

# Determination of Tiamulin Residue in Pork and Chicken by Solid Phase Extraction and HPLC

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## ABSTRACT

A high performance liquid chromatography (HPLC) was developed for determining tiamulin residue in chicken and pork. Samples were extracted with acetonitrile, purified by liquid partition separation, and extracted with *n*-hexane at last. The *n*-hexane extract was concentrated and eluted through a Bond Elut C18 cartridge for HPLC analysis. The HPLC system was performed on a Lichrospher 100 RP-18 column (5  $\mu$ m, 4.6 mm I.D.  $\times$  250 mm) using a mixture of 80% acetonitrile and 1% ammonia carbonate (90:10, v/v) as mobile phase, and detecting wavelength was set at 210 nm with an UV-Vis detector. The calibration curve ( $R^2 = 0.9995$ ) of tiamulin was highly linear at concentrations of 0.5~8.0 ppm, while the detection limit was 0.025 ppm. Recoveries of tiamulin spiked in chicken and pork samples ranged from 84.3~97.0% and 87.9~105.9%, respectively. Each 10 chicken and pork samples sold in retail markets were tested to detect tiamulin, while none of these samples contained tiamulin.

Key words: tiamulin, veterinary drug, high performance liquid chromatography (HPLC), pork, chicken

## INTRODUCTION

Tiamulin, 14-desoxy-14-(2-diethylaminoethyl) mercaptoacetoxy mutilin (Figure 1), is a semi-synthetic derivative of the naturally occurring diterpene antibiotic pleuromutilin<sup>(1)</sup>. It has obvious activity against anaerobic bacteria and is used exclusively in animal, largely in swine. It is used for the treatment of swine dysentery, swine enzootic pneumonia and chronic respiratory disease in poultry and for weight gain and feed efficiency<sup>(1-3)</sup>. The poisoning incidents due to ingestion of the feed mixed with ionophore antibiotic and tiamulin occurred in chicken<sup>(4)</sup>. Therefore, tiamulin is not used in animal except for swine<sup>(4)</sup>. In Taiwan, the regulation standard for tiamulin residue is 0.1 ppm in pork<sup>(5)</sup>.

There have been several methods to determine tiamulin, including cylinder plate method<sup>(4)</sup>, gas chromatography (GC)<sup>(6)</sup>, high performance liquid chromatography (HPLC)<sup>(2,3)</sup> and high performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS)<sup>(7)</sup>. The detection limit of these analytical methods was 0.0014 ppm for HPLC-MS/MS<sup>(7)</sup>, 0.05 ppm for cylinder plate method<sup>(4)</sup>, 0.4 ppm for GC<sup>(6)</sup> and 10 ppm for HPLC<sup>(2,3)</sup>. HPLC-MS/MS is a recently developed analytical method, but it needs high cost for instrument. Cylinder plate method is traditionally used to determine tiamulin in livestock; however, the lack of high specificity could cause unreliable results due to other antibiotic interferences<sup>(4)</sup>. The GC and HPLC methods have lower sensitivity than other methods.

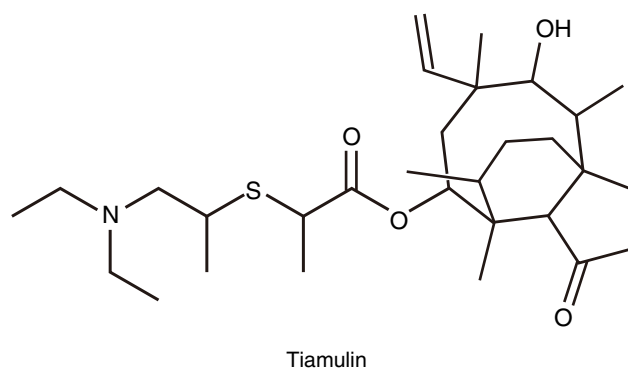


Figure 1. The chemical structure of tiamulin.

So far, there is no analytical method promulgated by the Department of Health, Taiwan, ROC. Meanwhile, it is an important issue to establish a standard analytical method for monitoring tiamulin in livestock. Therefore, the aims of this study are to establish a solid phase extraction (SPE) procedure to reduce matrix interference and to elevate the detection sensitivity by HPLC. In addition, several chicken and pork samples were purchased from Taiwanese traditional markets and analyzed for tiamulin.

## MATERIALS AND METHODS

### I. Samples

Each 10 samples of chicken and pork were purchased

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from local markets in Taiwan from March to September, 2004. All samples were homogenized and stored at -20°C before analysis.

## II. Reagents

Standard tiamulin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium carbonate, sodium carbonate and tartaric acid (analytical reagents), and acetonitrile, methanol and *n*-hexane (HPLC grade) were from Merck (Darmstadt, Germany).

