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Determination of gamithromycin in an injection by ultra-performance liquid chromatography-tandem quadrupole-time-of-flight mass spectrometry

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In this study, a sensitive and precise method was developed for the determination of gamithromycin in an injection using ultra-performance liquid chromatography-tandem quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS) and the results were compared with a similar analysis for the ion fragments of gamithromycin in MS/MS. The sample was dissolved in methanol then filtered and separated on a C18 column using acetonitrile-0.1% formic acid (containing 2 mmol/L ammonium acetate) as the mobile phase. The flow rate was 0.4 mL/min and the column temperature was 40 °C. The mass spectrometry conditions were electrospray ionization (ESI) operated in positive ion full scan mode and quantified using external calibration. Subsequently, ion fragments of the MS/MS were compared and analyzed. The linear range was 10 ~ 200 μg/L with a correlation coefficient of 0.9992. The limit of detection (LOD) was 0.77 μg/L and the limit of quantitation (LOQ) was 2.55 μg/L. The average recoveries of the intra-assay were 98.8%-105.6% with a relative standard deviation ranging from 1.79% to 2.38% and the inter-assay were 89.3%-110.7% with a relative standard deviation ranging from 4.93% to 6.27%. After the comparative analysis of the fragments with a Molecular Structure Correlator, the score of the total matching degree reached 83.19 and the scores of each ion fragment matching degree were all greater than 90, which supplied the basis for the confirmation of gamithromycin. The results indicated that the method was simple, sensitive and precise and could be applied in the determination of gamithromycin in real samples.

1. Introduction

Gamithromycin belongs to the 15-membered semi-synthetic macrolide antibiotics of the azalide sub-class and is a novel 7a-azalide that has recently been developed for treatment and prevention of bovine respiratory disease (BRD)(Huang et al. 2010) caused by *Mannheimia haemolytica*, *Mycoplasma mycoides subspecies mycoides*, *Mycoplasma*, *Streptococcus equi subsp. zooepidemicus* (*S. zooepidemicus*), *Rhodococcus equi* and so on (Forbes et al. 2011; Mitchell et al. 2013; Gautier-Bouchardon et al. 2014; Berghous et al. 2011). The uniquely positioned alkylated nitrogen at the 7a-position (Huang et al. 2010) of the lactone ring (Fig. 1) is conducive to rapid absorption of gamithromycin under physiological pH conditions that is beneficial to the long-term effect of the target tissue in the lung. The bacteriostatic and inhibiting activity of gamithromycin are similar to macrolides in that both inhibit bacterial protein synthesis by binding to 23S ribosomal RNA in the 50S-subunit of prokaryotic ribosomes and prevent protein elongation during the translocation process (Jain et al. 2004; Menninger et al. 1985). Because of its excellent antibacterial activity, gamithromycin has been extensively applied in veterinary medicine. At present, there are four synthetic methods used to fabricate gamithromycin and the ZACTRAN which are produced by Merial Limited whose major active component is gamithromycin. These have been sold in various EU countries.

Compared to traditional liquid chromatography, ultra performance liquid chromatography (UPLC) may improve the speed of analysis, separation efficiency, sample flux and sensitivity (Li et al. 2010). Quadrupole-time-of-flight mass spectrometry (Q-TOF-MS) offers the advantages of high resolution and high accuracy (Li et al. 2010). Using UPLC- Q-TOF-MS, we obtained the molecular ion information and abundant fragment ion information for gamithromycin, which may help provide accurate, quality information for this confirmation of the compound (Zhao et al. 2013).

In the present study, we developed a detection method for the determination of gamithromycin using ultra-performance liquid chromatography-tandem quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS) and primarily studied the general fracture pattern of protonated ions of gamithromycin.

2. Investigations, results and discussion

2.1. Qualitative mass spectrometry analyses

Detection of gamithromycin standard solutions was conducted according to the methods detailed in Section 3.3 and the mass spectrometry are shown in Figs. 2 and 3. The pseudo molecular ion peaks of gamithromycin in MS were $[M+2H]^+$(m/z389.2779) and $[M+H]^+$ (m/z777.5480). The intensity of $[M+2H]^+$ was higher than $[M+H]^+$, so the ion flow of $[M+2H]^+$ was extracted for quantitative analysis. The major fragments in MS/MS were m/z619.4534, m/z601.4446, m/z158.1177, m/z116.1072 and m/z83.0492. The gamithromycin standard solution extracted ion flow of $[M+2H]^+$ is shown in Fig. 3, where the retention time was 3.163 min with a sharp and symmetric peak, which met the requirements for gamithromycin detection.

