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Contributed Paper

Qualitative and Quantitative Analysis of Tildipirosin by Ultra-performance Hydrophilic Interaction Liquid Chromatography-tandem High Resolution Quadrupole Time-of-flight Mass Spectrometry

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ABSTRACT

Tildipirosin, a derivative of tylosin, is a semisynthetic 16-membered-ring macrolide antimicrobial. An ultra-performance hydrophilic interaction liquid chromatography-tandem high resolution quadrupole time-of-flight mass spectrometry method is reported herein to detect tildipirosin. Experiments were conducted to investigate the effects of the chromatographic column and the pH of the mobile phase on the separation of tildipirosin. Experimental results showed that the best detection conditions were as follows: a ACQUITY UPLC BEH Amide (100×2.1 mm, 1.7 μm) chromatographic column was used for the separation, with acetonitrile (containing 10 mmol/L ammonium formate, 0.125% formic acid, 5% water) and 0.125% formic acid aqueous solution (containing 10 mmol/L ammonium formate) as the mobile phases. The flow rate was 0.4 mL/min and the temperature of the column was set at 40 °C. Electrospray ionization mass spectrometry (ESI-MS) was used as the detector, applied in the full scan positive ion mode. The linear detection range was 62.5～1000 ng/mL with a correlation coefficient of 0.9962. The limit of detection and quantitation were 0.74 and 2.45 ng/mL. The developed method exhibited good repeatability and high accuracy. The tildipirosin fragments were analyzed and compared using the Molecular Structure Correlator. The overall matching score was 98.99 and the matching scores of five main fragments were above 90, which provided the necessary evidence for the structural confirmation of tildipirosin.

Keywords: ultra performance hydrophilic interaction liquid chromatography, high resolution quadrupole time-of-flight mass spectrometry, tildipirosin, qualitative analysis, quantitative analysis

1. INTRODUCTION

Tildipirosin, a derivative of tylosin, is a semisynthetic 16-membered-ring macrolide antimicrobial (Figure 1) [1, 2]. Sterile injection of tildipirosin has currently been approved for sale in Europe for the treatment of diseases of the respiratory system and is primarily used in bovines (180 mg/mL, subcutaneous injection) and swine (40 mg/mL intramuscular injection) [1-3]. The high incidence of mortality resulting from respiratory diseases in swine and bovines is an important problem in the global livestock breeding industry [4-7]. Tildipirosin can be used to prevent and treat trespiratory infections in pigs and cattle that originate from bacterial infection [1, 2, 3, 8]. Similar to the action of other macrolide antimicrobials, tildipirosin inhibits the protein synthesis in bacteria by binding to the 50S subunit of the ribosome in select strains of bacteria [9].

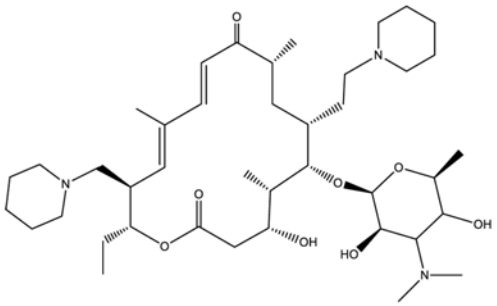


Figure 1. Chemical structure of tilipirosin.

Hydrophilic interaction liquid chromatography (HILIC) was proposed by Alpert in 1990 [10], and consists of the use of a polar stationary phase and a water-water soluble organic solvent (mostly acetonitrile) as the mobile phase. This method has been found to efficiently separate and provide accurate detection of strong polar and ionic compounds [11-13]. The high concentration of organic solvents benefits increased ionization efficiency in ESI-MS and can improve the detection sensitivity. In addition, HILIC is quite compatible with ESI-MS [14]. Compared to traditional liquid chromatography (LC), ultra-performance liquid chromatography (UPLC) offers the advantages ultrahigh pressure, high sensitivity and a high degree of separation [15]. High resolution quadrupole time-of-flight mass spectrometry can provide an accurate mass of mother ions and fragment ions that can help predict the possible molecular formula by further confirming the structure and fragmentation pattern [16, 17]. UPLC-Q-TOF-MS can improve the reliability of the antibiotic high speed qualitative screening, but can also provide good quantitative results with high sensitivity [18, 19]. HPLC-MS/MS has proven to be the best method for detecting tildipirosin [3, 20, 21]. However, there is a disadvantage in the current HPLC-MS/MS method for the detection of tildipirosin. For example, some methods [3] have provided quantitative results for tildipirosin, but no structural information could be obtained, while other methods [20, 21] were employed as screening processes to detect multiple-types of antibiotic, but none of these could be used to quantify tildipirosin.

