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Simultaneous Determination of Various Macrolides by Liquid Chromatography/Mass Spectrometry

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ABSTRACT

Macrolides are frequently used in veterinary medicine as therapeutic and preventive agents for various diseases. It is difficult to determine macrolides simultaneously with conventional methods due to their similar structures. A simultaneous analysis for erythromycin, roxithromycin, tiamulin and tylosin with LC/MS has been developed. Separation was performed on C18 reversed phase column. Mobile phase was gradiently flowed with 10 mM ammonium acetate and methanol. The mass spectrometer was run in the positive mode and selective ion monitoring mode. The molecular ions were [M+H]+ form at m/z 837.5 for erythromycin, at m/z 859.5 for roxithromycin, at m/z 494.2 for tiamulin and at m/z 916.7 for tylosin. Limits of detection were in the range from 0.001 to 0.01 μg/g lower than their MRLs.

Keywords : simultaneous determination; liquid chromatography/mass spectrometry; macrolides antibiotics

1. Introduction

Macrolide antibiotics have 12-, 14-, 16- or 17-membered macrocyclic lactone ring, which is bound to several amino and/or neutral sugars (fig.. 1). Because of their effective antimicrobial activity against Gram-positive bacteria, mycoplasma, chlamydia, they are frequently used in industrial animals to treat and prevent diseases or as growth promotants [1].

Incorrect use of these antibiotics may leave residues in edible tissues causing toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals, or indirectly, problems through the induction of resistant strains of bacteria [2]. Therefore, the Sourth Korea has set maximum residue limits (MRLs) for macrolide antibiotics in edible tissues of food-producing animals. The MRLs of erythromycin and tylosin are 0.1 g/kg in bovine and porcine. In case of poultry, those are 0.125 g/kg for erythromycin and 0.1 g/kg for tylosin. In order to monitor macrolide residues, simple, confirmatory and simultaneous analytical methods are required.

Microbiological assays were widely used for determination of macrolide antibiotics [3, 4]. Unfortunately, these methods could not be used for simultaneous analysis due to lacks of their specificities. Gas chromatography-mass spectrometry (GC-MS) supplies good sensitivity and selectivity [5], but direct analysis for macrolides antibiotics is difficult because of their thermal labile property and low volatility.

Liquid chromatographic methods have been reported for the determination of macrolide antibiotics: UV absorption [6-11], fluorimeteric [12-14], chemiluminescence [15] and electrochemical detection [16, 17] methods have been used for determination, but these methods have shown high limits of detection.

Recently, several simultaneous determination methods of macrolide antibiotics have been developed by mass spectrometry coupled with HPLC [18-20]. The determination methods of macrolides by LC/MS have advantages such as high specificity and selectivity due to each molecular mass.

The aim of this study is to develop a more simple, rapid and effective method for the simultaneous determination of three macrolide antibiotics (erythromycin, roxithromycin and tylosin) and a pleuromultlin antibiotic (tiamulin) by LC/MS with electrospray interface. Although tiamulin does not belong to a group of macrolide antibiotics, we determined this drug due to its similar structure to tylosin.

2. Materials and methods

2-1 Chemicals and reagents

Erythromycin, roxithromycin and tylosin were supplied by Sinil Chemicals (Seoul, Korea). Tiamulin was supplied by Daesung Microbials (Seoul, Korea). HPLC grade water and methanol were purchased from J.T. & Baker (New Jersey, USA). Reagent grade ammonium acetate was purchased from Sigma (Missouri, USA).

The individual stock standard solutions were prepared as 1 mg/㎖ in methanol and working standard solutions were prepared weekly by dilution of stock standard solutions with

