Determination of Valnemulin and Tiamulin in Feed by Solid Phase Extraction-Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry (Postprint)

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Abstract

This study aimed to establish an efficient and rapid solid-phase extraction-ultra-high performance liquid chromatography-tandem mass spectrometry (SPE-UPLC-MS/MS) method for the determination of valnemulin and tiamulin in feed. Valnemulin and tiamulin in feed were extracted with methanol, purified using an Oasis MCX solid-phase extraction cartridge, separated on a C18 chromatographic column with a mobile phase of water (containing 0.1% formic acid)-acetonitrile (containing 0.1% formic acid) under gradient elution, and detected using electrospray ionization (ESI) in positive ion mode under multiple reaction monitoring (MRM) mode, with quantification performed by the external standard method. The results showed that the limits of detection (LODs) of the established SPE-UPLC-MS/MS method for valnemulin and tiamulin in feed were 25.0 and 5.0 ng/g, respectively. Good linear relationships were obtained for valnemulin and tiamulin concentrations in the range of 0.01–5.00 g/mL, with correlation coefficients (R^2) greater than 0.99. At three spiked levels (2.5, 25.0, and 125.0 mg/kg), the recoveries of valnemulin ranged from 85.17% to 94.55% with relative standard deviations (RSDs) of 2.56%–7.65%, while the recoveries of tiamulin ranged from 84.69% to 99.36% with RSDs of 1.57%–4.95%. The results demonstrated that the established SPE-UPLC-MS/MS method is simple to operate, exhibits high accuracy, and can be applied for the rapid quantitative determination of valnemulin and tiamulin in feed.

Full Text

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Abstract

This study aimed to establish a rapid and efficient method for determining valnemulin and tiamulin in feed using solid phase extraction-ultra performance liquid chromatography-tandem mass spectrometry (SPE-UPLC-MS/MS). The analytes were extracted from feed samples with methanol, purified using an Oasis MCX solid phase extraction column, separated on a C18 chromatographic column with gradient elution using water (containing 0.1% formic acid) and acetonitrile (containing 0.1% formic acid) as mobile phases, and quantified by external standard calibration under multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI). The results demonstrated that the limits of detection (LOD) for valnemulin and tiamulin in feed were 25.0 ng/g and 5.0 ng/g, respectively. Both compounds exhibited good linearity in the concentration range of 0.01–5.00 g/mL with correlation coefficients (R^2) greater than 0.99. At three spiking levels (2.5, 25.0, and 125.0 mg/kg), the recoveries of valnemulin ranged from 85.17% to 94.55% with relative standard deviations (RSD) of 2.56%–7.65%, while tiamulin recoveries ranged from 84.69% to 99.36% with RSDs of 1.57%–4.95%. The established SPE-UPLC-MS/MS method is simple, accurate, and suitable for rapid quantitative determination of valnemulin and tiamulin in feed.

Keywords: solid phase extraction; ultra performance liquid chromatography-tandem mass spectrometry; feed; valnemulin; tiamulin

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Introduction

Since the 1950s, large quantities of veterinary drugs have been used in livestock production to promote animal growth and improve health. However, the abuse and overuse of these drugs in food-producing animals can lead to residues in meat, eggs, and milk products [1-5]. To ensure human and animal safety, the European Union completely banned the use of antibiotics as growth-promoting feed additives in food-producing animals starting in 2006 [6]. Nevertheless, antibiotics may still be illegally added to feed. Therefore, monitoring antibiotic

residues in feed is crucial for ensuring the safety of animal-derived food products, and analyzing drug content in feed has become an important means of guaranteeing feed and food safety [7].

Valnemulin and tiamulin (Figure 1 [Figure 1: see original paper]) belong to the same class of drugs and are new-generation semi-synthetic antibiotics of the pleuromutilin class [8-9]. As diterpene compounds, they are widely used in livestock, aquaculture, and feed additives due to their specific efficacy against Gram-positive bacteria and mycoplasma.