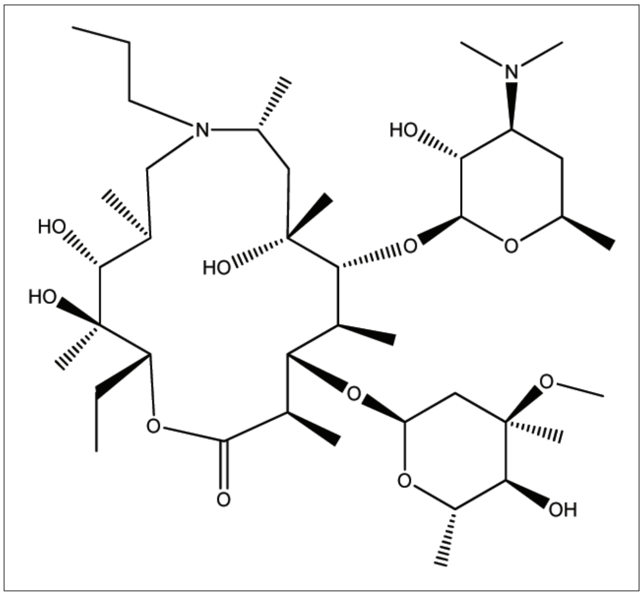


Fig.1: Structure of gamithromycin

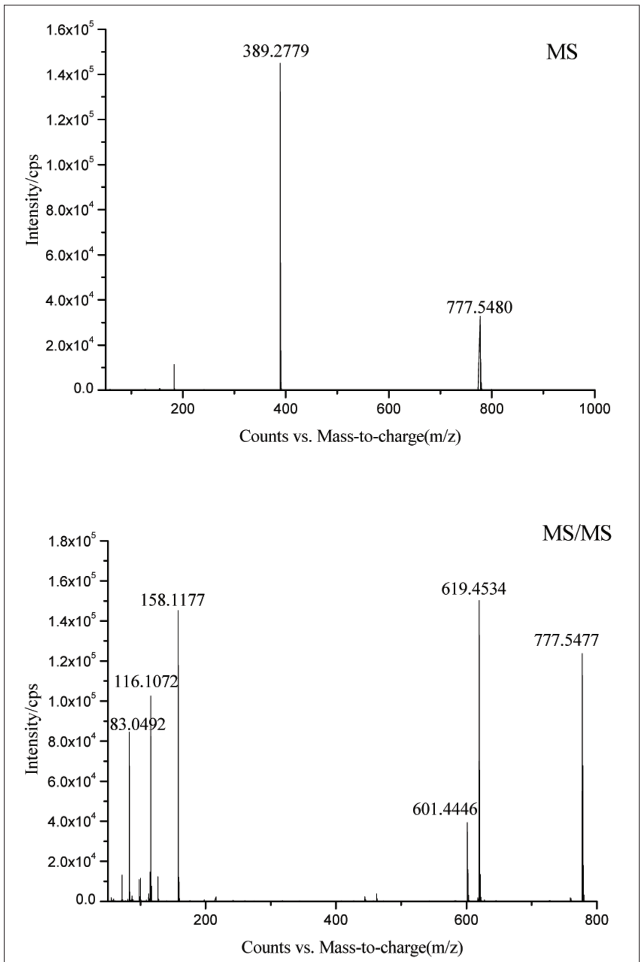


Fig.2: MS and MS/MS mass chromatogram of gamithromycin standard

2.2. Method validation

2.2.1. Standard curve

Using an appropriate amount of a gamithromycin standard, five standard samples were prepared using methanol to obtain a standard concentration curve. These standards were of the concentrations 10 μg/L, 25 μg/L, 50 μg/L, 100 μg/L and 200 μg/L. We chose to use the extracted ion flow peak area of the MS pattern of gamithromycin as the Y-coordinate and the concentrations of gamithromycin as the X-coordinate to plot the standard curve. The linear relationship for this curve was y=3849.5x+109606 with a correlation coefficient R=0.9992 and in the range of 10~200 μg/L. It was found that the linearity of the curve was acceptable.