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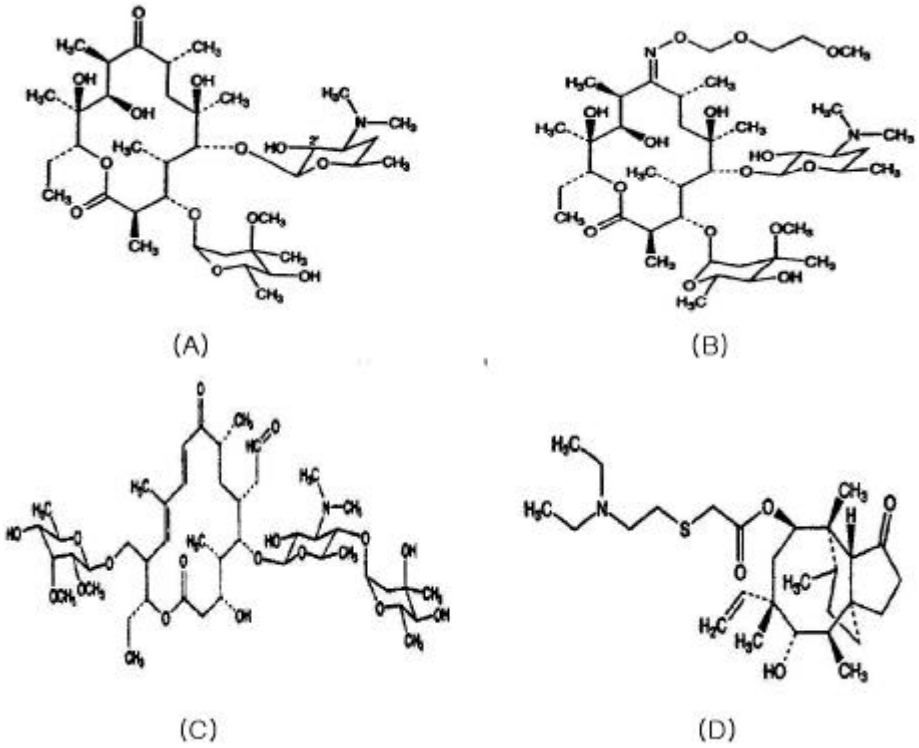


Fig. 1. The structure of erythromycin (A), roxithromycin (B), tylosin (C) and tiamulin (D).

methanol. All standard solutions were stored at 4℃ and were stable for at least 1 month under this condition. Deionized or distilled water of 18.2 MΩ cm-1 resistivity was used throughout the experiment.

2-2 Instrumentation and chromatographic conditions

Samples were analyzed by a Hewlett-Packard 1100 series LC/MSD system. It consisted of a G1322A degasser, a G1312A binary pump, a G1315A photo-diode-array detector, a 59987A electrospray interface and a 5989B mass spectrometer. The separation was performed on Nova-Pak C18 reverse phase column (4 μm, 3.9 mm x 150 mm I.D., Waters, USA). Analytical system was operated with a gradient elution at flow rate of 0.5 mℓ/min. The mobile phase consisted of 10 mM ammonium acetate (A) and methanol (B). Gradient runs were programmed as follows: 100% B for 3 min, decrease from 100% to 90% B for 6 min, decrease from 90% to 5% B for 6 min, 5% B for 5 min, re-equilibration with 100% B for 5 min, post-run with 100% B for 10 min, until the next sample injection.

The nebulizer gas was flowed at 45 p.s.i., 350℃ and 9.0 l/min and quadrupole was heated to 100℃. Mass spectrometer was run in the positive mode and scan mode from m/z 100 to 1000. Fragmentation voltage was 100 V. Analysis was carried out at the room temperature.

2-3 Calibration curves and the limit of detection

Calibration curves have been constructed by plotting area against the standard concentrations of macrolides in the range of 0.001 μg/mℓ ~ 5 μg/mℓ.

Limit of detection (LOD) and limit of quantitation (LOQ) were based on the signal-to-noise ratio based on their areas. The signal-to-noise ratio of 3 was accepted for the LOD and that of 10 for the LOQ.

3. Results

3-1. Chromatographic separation

All drugs used for the experiment were separated under the adopted conditions within 18 min (Fig. 2). Each separation of erythromycin (15 min), roxithromycin (16 min), tylosin (12 min) and tiamulin (14 min) was achieved successfully, on the same chromatogram.