Combining these features, a novel UPLC-Q-TOF-MS method was developed for tildipirosin separationand detection. The method is very fast, accurate and sensitive for tildipirosin. It was capable of measuring the mass of fragment ions as well as the analysis of the characteristic ion fragments and fragmentation patterns, which produces a fast, qualitative and quantitative analysis.

2. MATERIALS AND METHODS

2.1 Instrument and Reagents

For the UPLC analysis an Agilent 7890B Ultra Performance Liquid Chromatography

(Agilent, USA) equipped with binary gradient pump, vacuum degassing system and auto sampler was employed together with an Agilent 6540 Q-TOF mass spectrometry (Agilent, USA) for detection of the tildipirosin. An Agilent ChemStation system (Agilent, USA) was used to control the instrument, data acquisition and statistical evaluation of the data. Chromatographic grade formic acid, ammonium formate, ammonium acetate, and acetonitrile were purchased from Fisher Scientific (USA). Acetic acid and ammonia were analytical grade and were obtained from the Beijing Chemical Factory (China). Deionized water was used throughout the analytical process.

2.2 Standards and Samples

Standard samples of tildipirosin (structure confirmed by NMR, purity 98.0%) were prepared and purchased from the China Animal Husbandry Industry Co., Ltd. Various sizes of injection samples of Zuprevo®(The concentration of tildipirosin is 180 mg/mL.), 50, 100 and 250 ml were from obtained from Intervet International B.V., a member of the MSD Animal Health Group (Germany).

2.3 Chromatography

The analytical separation was conducted using an ACQUITY UPLC BEH amide column (100 × 2.1 mm, 1.7 mm) run at 40 °C. Mobile phase A and B were composed of acetonitrile (containing 10 mmol/L ammonium formate, 0.125% formic acid, 5% water) and water (containing 10 mmol/L ammonium formate, 0.125% formic acid). The flow rate was 0.4 mL/min and a 3 µL of sample was introduced into the UPLC system. The solvent gradient was as follows (A): 90% to 30% from 0.00 to 5.00 min, 30% to 90% from 5.00 to 5.10 min, and it was maintained at 90% to 8.00 min.

2.4 Mass Spectrometry

Positive mass spectra were obtained in the full scan mode (100-1000 amu). The spray and cone voltages were 3500V and 65V. The temperature and the flow rate of the drying gas were 300 °C and 8 L/min with 350 °C and 11 L/min for the sheath gas. The atomizing gas pressure was 35 psi.

2.5 Sample Preparation

The test and standard solutions were prepared by dissolving and diluting respective samples of tildipirosin in methanol to the required concentrations.

3. RESULTS AND DICUSSION

3.1 Optimization of Ultra-performance Hydrophilic Interaction Liquid Chromatography

3.1.1 Comparison of various chromatographic columns

Both the C_{18} column and HILIC column were used for the tildipirosin detection with water (containing 0.1% formic acid) and acetonitrile as the mobile phases. As shown in Figure 2, the pseudo molecular ion peaks of tildipirosin $[M+H]^+$ and $[M+2H]^{2+}$ were split and tildipirosin was barely retained on C_{18} column. As a result, the C_{18} column was deemed unsuitable for tildipirosin determination. However, tildipirosin was sufficiently retained on the HILIC column (Figure 3), which offered the possibility for use in the tildipirosin determination and analysis.

3.1.2 Influence of pH on the separation of tildipirosin

Since tildipirosin is an alkaline polar compound, pH has a significant influence on its chromatographic separation. Tildipirosin decomposes easily under strong acidic conditions, so the pH of the mobile phase was no less than 3.0. The HILIC column is

generally used in an acidic environment, so pH value of 3.5 and 5.3 were used to determine the relative separation effect (as shown in Figure 4 and Figure 5). Tildipirosin exhibited an effective retention at pH 3.5, and at this pH the asymmetry factor was much closer to 1 and a good peak shape resulted (Table 1). Therefore, it was concluded that a pH of 3.5 was a good choice for the analysis and detection of tildipirosin.