Currently, the main analytical methods for detecting valnemulin and tiamulin include high-performance liquid chromatography (HPLC) [10-12] and high-performance liquid chromatography-mass spectrometry (HPLC-MS) [13-16]. The EU non-mandatory act (2002/657/EC) introduced the concept of identification points (IP) for mass spectrometric analysis. As confirmatory methods for animal-derived samples, antibiotic detection should be based on more than one characteristic. LC-MS/MS operating in MRM mode generates one precursor ion and two product ions, providing 4 IPs for quantitative detection of antibiotics, whereas HPLC coupled with a diode array detector can only generate 1 IP [6]. Consequently, LC-MS/MS is predominantly used for quantitative detection of these two compounds. Most reported studies on valnemulin and tiamulin detection have focused on animal-derived foods such as livestock and poultry meat products, aquatic products, milk, and honey [15,17-22], with relatively few investigations targeting feed as the analytical matrix.

Feed composition may include cereals, sugars or fruits, fats, roots or tubers, legumes or oilseed plants, amino acids, and minerals. Thus, feed matrix is an extremely complex sample. During antibiotic detection, the main interfering substances include carbohydrates, lipids, proteins, vitamins, inorganic substances, additives, and contaminants or residues. These compounds may be co-extracted, causing signal suppression during LC-MS/MS analysis of valnemulin and tiamulin [6,23]. Effective extraction and purification are essential for accurate antibiotic determination.

In view of these challenges, this study optimized instrumental conditions and pretreatment methods for three types of feed samples—formula feed, concentrated feed, and premix feed—to successfully establish a rapid and effective SPE-UPLC-MS/MS method for monitoring valnemulin and tiamulin content in feed.

Materials and Methods

1.1.1 Reagents

Valnemulin hydrochloride (purity 99.95%) was purchased from Toronto Research Chemicals (Canada). Tiamulin (purity 98%) and HPLC-grade formic

acid (purity 98%) were obtained from Aladdin (USA). HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich (USA). MCX solid phase extraction cartridges (60 mg, 3 CC) were obtained from Waters Oasis (USA). Ammonia solution (analytical grade) was purchased from Sinopharm Chemical Reagent Co., Ltd. Feed samples were provided by Zhejiang Kesheng Feed Co., Ltd.

1.1.2 Instruments

The analytical system consisted of a Waters ACQUITYTM UPLC TQ Mass detector (Waters, USA), KQ-500E ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.), ultrapure water system (EMD Millipore, Germany), electronic analytical balance (Mettler Toledo, Switzerland), ST 40R centrifuge (Thermo, USA), and nitrogen evaporator (Organomation, USA).

1.2.1 Extraction

A 2.0 g feed sample was weighed into a 50 mL centrifuge tube, and 15 mL methanol was added [24]. The mixture was ultrasonicated for 30 min, then centrifuged at 5,000 r/min for 10 min. The supernatant was collected, and 15 mL methanol was added to the precipitate for a second extraction with ultrasonication for 30 min. After centrifugation, the two supernatants were combined, 0.2% formic acid aqueous solution was added, and the volume was adjusted to 50 mL with a volumetric flask for subsequent use.

1.2.2 Purification

One milliliter of the extract was loaded onto an MCX solid phase extraction column, which was sequentially washed with 2 mL 60% methanol aqueous solution (containing 0.1% formic acid), 2 mL 5% formic acid aqueous solution, and 2 mL methanol. The wash solutions were discarded. The analytes were then eluted with 2 mL 5% ammonia methanol solution. The eluate was collected and evaporated to dryness under nitrogen at 50 °C [24]. The residue was reconstituted in 2 mL 20% acetonitrile aqueous solution (containing 0.1% formic acid), filtered through a 0.22 m membrane, and analyzed by UPLC-MS/MS.