3-2. Mass spectra

For each molecule, the produced ions on mass spectra were the molecular related ion $[M+H]^+$, two adduct ions $[M+Na]^+$ and $[M+K]^+$, and several fragmentation ions (Fig. 3). The molecular ions, $[M+H]^+$, at m/z 734.5, 837.5, 494.4 and 916.5 for erythromycin, roxithromycin, tiamulin and tylosin were represented dominantly. Except tiamulin, other drugs produced two adduct ions, $[M+Na]^+$ and $[M+K]^+$, at m/z 756.5 and 772.5 for erythromycin, at m/z 859.5 and 875.5 for roxithromycin, at m/z 938.5 and 954.5 for tyloisin (Fig. 3). The appearance of fragmentation ions was due to the dissociation of amino or sugar moieties on the structure of drugs. The m/z 576.5 and 679.5 in mass spectra of erythromycin and roxithromycin were corresponding with

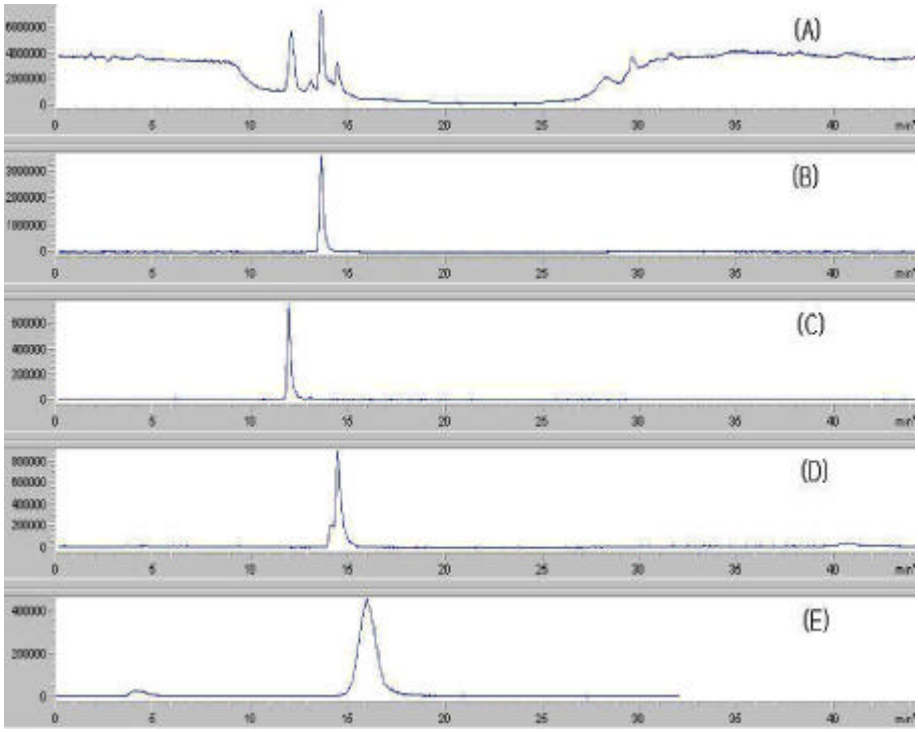


Fig. 2. Total ion chromatography (TIC, A) of macrolide and tiamulin. Extract ions chromatography (EIC) of tiamulin (B), tylosin (C), erythromycin (D) and roxithromycin (E).

a [M-desosamine+H]+. The *m/z* 158.1 of erythromycin and roxithromycin was corresponding with a [desosamine + H]+, and the *m/z* 115.1 of roxithromycin was corresponding with a [cladinose-OCH3 + H]+. The fragment ions of tiamulin, *m/z* 192.1 was a moiety of [2-(diethylamino)-ethyl, thio] acetic acid dissociating from molecular ion. The fragment ions of tylosin, *m/z* 742.5 and 772.5 were corresponding with [M-mycinose+H]+ and [M-mycarose+H]+, respectively. These results were summarized in Table 1.

