



Liquid to liquid extraction and liquid chromatography–tandem mass spectrometry determination of hainanmycin in feed



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ABSTRACT

Hainanmycin is a new veterinary polyether antibiotic and has few sensitive analytical method in present days. In this study, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) relying on multiple reaction monitoring (MRM) detection was developed for analysis of hainanmycin in animal feed. Feed samples were extracted with ethyl acetate and purified by two steps of liquid–liquid extraction (LLE) to get rid of water solvable matrix and lipids one by one. The final simple was analyzed by LC–MS/MS. The LC mobile phase was composed of 0.1% aqueous formic acid and 0.1% formic acidified acetonitrile by gradient elution. Average recoveries ranged from 74.22% to 87.85%, as determined by spiking with 2.0 (LOQ) \sim 2500 $\mu\text{g kg}^{-1}$ of hainanmycin. The inter-day and intra-day coefficient of variation was 9.21% to 11.77% and 7.67% to 13.49%, respectively. The limit of detection (LOD) and the limit of quantitation (LOQ) were 0.36 $\mu\text{g kg}^{-1}$ and 2.0 $\mu\text{g kg}^{-1}$, respectively.

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1. Introduction

Hainanmycin (Fig. 1) is a new veterinary polyether antibiotic, a digestion promoter and metabolic conditioner [1–3]. It is primarily effective for inhibiting gram-positive bacterium, and is used for the prevention and treatment of poultry coccidiosis and added to cattle, sheep, and other livestock feed as growth promoting agent [4]. Toxicological studies on hainanmycin show that it can have certain negative effects. Losing of appetite and paralysis can occur in animals fed with high concentrations of hainanmycin and drug residue has also been detected in human food [5]. Fast, simple and effective analytical methods are urgently needed to monitoring the content of hainanmycin in animal feed.

Only two Chinese published papers have been published on the analysis of hainanmycin [6,7]. Wen et al. identified the molecular weight and molecular structure of hainanmycin by time of flight mass spectrometry (TOF) [6], and also determined its purity by high-performance liquid chromatography (LC) [7]. There are a number of other commercialized polyether antibiotics, semduramicin and maduramicin have the closest chemical structure to hainanmycin (Fig. 1) [8,9]. Some related analytical methods have

been reported for these polyether antibiotics [9–13]. Chang et al. established an online sample pretreatment technique coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the determination of maduramicin residue in chicken [9]. Cronly et al. analyzed eleven coccidiostats (including semduramicin and maduramicin) in animal feed by LC–MS/MS [10]. Ha et al. determined semduramicin, maduramicin and other polyether antibiotics in food samples by LC–MS/MS with a limit of quantitation (LOQ) value of 0.4 $\mu\text{g kg}^{-1}$ for milk and 1 $\mu\text{g kg}^{-1}$ for chicken, for chicken liver and for eggs [11]. Pereira et al. analyzed maduramicin and semduramicin in ultra-high-temperature processed milk using quick, easy, cheap, effective, rugged and safe method of extraction and followed by LC–MS/MS [12]. Piatkowska et al. reported an LC–MS/MS method for determination of different class of analytes including maduramicin and semduramicin in eggs, with purification process of liquid–liquid extraction and Hybrid solid phase extraction [13]. All these published method provide good insight for the development of a LC–MS/MS method for analyzing hainanmycin in animal feed.

2. Material and methods

2.1. Chemicals and reagents

Hainanmycin (Fig. 1) was purchased from the Chinese Institute of Veterinary Drug Control (Beijing, China). Ethyl acetate and

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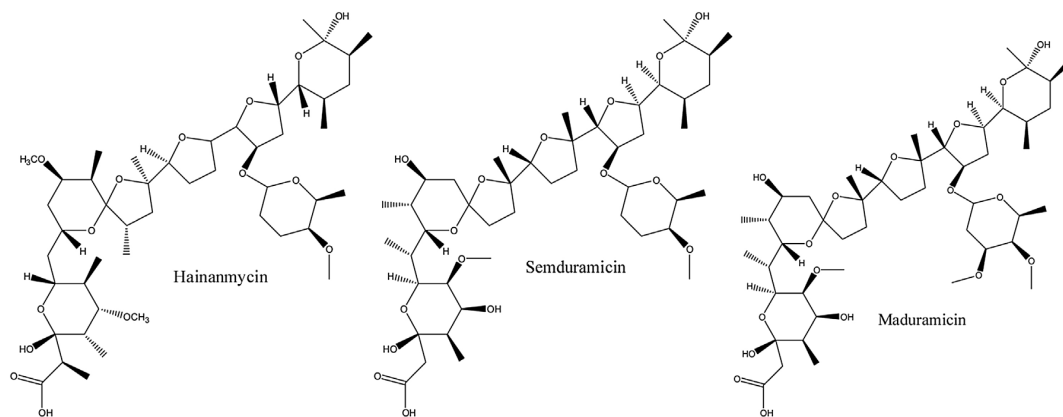