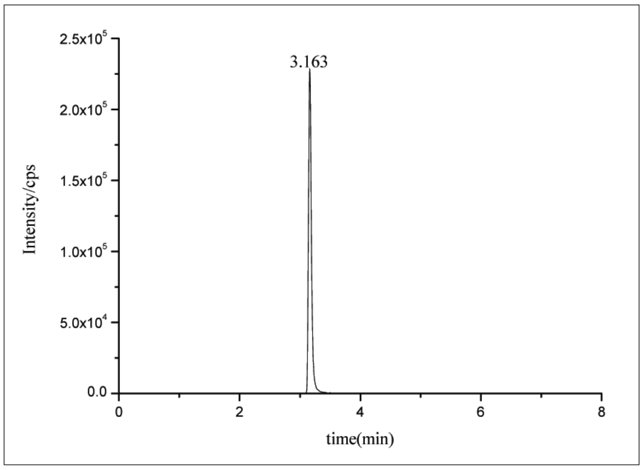


Fig.3: Extracted ion flow chart of gamithromycin standard

2.2.2. LOD and LOQ

For the samples appropriate amounts of the injectable gamithromycin was diluted with methanol. The detection limit (LOD) was 0.77 μg/L with S/N=3 and the quantitation limit (LOQ) was 2.55 μg/L with S/N=10.

2.2.3. Recovery, accuracy and precision of the analytical method

The injection matrix was spiked with standard concentrations of 30 μg/L, 50 μg/L, 100 μg/L to detect gamithromycin in the injection matrix (the main ingredient was glycerolformal) to confirm its performance for target analysis in the sample test. The average recoveries of the intra-assay (n=3) were 98.8-105.6 % with a relative standard deviation ranging from 1.79 % to 2.38 % and the inter-assay (n=3) were 89.3-110.7 % with a relative standard deviation ranging from 4.93% to 6.27%. The results indicated the acceptable accuracy and precision of the developed UPLC-Q-TOF-MS method.

2.3. Comparative analyses of fragments of MS/MS

There are no recent reports of the fracture laws of gamithromycin, so we compared the gamithromycin ion fragments in MS/MS utilizing the Molecular Structure Correlator. As shown in Table 2, the overall score was 83.19 and the matching score for the ion fragment structure was more than 90, which demonstrated a good correlation between ion fragments in MS/MS and expected fragments. The results clarified the gamithromycin general fracture law and supplied a good basis for the confirmation of gamithromycin in the standard samples.

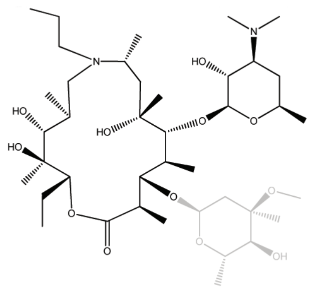
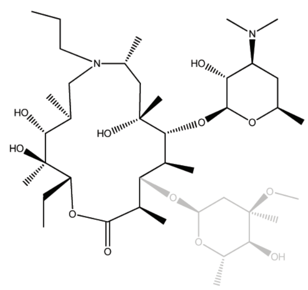
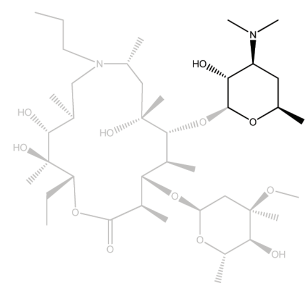
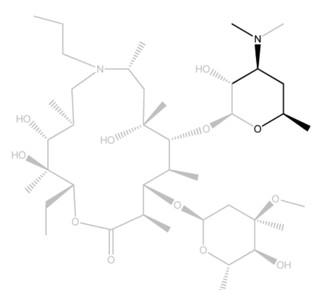
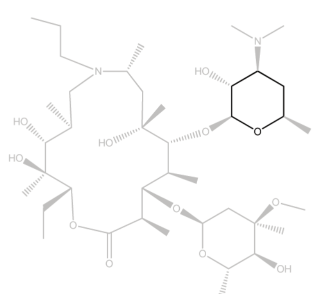
2.4. Analysis of gamithromycin in real samples

To demonstrate the applicability of the developed method, real samples were tested. The extracted ion flow chart in MS is shown in Fig. 4, the retention time for the real sample was consistent with that for the gamithromycin standards and the peak-shape was good, which demonstrated that UPLC-Q-TOF-MS could be used for the analysis of injectable gamithromycin.