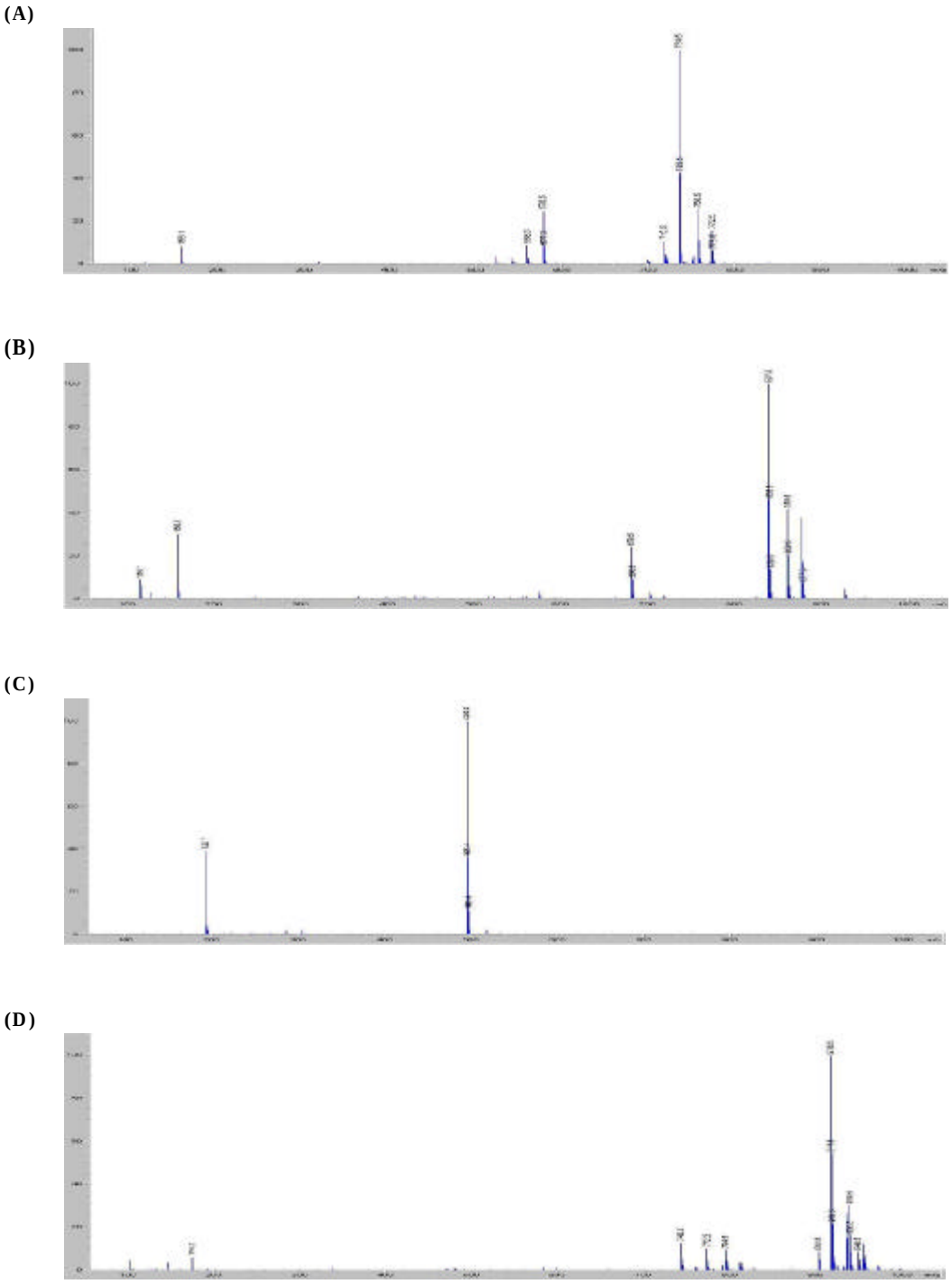
3-3. Linearity and the limit of detection

All experimented drugs in the range of 0.001 μg/g ~ 5μg/g showed good linearity, with correlation coefficient of 0.99(Table 2). The limit of detection and limit of quantitation ranged from 0.001 to 0.01 μg/g and from 0.005 to 0.05 μg/g (Table 3), respectively. These figures were much lower than the MRLs set up by the South Korea.

4. Discussion

LC/MS was highly sensitive and selective for the simultaneous determination of macrolides comparing with other published methods. Several methods were reported for simultaneous determination of macrolides. Simultaneous determination methods by HPLC with UV detector [6-11] have been developed, but these methods are difficult to detect macrolides such as erythromycin and roxithormycin due to their weak UV absorbance. The fluorimetric detection with pre-column derivatization procedures requires long separation times and is less sensitive than LC/MS [12-14]. In addition, fluorimetric detection is limited for simultaneous determintion because of the different derivatization method of each drug. Kees *et al.* [16] and Dreassi *et al.* [17] have reported for the determination methods of erythromycin and roxithromycin using HPLC with electrochemical detector, which is more sensitive than UV detector. But, these methods are difficult to set up analytic condition because the determination methods by electrochemical detection are very sensitive to environmental condition.