Fig. 1. Chemical structure of hainanmycin, semduramicin and maduramicin.

methanol were chromatographic pure grade; both obtained from Dima Technology Inc. (Muskegon, MI, USA). Analysis pure grade of hexane was purchased from Sigma-Aldrich Corporation (St. Louis, Mo, USA). Deionized water was generated from a Milli-Q filtration system (Millipore, Bedford, MA, USA). All other chemicals and reagents were analytical grade and obtained from the Beijing Chemical Company (Beijing, China).

Stock solutions of hainanmycin were prepared in acetonitrile at concentrations of 0.01, 0.1 and 1.0 mg mL⁻¹. The working solutions were prepared by further diluting with 90% methanol as required. All solutions were stored at -20 °C.

2.2. Instrumentation conditions

An ACQUITY UPLC™ system (Waters Co., MA, USA) coupled with a Quattro LC triple quadrupole tandem mass spectrometer equipped with a Z-spray ESI interface (Micromass British Co, Manchester, UK) was used for separation and analyses.

Chromatographic separation was achieved through an ACQUITY UPLC® C₁₈ column (50 mm long, 2.1 mm i.d., 1.7 μm particle size; Waters, Milford, MA, USA) at the column temperature of 35 °C. The flow rate was 0.2 mL min⁻¹. Solvents used were 0.1% aqueous formic acid (A) and 0.1% formic acidified acetonitrile (B) with an elution gradient shown in Table 1. The injection volume was 2 μL.

Mass quantification set on multiple reaction monitoring (MRM) in the positive ion mode. The desolvation temperature was 300 °C and the source temperature was 120 °C. Nitrogen was used as the desolvation gas at a flow rate of 275 L h⁻¹. Collision-induced dissociation (CID) was performed using the collision gas argon, at the pressure of 2.7 × 10⁻³ mbar. The precursor ion selected was *m/z* 907.5, and the product ions detected were *m/z* 845.3 and *m/z* 863.4, using optical mass spectrometric parameters.

2.3. Sample preparation

One gram of ground feed sample was weighed in a polypropylene centrifuge tube (50 mL). Ethyl acetate (2 mL) was added to

the tube and the contents of the tube mixed by vortexing for 1 min. Then the sample was centrifuged at 3500 r min⁻¹ for 3 min. The extraction step was repeated twice and each supernatant was collected, combined and transferred to a 10 mL centrifuge tube.

Water (4 mL) was added in the ethyl acetate extract and mixed by vortexing for 1 min, followed by centrifugation for 5 min at 3500 r min⁻¹. The upper phase was transferred into a new 10 mL tube and dried under a stream of nitrogen while warming in a water bath temperature at 40–50 °C. A mixture of 2.0 mL of 90% methanol and 1.0 mL of hexane was added to the tube containing the extract. The mixture was vortexed by 2 min to ensure that all drug including that coating wall of the tube had been fully dissolved.

The phases were separated by centrifugation for 2 min at 3500 r min⁻¹. The upper layer (hexane) was discarded and remaining lower methanol layer (1 mL) was withdrawn and filtered through a 0.22 μm methanol compatible membrane for LC–MS/MS analysis.

2.4. Calibration and quantification

Matrix-matched calibration curves of 0.1, 1, 5, 10, 50, 100, 500 μg L⁻¹ of hainanmycin were prepared, the peak area ratios obtained were used to prepare a calibration curve to hainanmycin concentration (transferred to concentration in feed).