1.3.1 Chromatographic Conditions

Chromatographic separation was performed on a BEH C18 column (50 mm × 2.1 mm, 1.7 m particle size) with an injection volume of 2 L and flow rate of 0.5 mL/min [25]. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The gradient elution program is shown in Table 1 .

1.3.2 Mass Spectrometry Conditions

Mass spectrometric detection was conducted using an electrospray ionization source in positive ion mode with MRM. The optimized parameters were: capillary voltage 3.5 kV, source temperature 150 °C, desolvation temperature 500 °C. Nitrogen was used as nebulizer gas, curtain gas, and auxiliary gas, while argon served as collision gas.

In accordance with EU regulations (2002/657/EC), one precursor ion and two product ions were selected for each analyte, providing 4 identification points. The MS/MS parameters are summarized in Table 2 .

1.4 Preparation of Standard Curves

Accurately weighed 13.31 mg of valnemulin hydrochloride standard was dissolved in acetonitrile and diluted to 25 mL to prepare a stock solution of 500.0 g/mL. Similarly, 12.50 mg of tiamulin standard was dissolved in acetonitrile and diluted to 25 mL to obtain a 500.0 g/mL stock solution. Equal volumes of the two stock solutions were mixed to prepare a mixed standard working solution containing 250.0 g/mL of each compound.

This mixed standard working solution was diluted to seven concentration levels (0.25, 1.25, 2.50, 12.50, 25.00, 62.50, and 125.00 g/mL). Seven portions of blank feed samples (2.0 g each) were spiked with 4 mL of each concentration level and processed according to section 1.2, yielding matrix-mixed standard working solutions with final concentrations of 0.01, 0.05, 0.10, 0.50, 1.00, 2.00, and 5.00 g/mL for both analytes. Each concentration was prepared in duplicate, and each sample was injected twice. Additionally, six blank formula feed samples were processed similarly without standard addition to serve as blank matrix controls. All final solutions were filtered through 0.22 m membranes before UPLC-MS/MS analysis.

1.5 Accuracy and Precision

Considering the clinical supplementation levels of valnemulin and tiamulin in feed (5–200 g/g) and the potential for instrument contamination at high concentrations, mixed standard working solutions at 1.25, 12.5, and 62.5 g/mL were added to feed matrices to achieve final spiking levels of 2.5, 25.0, and 125.0 mg/kg. Six replicates were prepared for each feed type at each concentration level, with each sample injected twice to calculate recoveries and relative standard deviations.

1.6 Data Processing

Data were processed and calculated using Excel 2010 statistical software, with results expressed as mean values.

Results and Analysis

2.1 Linear Range of the Method

Following the procedure described in section 1.4, the results showed good linearity for valnemulin (y = 315.8x - 21,855.8, R^2 = 0.9988) and tiamulin (y = 552.9x + 59,124.5, R^2 = 0.9961) in the concentration range of 0.01–5.00 g/mL.

2.2 Limits of Detection and Quantification

The limits of detection (LOD) were established at 25.0 ng/g for valnemulin and 5.0 ng/g for tiamulin, both exceeding a signal-to-noise ratio of 3:1. The limits of quantification (LOQ) for both compounds were set at 0.5 g/g, corresponding to a signal-to-noise ratio greater than 10:1, which satisfies international requirements for analytical method performance.

2.3 Accuracy and Precision of the Method

Using formula feed, concentrated feed, and premix feed as test matrices, 2.0 g samples were spiked with 4 mL of mixed standard working solutions at 1.25, 12.5, and 62.5 g/mL to achieve final concentrations of 2.5, 25.0, and 125.0 mg/kg. Samples were prepared according to section 1.2, with six replicates per spiking level. As shown in Table 3 , recoveries of valnemulin ranged from 85.17% to 94.55% with RSDs of 2.56%–7.65%, while tiamulin recoveries ranged from 84.69% to 99.36% with RSDs of 1.57%–4.95% across the spiking range of 2.5–125 mg/kg. Chromatograms of blank feed (Figure 2 [Figure 2: see original paper]) showed no interference peaks for either analyte, while matrix-spiked standard solution chromatograms (Figure 3 [Figure 3: see original paper]) displayed sharp peaks without interfering signals, demonstrating the method' s stability and compliance with international performance criteria for veterinary drug residue analysis.