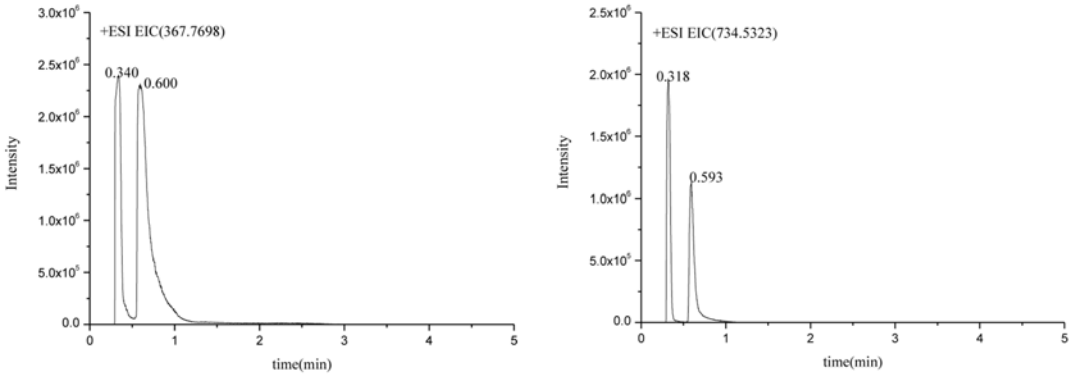


Figure 2. Typical extracted ion chromatograms of tildipirosin separated by C18 column in primary mass spectrum.

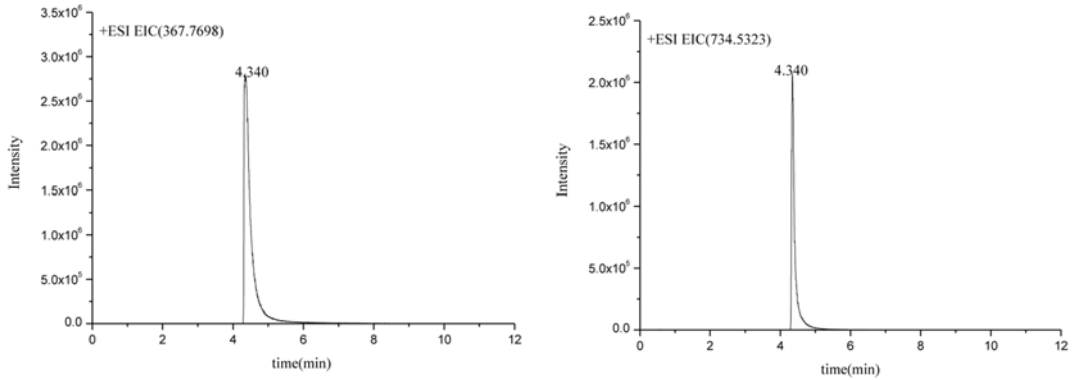


Figure 3. Typical extracted ion chromatograms of tildipirosin separated by HILIC column in primary mass spectrum.

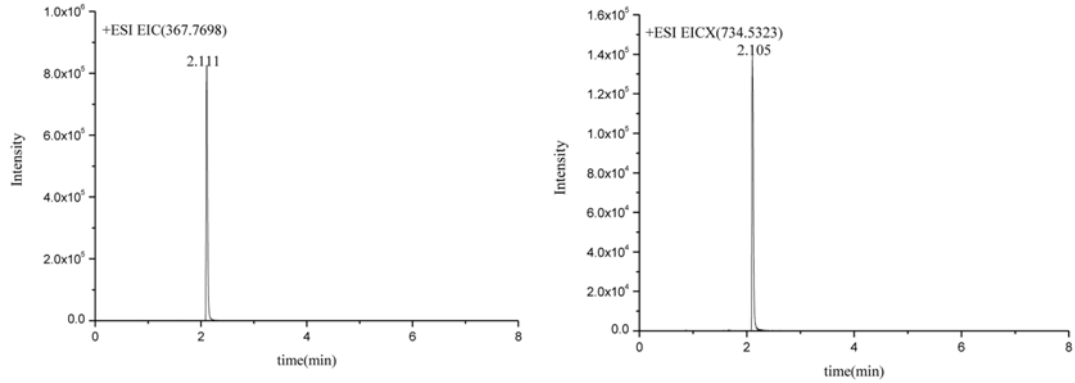


Figure 4. Typical extracted ion chromatograms of tilipirosin in primary mass spectrum at pH 3.5.