## III. Instruments

A blender (Polytron Pt-3100, Kinematica AG, Littanluzern, Switzerland) and Whatman No. 2 filter paper (Whatman, Maidstone, UK) were used. The C18 SPE cartridge column (Bond Elut C18, 3 mL/500 mg, pre-treated with 10 mL of methanol and 10 mL of distilled water) was purchased from Varian Company (CA, USA). The high performance liquid chromatographic equipment used was Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan), consisting of a Shimadzu LC-10 AT pump, a Shimadzu SPD-10A UV-Vis detector (set at 210 nm), and a Shimadzu C-R4A Chromatointegrator.

## IV. Preparation of Standard Solutions

One hundred milligrams of tiamulin were taken and dissolved into 100 mL of 0.1% tartaric acid as a stock solution. Then, the stock solution was diluted with distilled water into a series of standard solutions (0.5, 1.0, 2.0, 4.0, 8.0 ppm).

## V. Analytical Procedure

### (I) Extraction

Each ground sample (10 g) of chicken and pork was extracted with 30 mL of acetonitrile and filtered. The residue was extracted for two times. The filtrates were combined and evaporated to dryness under vacuum at 45°C. The residue was dissolved into a separation funnel with 5 mL of *n*-hexane and 5 mL of 0.1% tartaric acid, and shaken for 3 min. The *n*-hexane phase was discarded, and the aqueous phase was shaken again with 5 mL of *n*-hexane. The aqueous layer was collected into another separation funnel, followed by adding 5 mL of 0.1% sodium carbonate and 5 mL of *n*-hexane, and shaking for 1 min. The *n*-hexane phase was again added with 5 mL of 0.1% sodium carbonate, shaken for 1 min, and then the aqueous layer was discarded. The *n*-hexane phase was evaporated to dryness.

### (II) Cleanup

The dried extract was dissolved with 0.2 mL of 0.1% tartaric acid and 0.8 mL of distilled water, and applied onto

a C18 SPE cartridge column, which was preconditioned with a mixture of 5 mL of methanol and 5 mL of distilled water. The column was eluted with 0.5 mL of 0.1% tartaric acid. The eluate (1.5 mL) was collected and a 20- $\mu$ L aliquot was used for HPLC analysis.

## VI. HPLC Analysis for Identification and Quantitative Test of Tiamulin

A Lichrospher 100 RP-18 reversed-phase column (5  $\mu$ m, 250  $\times$  4.6 mm, E. Merck) was used for separation, while a mixture of 80% acetonitrile and 1% ammonium carbonate (90:10, v/v) was used as the mobile phase at a flow rate of 1.0 mL/min. Each 20  $\mu$ L of the sample solution and standard solutions were injected into HPLC, respectively. The peak retention time and peak area of the sample solution were compared with those of the standard solutions. The concentration of tiamulin in the sample solution was calculated according to the following equation.

$$\text{Tiamulin concentration (ppm)} = \frac{C \times V}{W}$$

C: tiamulin concentration ( $\mu$ g/mL) in sample solution determined from standard curve

V: eluted volume (mL)

W: sample weight (g)

## VII. Recovery

The recovery of tiamulin was determined by fortifying homogenized chicken and pork samples with 0.025, 0.2, 0.4 and 0.8 ppm tiamulin. Each spiked amount was analyzed in triplicate including a blank test to evaluate the average recovery.

## VIII. Detection Limit

The detection limit of tiamulin was determined by extracting chicken and pork samples fortified at 0.025, 0.05 and 0.1 ppm levels. The procedure was the same as described above. The detection limit was evaluated according to the ratio of sample peak area to noise peak area for more than 3 times<sup>(7)</sup>.

## RESULTS AND DISCUSSION

### I. The Mobile Phase of HPLC and Linearity of the Standard Curve

Markus and Sherma<sup>(2)</sup> used the mobile phase of methanol/acetonitrile/1% ammonia carbonate solution (60:30:25, v/v/v) to determine tiamulin in feed premixes. In this study, the response of the chromatogram for standard solution of tiamulin decreased as the methanol ratio in the mobile phase increased. The best response was obtained by using a mixture of 80% acetonitrile and 1% ammonia carbonate (90:10, v/v) as the mobile phase.

Figure 2 was the standard curve of tiamulin determined by HPLC. The linear regression equation of tiamulin standard curve was calculated as  $y = 35316x + 5424$  ( $R^2 = 0.9995$ ), where  $y$  was the peak area and  $x$  was the concentration of tiamulin. The correlation coefficient was higher than 0.999, which showed a good linearity within the range of 0.5 to 8 ppm.