Five replicates of drug-free feed samples were extracted and performed by LC–MS/MS analysis. The signal-to-noise ratio of 3 was used to calculate the limit of detection (LOD) and the concentration of LOD was transferred to the drug amount in feed. The limit of quantification (LOQ) was considered acceptable by at least 10 times response of signal-to-noise ratio (transferred to the drug amount in feed too) and was proven by 5 replicates of reasonable spiking samples.

Five replicates feed samples containing hainanmycin at 100, 250, 2500 μg kg⁻¹ were extracted and analyzed in triplicate to determine method accuracy, intra-assay precision and inter-assay precision. The highest spiking concentration of hainanmycin were diluted a ten-fold for above the up limit of determination before performed to LC–MS/MS.

2.5. Actual sample preparation

Eight actual feed samples from different feed production factories were collected. These actual feed samples were extracted and analyzed by LC–MS/MS and amount of hainanmycin in each was determined using the calibration curve. If the drug concentration was over the up limit of quantification, it should be diluted

Table 1
Gradient elution proceed for analysis of hainanmycin.

Time (min)	A	B
0.00	90.0	10.0
2.00	1.0	99.0
3.00	1.0	99.0
4.00	90.0	10.0
6.00	90.0	10.0

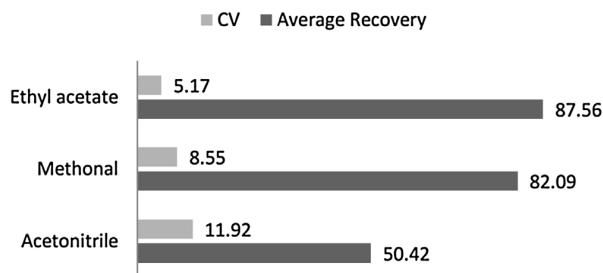


Fig. 2. Average extractive recoveries (%) of three solvents for extracting hainanmycin in feedstuff.

3. Results analysis and discussion

3.1. Sample preparation

Hainanmycin has similar structure to other two polyether antibiotics, semduramicin and maduramicin (Fig. 1). Primarily we intended to develop its preparation proceed from those two antibiotics on the cases of few publishes about Hainanmycin. Acetonitrile is the most popular extraction solvent for analysis of them in animal feed [10]; milk [11,12]; chicken, chicken liver and egg samples [11,13]. Water and methanol extraction was reported for analysis of them in chicken meat [9]. We also considered another solvent, ethyl acetate, which is well-known as a green solvent. Hainanmycin has the chemical structure of tetrahydrofuran and tetrahydropyran rings and has no-polar features, we thought that it might be easy to dissolve in ethyl acetate. A one-time extraction efficiency of acetonitrile, methanol and ethyl acetate at identically 10 μL volumes for 100 $\mu\text{g mL}^{-1}$ of hainanmycin in 1 g feed were investigated in five replicate samples. The results shown in Fig. 2, clearly demonstrated that ethyl acetate shows the highest average recovery among these three solvents, 5% and 37% greater than methanol and acetonitrile, respectively. Furthermore, an extraction efficiency of 87% is sufficient when combined with an LC–MS/MS. Then ethyl acetate, without additives, was selected as the extraction solvent.

3.2. Optimization of UPLC–MS/MS conditions

Methanol-water-trifluoroacetic acid (90:10:0.05, v/v/v) and 0.1% aqueous formic acid-0.1% formic acidified methanol (10:90, v/v) had been previously used for analysis of hainanmycin by LC and LC–MS [6,7]. The application of a 0.1% aqueous formic acid-0.1% formic acidified methanol mobile phase gave better peak shape than methanol-water-trifluoroacetic acid in this experiment, so we kept it as an option for further optimization. Generally, methanol and acetonitrile afford similar separation resolution on chromatography, but for some analyte, they show large differences in the peak shape or resolution. Here many different isocratic and gradient elution systems relying on 0.1% aqueous formic acid-0.1% formic acidified methanol and 0.1% formic acid-0.1% formic acidified acetonitrile were tested to optimize the analysis of hainanmycin by LC–MS/MS. The gradient elution presented in Table 1 was selected as the final mobile phase as it afforded the best peak shape, the sharpest elution time and the fewest number of peak disturbances. LC–MS/MS parameters also optimized with the MRM detection in ESI positive ion mode, parent ion of m/z 907.5 and product ions of m/z 845.3 and m/z 863.4 were selected to be the quantification ion pairs. Under these optimized conditions, chromatograms of hainanmycin in feed were analyzed (Fig. 3).

