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Determination of macrolide antibiotics by liquid chromatography

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Abstract

The liquid chromatographic separation of seven macrolides used in food producing animals in the European Union has been studied. Separation was performed by using an end-capped high-purity silica-based C_{18} column and mobile phases consisting of phosphate buffer (pH 2.5)–acetonitrile mixtures. The effect of pH and acetonitrile percentage on the separation was examined. Two UV-based detection systems, wavelength programming and diode array, were assayed. Detection limits were in the range 6–33 μg l^{-1} for spiramycin, tilmicosin, tylosin, kitasamicin and josamicin and about 400 μg l^{-1} for erythromycin and oleandomycin. The suitability of the method for multiresidue determination of the five macrolides is demonstrated by the analysis of spiked samples of chicken muscle.

Keywords: Antibiotics; Macrolide antibiotics

1. Introduction

Macrolide antibiotics, which are active agents against Gram-positive bacteria, are frequently used as veterinary drugs in food-producing animals. They target the bacterial ribosome and inhibit bacterial protein biosynthesis. Residues of these antibiotics in foods could lead to allergic reactions in sensitive or sensitised individuals and indirect effects such as the development of resistant strains of bacteria. Consequently, the European Union (EU) has developed regulations and set maximum residue limits (MRLs) for most of the veterinary drugs used in food-producing animals. The status of MRLs for macrolides is summarised in Table 1. In order to carry out monitoring programs, screening and confirmatory analytical methods are needed.

The determination of antibiotics is commonly carried out by microbiological assays, but they are often lengthy and lack the specificity and precision required for regulatory purposes. Chemical methods such as liquid chromatography (LC) are appropriate alternatives. Reversed-phase LC with silica-based columns is the most usual approach for macrolide separation. Current mobile phases consist of a mixture of acetonitrile (ACN) and an aqueous phosphate or acetate buffer [1]. Methanol or acetonitrile–methanol mixtures have occasionally been used [2,3]. Separation is usually carried out in acidic medium, except for erythromycin, for which neutral media is preferred due to its instability in acidic medium.

Although LC methods have been extensively used for individual compounds, multiresidue methods, which offer great advantages for monitoring pur-

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Table 1
Status of macrolides in EU regulations

Substance	Main component	MRLs[a] ($\mu g\ kg^{-1}$)	Regulation[b]
Erythromycin	Erythromycin A	40–400	1442/95
Josamycin	Leucomycin A_3	200–400	282/96; 953/99
Kitasamycin	Leucomycin A_5		Under evaluation
Oleandomycin		300[c]	
Spiramycin	Spiramycin I	200–600	675/92; 1442/95
Tilmicosin	*cis*-Tilmicosin	75–1000	955/94; 1102/95; 1917/98
Tylosin	Tylosin A	50–100	3093/92; 1442/95; 1838/97

[a] These values depend on the tissue and the animal. For detailed data see http://www.eudra.org/vetdocs/vets/MRL.htm
[b] These regulations amending annex of Regulation 2377/90.
[c] This value corresponds to UN Food and Agriculture Organization (FAO) recommendations, it is not included in the EU list.

poses, have been scarcely reported [4,5]. UV is the most common detection system. However, macrolides like erythromycin and oleandomycin lack a suitable chromophore and non-selective low-UV wavelength must be used. Other proposed systems include electrochemical detection for erythromycin [6–8] and pre-column derivatization combined with fluorimetric detection for erythromycin [9,10] and josamycin [11,12], but they have not been applied to multiresidue analysis. More recently, mass spectrometry has been proposed [13].

The aim of this study is to develop a new multiresidue method for the separation by reversed-phase LC of seven macrolides authorised for food-producing animals in the EU: erythromycin (ERY), spiramycin (SPI), oleandomycin (OLE), tylosin (TYL), tilmicosin (TILM), kitasamycin (KIT) and josamycin (JOS). UV absorption was chosen as detection system since it is the most universal and is available in most laboratories. Two modes have been applied: UV detection with wavelength programming and diode array detection (DAD).

2. Experimental

2.1. Chemicals

Spiramycin, tylosin tartrate, oleandomycin phosphate and erythromycin were supplied by Sigma (St. Louis, MO, USA). Tilmicosin, kitasamycin and josamycin were kindly supplied by Elanco Valquímica (Madrid, Spain), Laboratorios Dr. Esteve (Barcelona, Spain) and Laboratorios Virbac (Esplugues de Llobregat, Spain), respectively. The structures of these compounds are shown in Fig. 1.

Stock solutions (1 g l^{-1} of SPI, TILM, TYL, KIT and JOS, and 10 g l^{-1} of ERY and OLE) were prepared by dissolving the compounds in methanol (HPLC; Baker, Deventer, The Netherlands). These solutions were stored in dark glass bottles at 4°C; under these conditions they were stable for at least 4 months. A mixture solution (100 mg l^{-1} of SPI, TILM, TYL, KIT and JOS and 1 g l^{-1} of ERY and OLE) was prepared weekly by dilution with water and also stored at 4°C. Working standard solutions were freshly prepared by dilution with a mixture 0.05 *M* NaH_2PO_4 (pH 4.4)–ACN (70:30, v/v).

Bond Elut SCX (500 mg) cartridges were purchased from Varian (Harbor City, CA, USA).

Hyflo Super-Cel was kindly supplied by World Minerals Division Española (Rubí, Barcelona, Spain).