Table 1: Recoveries in injection matrix

Spike levels (μg·L^{-1})	Recoveries (%)		Coefficient of variation(%)	
	Intra (n=3)	Inter (n=3)	Intra (n=3)	Inter (n=3)
30	98.8	108.8	1.79	4.93
50	102.7	89.3	1.97	6.27
100	105.6	110.7	2.38	5.76

Table 2: Comparative analysis of ion fragment structure

Num.	m/z	Ion fragment structure	Score
1	619.4534		99.6
2	601.4446		97.9
3	158.1177		98.5
4	116.1072		94.1
5	83.0492		92.7

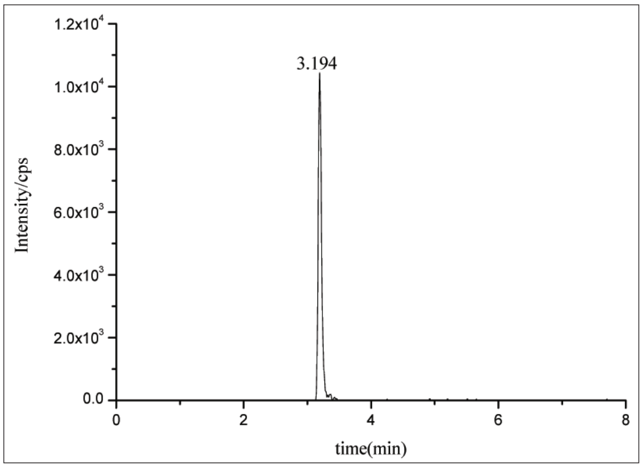


Fig.4: Extracted ion flow chart of gamithromycin in real samples

3. Experimental

3.1. Apparatus

For the analyses, an Agilent 7890B Ultra performance Liquid Chromatography (UPLC) was used equipped with binary gradient pump, vacuum degassing machine and autosampler, an Agilent 6540Q-TOF mass spectrometer, with instrumental control, data acquisition and data analysis employed the Agilent "Mass Hunter" system and the fragment analysis software used for this study was the Molecular Structure Correlator.

3.2. Chemicals and reagents

Gamithromycin standards were obtained from the China Animal Husbandry Industry Co., Ltd (the structure of the gamithromycin in the standard was confirmed by nuclear magnetic resonance and the chemically purity was found to be 96.2%). A gamithromycin injection was used (150 mg/ml, Merial Limited), chromatographically pure formic acid and massmatographic pure acetonitrile were obtained from Fischer Scientific and used as received.

3.3. UPLC-Q-TOF-MS analyses

3.3.1. UPLC conditions

An Agilent Extend-C18 column (50 x2.1 mm, 1.8 μm) was used for the analysis with a column temperature of 40 °C. Mobile phase A consisted of 0.1% formic acid aqueous solution (2 mmol/L ammonium acetate), mobile phase B was acetonitrile with a flow-rate was 0.4 mL/min, the injection volume was 3 μL; The following gradient was applied: 0.00→5.00 min, 90%→70% A; 5.00→5.10 min, 70%→90% A; 5.10→8.00 min, 90%→90% A.

3.3.2. Mass spectrometry conditions

The electro-spray positive ion source (ESI+) had a spray voltage of 3500 V, a vaporizer temperature of 300 °C, cone voltage: 65 V. The sheath gas temperature was 350 °C and dry gas and sheath gas flow were 8 and 11L/min. The nebulizer gas pressure was 35 psi and the full scan mode (50~1200amu) was employed.

3.4. Solutions

The gamithromycin injection was diluted for testing: by dissolving the full volume of the injection in methanol to a final concentration of 1000 μg/L. The gamithromycin standard was diluted for testing: by dissolving the standard in methanol to a final concentration of 1000 μg/L.

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