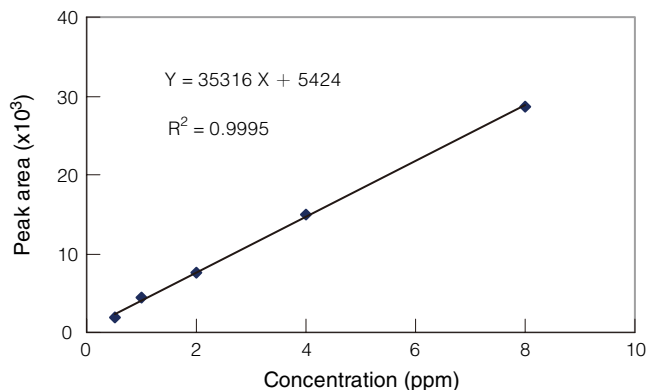


Figure 2. The standard curve and correlation equation of tiamulin determined by HPLC.

## II. Extraction and Cleanup Conditions

Schlusenser *et al.*<sup>(7)</sup> reported that ethyl acetate was the suitable solvent for extraction of tiamulin in liquid manure. Markus and Sherma<sup>(2,3)</sup> demonstrated that hexane/ethyl acetate (3:1, v/v) solution was used for tiamulin extraction in feed premixes and swine feeds. However, our study showed that extraction of tiamulin from pork and chicken with ethyl acetate or hexane/ethyl acetate solution caused persistent emulsion, and the recovery of tiamulin was low (40~50%, data not shown). Therefore, acetonitrile was used as an extracting solvent to eliminate this problem in this study.

It was found that many impurities interfered with the determination of tiamulin, when Bond Elut C18 cartridge was not used to cleanup the impurities from the extracts from chicken and pork samples (Figures 3 and 4). Although sample preparation with a different cartridge (diol SPE cartridge) has been reported by Schlusenser *et al.*<sup>(7)</sup>, we found that partition with *n*-hexane followed by passing the extract through a Bond Elut C18 cartridge could effectively remove the impurities from the extracts from chicken and pork samples (Figures 3 and 4).

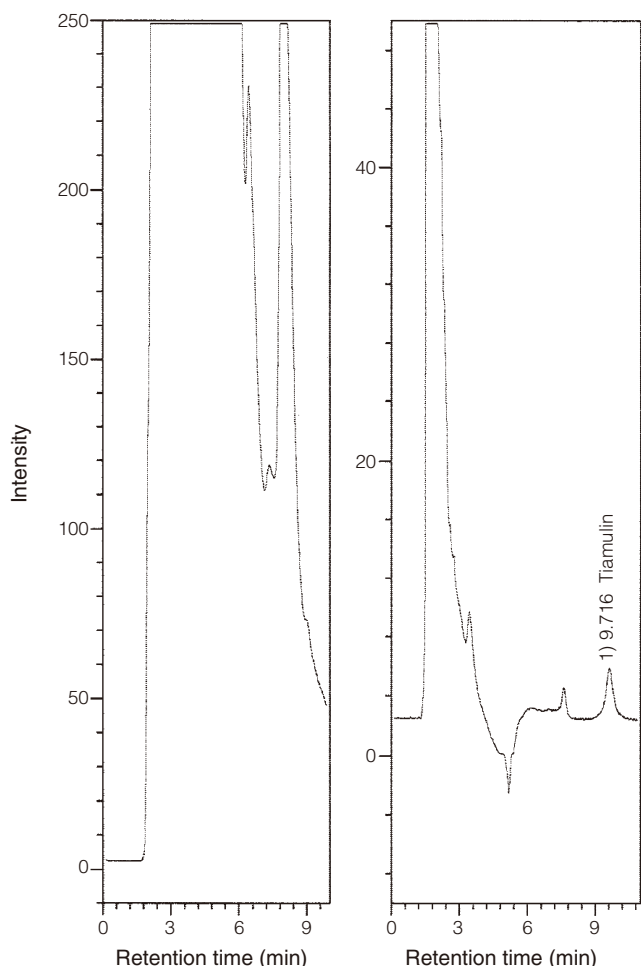


Figure 3. The high performance liquid chromatograms of chicken spiked with 0.8 ppm tiamulin and prepared without (A) and with (B) C18 SPE cartridge.

