calculating the average.

Sample Extraction. In the previous papers, ammoniac acetonitrile/methanol[28] and 95% dimethylformamide[29] were used to extract NTV in feeds with recoveries of lower than 70%. A mixture

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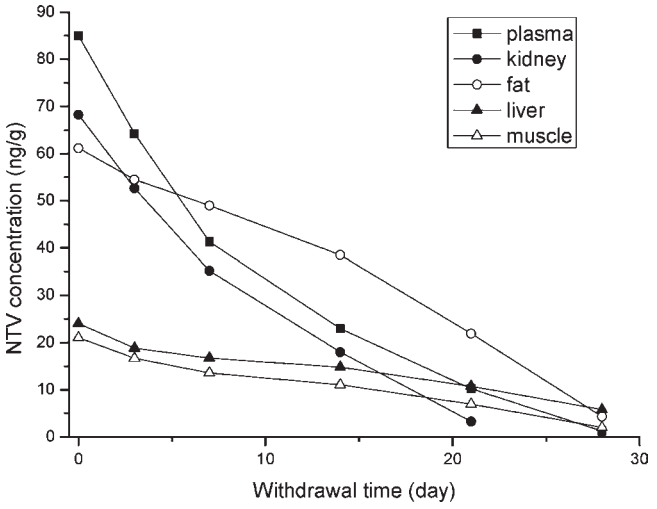

Figure 3. Depletion curves of NTV in different samples after cessation of NTV at 0, 3, 7, 14, 21, and 28 days.

of dimethylformamide/acetonitrile/methanol (50:25:25, v/v/v)[27] was reported to obtain good recoveries (>85%). Only one paper reported that the NTV residues in animal tissues were extracted with acetonitrile under the assistance of ultrasound.[30] In this study, acetonitrile/methanol (1:1, v/v) was used to extract NTV from tissue samples, and the recoveries were acceptable. The representative chromatograms of blank samples, NTV-fortified blank samples, and the real samples from NTV-treated birds showed no impurity peak around the NTV peak (Figure 2), indicating that the sample preparation could almost remove the sample impurities completely.

This is the first paper describing the development of a HPLC method for the determination of NTV in animal tissues, and this method could be a practical method for the routine monitoring of NTV residues in animal-derived products.

Residue Depletion. By now, the metabolisms of other nitrofurans have been reported in several papers,[7,9,13,17] but there has been no study reporting the metabolism of NTV. Although NTV is also a nitrofuran drug, it is different from the five banned nitrofurans because of its stable molecular structure. To verify that NTV is not metabolized *in vivo* and is accumulated in animal tissues as the parent drug, this study was designed. A total of 30 broiler chickens were fed with NTV-added feeds (10 mg/kg) for 7 consecutive days, and the residue concentrations of parent NTV in muscle, fat, liver, kidney and plasma were determined. The residue concentrations of NTV in these samples at the withdrawal period of 0, 3, 7, 14, 21, and 28 days are shown in Table 2. The depletion curves of NTV in these samples are shown in Figure 3 by plotting the withdrawal times verse the average concentrations in different sample matrixes.

At 0 withdrawal time, the NTV concentrations averaged 84.98 ng/mL in plasma, 68.28 ng/g in kidney, 61.18 ng/g in fat, 24.04 ng/g in liver, and 21.04 ng/g in muscle (Table 2). The highest level of parent NTV was in plasma immediately after cessation of NTV, which is different from the metabolism of other nitrofurans. Zuidema et al.[9] have shown that the concentrations of the metabolites of furazolidone, furaltadone, and nifursol in kidney were the highest and that the concentrations in plasma were comparable to those in liver. The NTV concentrations in plasma decreased quickly (Figure 3), and the concentrations were the lowest at the withdrawal time of 28 days (Table 2).

The residue level of NTV in kidney was high at 0 withdrawal time (only lower than that in plasma); however, the concentration decreased quickly (Figure 3), and NTV was not detectable after 28 days of cessation (Table 2).

The NTV concentrations in fat were medium at the withdrawal time of 0 days, and the depletion of NTV in fat was slow (Figure 3). At cessation of 7, 14, and 21 days, fat contained the highest NTV concentrations compared to other samples, and at cessation of 28 days, the NTV concentrations in fat still averaged 4.3 ng/g (only lower than that in liver) (Table 2). Therefore, fat could be used as a target tissue for the inspection of the NTV residue.

The residue level of NTV in liver was low at 0 day of cessation (only higher than that in muscle), which was also different from the metabolism of other nitrofurans.[11] The concentrations decreased slowly (Figure 3), and the concentrations still averaged 5.8 ng/g at the withdrawal time of 28 days (Table 2), which is similar to other nitrofurans.[11] The residue level of NTV in muscle was the lowest at 0 day of cessation, which was similar to the report.[11] The concentrations decreased slowly (Figure 3), and NTV was still detectable after 28 days of cessation (Table 2).

From the observation of Figure 3, it can be said that NTV was eliminated very slowly in chicken, although the elimination of NTV in different samples was varied. After 21 days of cessation, the concentrations of NTV were still higher than 1 ng/(g or mL) in all of the selected samples and NTV was still detectable in liver, muscle, fat, and plasma after 28 days of cessation. Therefore, a withdrawal time of at least 28 days for NTV is proposed if NTV is licensed as a feed additive in other countries. Although the depletions of NTV in plasma and kidney were faster than that in liver, muscle, and fat, all of these samples could be used to inspect the residue of NTV. However, the best target tissue was liver.

The parent drug of other nitrofurans cannot be detected even within a few hours after cessation of treatment, but the parent NTV can be detected in all of the samples after 21 days of cessation. On the basis of these findings, it can be said that, even though not all of the NTV residues, the overwhelming majority of NTV residues were in the format of the parent drug. If this point has to be proven thoroughly, the ^{14}C-labeled NTV may be used to perform a residue depletion experiment, as described by Hoogenboom et al.[10] At present, the results obtained in this study were in support of the assumption that nitrovin is not metabolized *in vivo* and is accumulated in animal tissue as the parent drug. For verification if there were tissue-bound NTV residues, the muscle samples at 0 day of withdrawal were extracted as the sample preparation for the tissue-bound metabolites of other nitrofurans, treated with hydrochloric acid (0.2 mol/L) and protease, and incubated overnight at 37 °C.[9,14−17,20] As expected, the residue levels with and without the additional treatment were almost the same, indicating that there is no tissue-bound NTV residue. Therefore, the marker residue of NTV in animal tissues was the direct extractable parent NTV, which is in accordance with the previous report.[30]

As a nitrofuran drug, NTV has been banned from use as a feed additive in food-producing animals in China because of its potential mutagenic effect. However, it has not been forbidden in other countries. This paper first reported the residue depletion of NTV in broiler chickens. Results showed that NTV was eliminated slowly *in vivo* and persisted for 28 days in chicken liver, muscle, fat, and plasma. Therefore, the results of this study are in support of the ban of NTV in food-producing animals and

the use of parent NTV as the target residue in animal-derived products. The residue depletions of NTV in other food-producing animals remain to be studied.

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