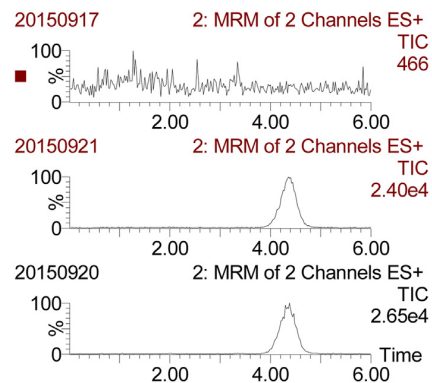


Fig. 3. Chromatograms of hainanmycin in feedstuff (A. Blank sample, B. 2.0 $\mu\text{g kg}^{-1}$ spiked sample, C. 1.0 $\mu\text{g L}^{-1}$ standard solution).

Table 2
Recoveries and CVs of spiked hainanmycin in feed.

Fortification ($\mu\text{g kg}^{-1}$)	Recovery (n = 5, %)	Iner-day CV (n = 5, %)	Inter-day CV (n = 3, %)
2.0 (LOQ)	87.85	11.77	13.49
100	81.97	9.21	10.52
250	87.09	9.92	5.33
2500	74.22	10.95	7.67

3.3. Method validation

Analyte spiked samples were used as calibration standards for compensating the influence of matrix, and satisfactory recoveries were obtained by quantification using the analyte-fortified calibration curve. The chromatographic response values of all analyte-matched standards containing from 0.1 to 500 $\mu\text{g L}^{-1}$ of hainanmycin were calculated and the following linear regression equation was obtained: $y = 4097.5x - 6358$, with the correlation coefficient of 0.9999. Thus, an excellent linear correlation was obtained between chromatographic peak area and hainanmycin concentration. Thus, 500 $\mu\text{g L}^{-1}$, which equals to 1000 $\mu\text{g kg}^{-1}$ of target drug in feed was regarded as the upper limit of quantification (ULOQ).

The selectivity of the method was evaluated using five drug free samples and five spiked samples. No interference was observed at the retention time of the hainanmycin (see Fig. 3). The LOD was 0.36 $\mu\text{g kg}^{-1}$ calculated as signal to noise rate of 3 (transfer to drug concentration in feed). The acceptable LOQ was decided to be 2.0 $\mu\text{g kg}^{-1}$ compared to 10 times of signal to noise (1.2 $\mu\text{g kg}^{-1}$) after we analyzed 5 replicates of the same concentration of spiked samples (Table 2).

Accuracy and precision were evaluated by determining recoveries and the coefficient of variation values (CV) of hainanmycin in spiked feed samples at the levels of 2.0 (LOQ), 100, 250, 2500 $\mu\text{g kg}^{-1}$ for three days. Mean recoveries of hainanmycin ranged from 74.22% to 87.85%, with the intra-day CV of 9.21–11.77% and inter-day CV of 7.67–13.49%. These results demonstrated gave good method stability and reproducibility.

3.4. Actual sample detection

Three swine premix feeds, three middle swine feeds and two swine concentrated feeds were pretreated by the preparing method and detected by the current method. Hainanmycin at 1071 $\mu\text{g kg}^{-1}$ was detected in one premix feed and at 100 $\mu\text{g kg}^{-1}$ was detected in one middle swine feed. No hainanmycin was detected in other feed

sample tested. These results suggest that our method can be applied to actual feed samples to determine the presence of hainanmycin.

4. Conclusion

A quantification method of LC–MS/MS for hainanmycin in feed was developed according to the intensive feed monitoring require of present time [1–5]. The analyte was extracted from feed by ethyl acetate and then purified by two steps of LLEs to remove water soluble matrix and lipids one by one. The final sample was solved in methanol and analyzed by LC–MS/MS. The quantifiable range for hainanmycin was 1–500 $\mu\text{g kg}^{-1}$. Samples with concentrations above the ULOQ can be reliably diluted 10 times using control matrix to fall within the validated calibration range. This method affords good reproducibility, sensitivity and works well on hainanmycin in actual arrival feed samples.

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