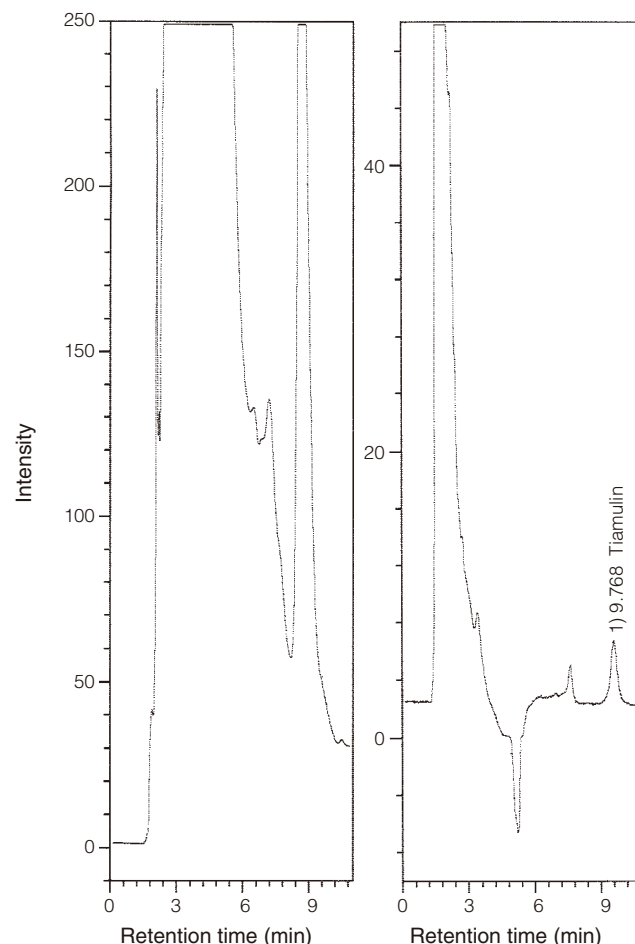


Figure 4. The high performance liquid chromatograms of pork spiked with 0.8 ppm tiamulin and prepared without (A) and with (B) C18 SPE cartridge.

### III. Recovery and Detection Limit

The recoveries of tiamulin spiked into chicken and pork samples with four amounts (0.025, 0.2, 0.4 and 0.8 ppm) determined by HPLC were shown in Table 1. It was found that the recoveries of tiamulin spiked into chicken and pork samples ranged from 84.3 to 97.0% and 87.9 to 105.9%, respectively, while the coefficients of variation (CV) were lower than 8.3%. This result indicated the analytical method was quite accurate for the determination of tiamulin. The detection limit of tiamulin was 0.025 ppm in both chicken and pork samples according to the signal-to-noise ratio of 3:1 reported by Schlusener *et al.*<sup>(7)</sup>. This level (0.025 ppm) is lower than the residue limit (0.1 ppm) of veterinary drugs set by Department of Health<sup>(5)</sup>. In addition, the detection limits of tiamulin reported for other analytical methods were 0.0014 ppm for HPLC-MS/MS<sup>(7)</sup>, 0.05 ppm for cylinder plate method<sup>(4)</sup>, 0.4 ppm for GC<sup>(6)</sup> and 10 ppm for HPLC<sup>(2,3)</sup>. The higher detection limit (10 ppm) of tiamulin by HPLC reported by Markus and Sherma<sup>(2,3)</sup>, compared to 0.025 ppm in this study, could be due to the poor absorption at 254 nm in HPLC system. After UV spectrum scanning at the range of 200~350 nm for tiamulin, the maximum UV absorption at 210 nm was selected for tiamulin detection in this study. Although the detection limit in this study was higher than that of the HPLC-MS/MS method<sup>(6)</sup>, it was still lower than those of other methods<sup>(2-4,6)</sup>.

### IV. Survey of Tiamulin in Commercial Chicken and Pork Muscle Samples

In this study, 10 each chicken and pork samples sold in local retail markets were tested to detect tiamulin residue. All of them were below the detection limit (< 0.025 ppm).

### CONCLUSIONS

Judging from the above data, the HPLC method with a mixture of 80% acetonitrile and 1% ammonia carbonate (90:10, v/v) as mobile phase was valid, accurate and precise for the determination of tiamulin residue in chicken and pork samples. The detection limit was as low as 0.025 ppm and the average recovery was higher than 84.3%. The sample preparation using acetonitrile extraction and C18 SPE cartridge cleanup was appropriate for the determination of tiamulin residues in chicken and pork samples. None of the 10 each commercial chicken and pork samples contained the tiamulin residue.

**Table 1.** Recoveries of different amounts of tiamulin spiked into chicken and pork samples as determined by HPLC

Sample	Spiked level (ppm)	Recovery (%) <sup>a</sup>
Chicken	0.025	84.3 (7.1) <sup>b</sup>
	0.2	91.0 (5.4)
	0.4	97.0 (8.3)
	0.8	91.0 (4.6)
Pork	0.025	87.9 (5.2)
	0.2	105.1 (7.1)
	0.4	99.2 (4.3)
	0.8	105.9 (4.7)

<sup>a</sup>Average of triplicate determinations.

<sup>b</sup>Values in parentheses are coefficients of variation (CV, %).

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