The determination method by gas chromatography-mass spectrometry (GC-MS) has been reported [5]. This method needs the derivatization procedures for each macrolide, thus taking a long time for the determination of macrolides. LC/MS which omits the derivatization procedures was successfully applied to determine several macrolides. Simultaneous determination methods by HPLC with UV detector [6-11] have been developed, but these methods are difficult to detect macrolides such as erythromycin and roxithormycin due to their weak UV absorbance. The fluorimetric detection with pre-column derivatization procedures requires long separation times and is less sensitive than LC/MS [12-14]. In addition, fluorimetric detection is limited for simultaneous determintion because of the different derivatization method of each drug. Kees *et al.* [16] and Dreassi *et al.* [17] have reported for the determination methods of erythromycin and roxithromycin



ig. 3. The mass spectra of erythromycin (A), roxithromycin (B), tylosin (C) and tiamulin (D).

Table 1. Summarized mass spectra of the drugs used experiments

Drugs	Molecular mass	Molecular ion [M + Na]+	Adduct ions [M + Na]+ ; [M + K]+	Fragmentation ions
Erythromycin	733.5	734.5	756.5; 772.5	158.1; 558.3; 576.5
Roxithromycin	836.5	837.5	859.5; 875.5	115.1; 158.1; 679.5
Tiamulin	493.4	494.4	—	192.1
Tylosin	915.5	916.5	938.5; 945.5	742.5; 772.5; 794.5

Table 2. The linearity of the drugs

Drugs	Equationa		RSDb	Linearity(r)
	Slope(106)	Intercept(104)		
Erythromycin	7.02	-1.93	1.45	0.99
Roxithromycin	4.90	-73.49	8.43	0.99
Tiamulin	43.3	-65.12	4.2	0.99
Tylosin	3.9	-32.2	4.2	0.99

a is used for the back-calculation of the drug concentration in the sample, y=mx + b (m, the slope; b, the intercept; x, the amount; y, the area)

b is the residual standard deviation of calibration curve in the regression analysis and is represented as 105

Table 3. LOD, LOQ and reproducibility of four drugs

Drugs	LOD(μg/mℓ)	LOQ(μg/mℓ)	Reproducibility(r)
Erythromycin	0.005	0.02	0.99
Roxithromycin	0.01	0.05	0.99
Tiamulin	0.001	0.005	0.99
Tylosin	0.001	0.01	0.99

using HPLC with electrochemical detector, which is more sensitive than UV detector. But, these methods are difficult to set up analytic condition because the determination methods by electrochemical detection are very sensitive to environmental condition.

As described in above as to the simultaneous determination of macrolides, there are several problems such as weak UV absorption, long separation time and difficult derivatization procedure. Our method has solved previous problems by application of liquid chromatorygraphy/mass spectrometry (LC/MS). LC/MS minimizes chromatographic separation and method development time in confirming the molecular identities of the target substance.

The partially overlapped peak in erythromycin observed in Fig. 3 needs some discussion in this study. Macrolides were generally composed of more than one structural component. In determination of macrolides, major components were generally used as indicators to evaluate the residue levels [7]. However, minor components could be also remained in edible tissues. This peak in erythromycin indicates a major component combined with a minor component. Its mass spectrum pattern was different from that of major component. Even changing the mobile phase, the minor component was not separated and was moved together with the major component. The chromatographic property of partially overlapped peak in erythromycin is similar to that of the major component. As this overlapped peak resulted from the addition of the minor component, LC/MS based on their molecular weight could identify the minor component.

5. Conclusion

LC/MS with electrospray is a simple, rapid and effective technique for the simultaneous determination of macrolides. The fragmentation patterns provide the confirmatory information of macrolides. The relevance of these studies for the determination of macrolide in biomatrices remains further investigated.

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