Discussion

3.1 Sample Matrix Effects

Matrix components can cause signal suppression or enhancement [24]. In this study, mixed standard solutions and matrix-matched standard solutions at 0.05 g/mL were analyzed. The average peak area of the valnemulin product ion (m/z 263.02) was 12,353 in pure standard solution but decreased to 11,680 in matrix-matched solution. Similarly, the tiamulin product ion (m/z 192.01) showed an average peak area of 51,487 in standard solution versus 44,116 in matrix-matched solution. The reduced peak areas in both cases indicated that the feed matrix exerted a suppressive effect on both analytes. Therefore, this study employed blank feed samples spiked with valnemulin and tiamulin to construct matrix-matched calibration curves for external standard correction.

3.2 Selection of Solid Phase Extraction Column

Unpurified samples exhibited strong matrix effects [26], leading to column contamination, peak broadening, increased column pressure, and reduced sensitivity. Based on references [10-11], three MCX SPE cartridge specifications were evaluated: 3CC (60 mg), 6CC (150 mg), and 6CC (500 mg). The 6CC cartridges required larger elution volumes, while the 3CC (60 mg) cartridge completely eliminated matrix effects and provided reliable results. Therefore, considering efficiency and cost-effectiveness, the 3CC (60 mg) MCX cartridge was selected for feed sample purification.

3.3 Selection of Purification Conditions

Oasis MCX sorbent is a mixed-mode cation-exchange reversed-phase adsorbent offering high selectivity and sensitivity for basic compounds through combined ion-exchange and reversed-phase retention mechanisms. While typical protocols involve preconditioning with methanol, water, and formic acid solution before sample loading [10,14], this study found that direct sample loading onto the Oasis MCX column without preconditioning, followed by immediate elution, effectively eliminated matrix effects while achieving high recoveries.

Literature references report various water bath temperatures (37 °C [25], 40 °C [27], and 50 °C [24]) for concentrating extracts or eluates containing valnemulin and tiamulin using nitrogen evaporation. This study evaluated these three temperatures for concentrating a 0.10 g/mL mixed standard solution. Compared with directly prepared 0.10 g/mL standard solution, no significant differences were observed among the three temperatures (Table 4). Therefore, 50 °C was selected to improve experimental efficiency.

Conclusion

1. This study successfully established a solid phase extraction purification procedure using sequential washing with 2 mL 60% methanol aqueous solution (containing 0.1% formic acid), 2 mL 5% formic acid aqueous solution, and 2 mL methanol, followed by elution with 2 mL 5% ammonia methanol solution to obtain purified extracts containing valnemulin and tiamulin.
2. Matrix interference was corrected by external standard calibration using UPLC-MS/MS analysis, establishing a quantitative method for determining valnemulin and tiamulin in feed. The limits of detection were 25.0 ng/g for valnemulin and 5.0 ng/g for tiamulin.
3. Both compounds showed good linearity in the range of 0.01–5.00 g/mL with $R^2 > 0.99$. At three spiking concentrations (2.5, 25.0, and 125.0 mg/kg), valnemulin recoveries were 85.17%–94.55% (RSD: 2.56%–7.65%) and tiamulin recoveries were 84.69%–99.36% (RSD: 1.57%–4.95%).

4. In summary, the developed SPE-UPLC-MS/MS method is simple, accurate, and applicable for rapid quantitative analysis of valnemulin and tiamulin in feed.

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Note: Figure translations are in progress. See original paper for figures.