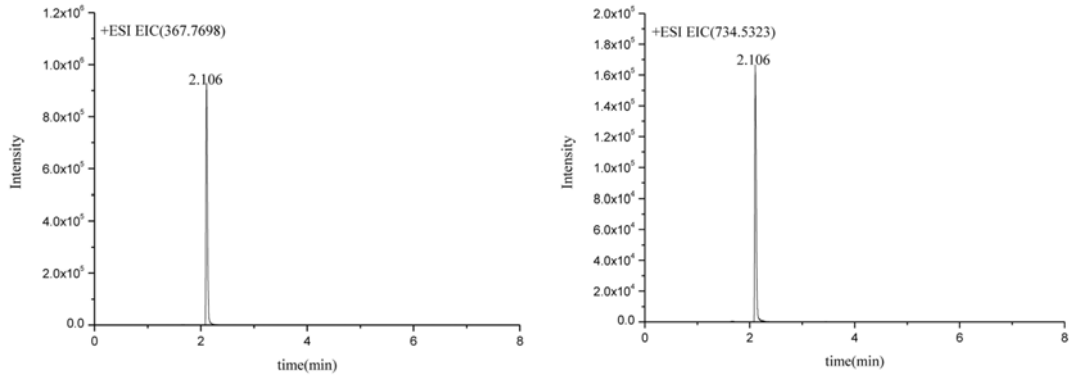


Figure 5. Typical extracted ion chromatograms of tilipirosin in primary mass spectrum at pH 5.3.

Table 1. Asymmetry factors of extracted ions in mass spectrum under different separation conditions.

pH	Extracted ions in MS	Asymmetry factors
3.5	$[M+2H]^{2+}$	1.19
	$[M+H]^{+}$	1.27
5.3	$[M+2H]^{2+}$	1.89
	$[M+H]^{+}$	1.83

3.2 Qualitative Analysis of the Mass Spectrum

The reference solution of tildipirosin was analyzed using the method previously described in Section 1.3 and the resulting mass spectra are shown in Figure 6. The pseudo molecular ion peaks of tildipirosin were observed at m/z 367.7698 and 734.5323, which corresponded to $[M+2H]^{2+}$ and $[M+H]^{+}$. In addition, the peak $[M+2H]^{2+}$ had a higher intensity than $[M+H]^{+}$, suggesting that the extraction ion intensity of $[M+2H]^{2+}$ could be used for the quantitative analysis of tildipirosin. The main fragments m/z 98.0965, m/z 174.1125, m/z 561.4263, m/z 132.1018 and m/z 88.0757 in tandem mass spectrum were considered to be characteristic fragments of tildipirosin.

3.3 Methodology Validation

3.3.1 Linearity, limits of detection, quantitation

A set of calibration standards at concentrations of 62.5 ng/ mL, 125 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL were individually prepared. A subsample volume of 3 µL was introduced into UPLC-Q-TOF-MS and the chromatogram was recorded. The areas of extracted ion peak $[M+2H]^{2+}$ exhibited good linearity with concentrations of tildipirosin in the range of 62.5-1000 ng/mL. The linear equation for these results was Y=9372.7X+165763 and the coefficient of correlation was 0.9962. Further research indicated that the limit of detection (LOD) was 0.74 ng/mL (S/N=3) and the limit of quantitation (LOQ) was 2.45 ng/mL (S/N=10).

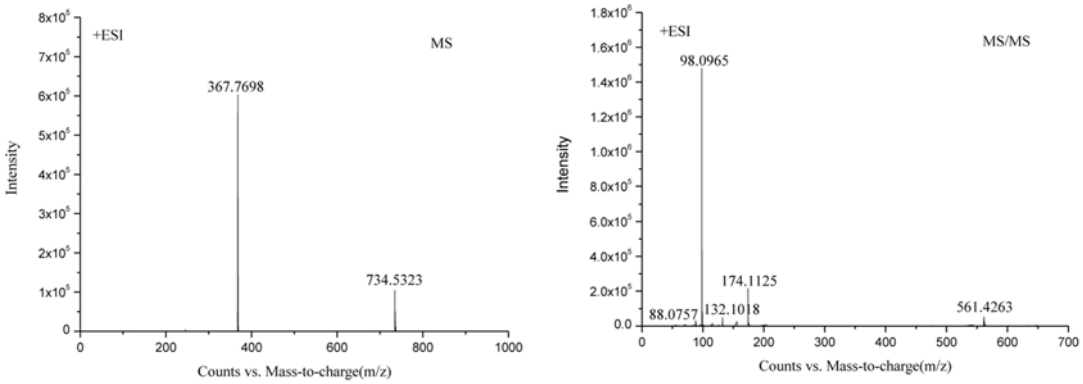


Figure 6. Primary mass spectrum and tandem mass spectrum of working reference sample of tildipirosin.

3.3.2 Recovery, accuracy and precision

Recoveries of tilipirosin were assessed at three independent spike levels, which were 250, 500, 1000 ng/mL. Reference samples of tilipirosin were added to methanol to evaluate the relative recoveries. Experiments were conducted three times at each level, and three different samples were determined continuously. The average recovery, inter- and intra- coefficients of variation were calculated using these analyses. As shown in Table 2, the inter-assay recoveries varied between 93.6 to 102.6% and the coefficients of variation ranged between 2.72 to 4.30%. The intra-assay recoveries varied between 91.8 to 106.7% and the coefficients of variation ranged between 4.97 to 8.01%. This indicated that the proposed method possessed both good repeatability and high accuracy.

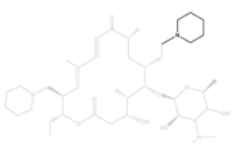
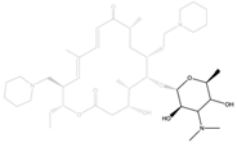
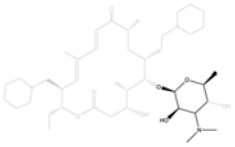
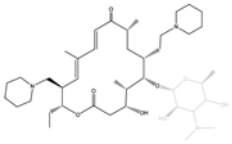
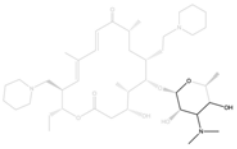
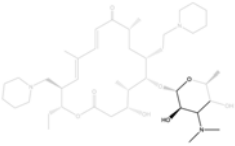
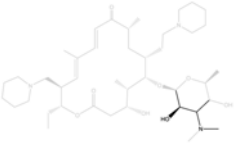
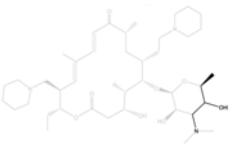
Table 2. Inter- and intra- recoveries and coefficients of variation at three spike levels.

Spike levels	% Recoveries		% Coefficients of variation	
(ng/L)	Intra (n=3)	Inter (n=3)	Intra (n=3)	Inter (n=3)
250	98.8	106.7	4.30	4.97
500	93.6	98.1	2.72	5.04
1000	102.6	91.8	2.91	8.01

3.4 Analysis and Comparison of Ion Fragments in High Resolution Quadrupole Time-of-flight Mass Spectrometry (MS/MS)

The general fragmentation pattern of tildipirosin has, as yet, not been reported in the literature. The five characteristic fragment ions in the MS/MS analysis conducted in this reported study were analyzed and compared to the Molecular Structure Correlator (Table 3). The overall matching score that was obtained was 98.99 and each matching score of the five main fragments was above 90. These results illustrated that the fragmentation ions in the MS/MS analysis correlated to those predicted and provided a general fragmentation pathway for tildipirosin.

Table 3. Fragment ions observed in tandem mass spectrum.

Serial number	m/z	Structures of fragmentation	Molecular formula	Scots
1	98.0965		$C_6H_{12}N$	97.7
2	174.1125	 or 	$C_8H_{16}NO_3$	98.5
3	561.4263		$C_{33}H_{57}N_2O_5$	99.6
4	132.1018	 or 	$C_6H_{14}NO_2$	94.2
5	88.0757	 or 	$C_4H_{10}NO$	94.2

3.5 Determination of Practical Samples

The commercial injection sample of tildipirosin (Zuprevo®) was diluted with methanol to the recommended concentration. The average concentration was calculated from the chromatographic peak area based on the internal standard method. The concentrations of the three different tildipirosin samples were determined according to the above method and three parallel determination results are shown in Table 4.

Table 4. Concentration determination results (N=3).

Packaging Quantities	50 mL	100 mL	250 mL
Content (mg/mL)	179.48	181.31	180.39
RSD%	2.13	1.83	2.44

4. CONCLUSIONS

In this study, ultra-performance hydrophilic interaction liquid chromatography-tandem high resolution quadrupole time-of-flight mass spectrometry method was used to analyze for tildipirosin. The optimized experimental conditions were obtained after a systematic study and the methodology was validated. The primary research on the fragmentation pattern of tildipirosin provided evidence for the confirmation of the material's structure. In summary, the developed analytical method proved to be a fast and accurate technique for analysis of tildipirosin and produced improved qualitative confirmation, which validated its use for fulfilling the demands of tildipirosin detection in industrial drug production processes.

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