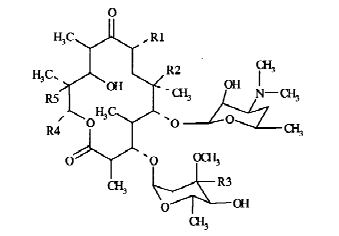
Double-deionized water (Milli-Q; Millipore, Molsheim, France) of 18.2 $M\Omega\ cm^{-1}$ resistivity was used throughout.

2.2. Samples

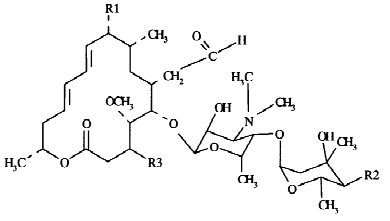
Chicken samples used for the preparation of spiked muscle were purchased from local groceries. The samples chosen contained no macrolides. Skin and bones were removed prior grinding the muscle. Mince muscle was kept at −20°C and before analysis the sample was thawed.

2.3. Apparatus

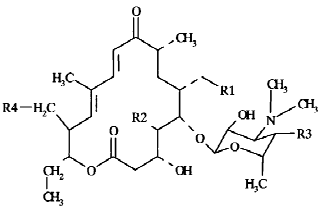
A HP-1050 liquid chromatograph with a UV



Compound	R1	R2	R3	R4	R5
Erythromycin (ERY)	-CH_3	-OH	-CH_3	-CH_2-CH_3	-OH
Oleandomycin (OLE)	O	-H	-H	-CH_3	-H



Compound	R1	R2	R3
Spiramycin (SPI)		-OH	-OH
Kitasamycin (KIT)	-OH	-O-CO-CH_2-CH_2-CH_3	-OH
Josamycin (JOS)	-OH	-O-CO-CH_2-CH(-CH_3)-CH_3	-O-CO-CH_3



Compound	R1	R2	R3	R4
Tylosin (TYL)	-COH	-CH_3		
Tilmicosin (TILM)		-OCH_3	-OH	

Fig. 1. Structures of the macrolides studied.

detection system (Hewlett-Packard, NY, USA) was used. Response was monitored at the following wavelengths: 232 nm from start to min 8.3, 287 nm from min 8.3 to 11, 204 nm from min 11 to 12.7, 287 nm from min 12.7 to 14.3, and 232 nm from min 14.3 to the end.

A Waters-2690 liquid chromatograph (Waters, Milford, MA, USA) with a Waters 996 diode array detector was used.

A Crison (Alella, Barcelona, Spain) GLP 21 pH meter equipped with a Crison 52-02 Ag/AgCl combined glass electrode was used for pH measurements.

A peristaltic pump (Gilson, Villiers le Bel, France) and a LaboRota 300 rotavapor (Resona, Germany) were used.

2.4. Procedures

2.4.1. LC–UV

The separation was performed on a Hypurity Elite C_{18} (Hypersil, Cheshire, UK) analytical column (5 μm particle size, 25 cm×4.6 mm I.D.) equipped with a HyPurity Elite C_{18} (10 mm×4 mm) guard column, using binary gradient elution. Mobile phase A was a 0.025 *M* aqueous phosphate buffer, pH 2.5 and mobile phase B consisted of a mixture of this buffer solution–ACN (60:40, v/v). Aqueous solutions were filtered through a 0.22-μm nylon membrane filter (Lida, Kenosha, WI, USA). A gradient elution of 50 to 100% of mobile phase B in 15 min, followed by a hold time of 5 min and a post-time of 10 min was applied. The mobile phase flow-rate was set at 1.5 ml min^{-1} and the chromatograph was operated at room temperature. Samples and standard solutions were also filtered through a 0.22-μm nylon membrane and injected using a 100-μl injection loop.

2.4.2. Sample treatment

A 2.5-g amount of tissue was manually shaken for 5 min with 17 ml of 0.3% metaphosphoric acid–methanol (7:3, v/v) in a glass tube. The extract was filtered through a 2-mm layer of Hyflo Super-Cel coated on a suction funnel. The filtrate was rotary evaporated to about 12 ml and loaded on a Bond-Elut SCX 500-mg cartridge, previously conditioned with 5 ml of methanol and 10 ml of 0.1 *M* KH_2PO_4 (pH 4.4). The cartridge was then washed in 10 ml of water and 3 ml of 0.1 *M* K_2HPO_4 (pH 8.8). Elution was carried out with 10 ml of methanol. The extract was rotary evaporated to dryness at 45°C, reconstituted with 1 ml of 0.05 *M* NaH_2PO_4 (pH 4.4)–ACN (70:30, v/v), filtered and injected into the chromatographic system.

3. Results and discussion

The fermentation that produces macrolides is not entirely selective, and in addition to the major components, small quantities of other components

are present. Due to the low level of residues present in animal tissues, it is difficult to monitor all components. A common practice is to use the main component as an indicator to evaluate the residual level [4] and it is adopted here. In Table 1 the main component of each macrolide studied is indicated.

3.1. Optimisation of the chromatographic separation

Preliminary studies showed that end-capped columns offer advantages over non-end-capped ones with regard to peak symmetry. Therefore, several end-capped C_{18} and C_8 (Kromasil 100, Hypurity elite, Hypersil Green Env., and Inertsil) columns were tested using acetonitrile–water mobile phases. On the whole, the Hypurity column provided the best results, since it showed the highest efficiency and acceptable asymmetry factors, and thus, it was selected for further experiments.

The acetonitrile content and pH of the mobile phase strongly affect the chromatographic behaviour of the analytes. Although no significant effect on the retention times was observed in the pH range 2–4, peak shape improved as pH decreased and pH 2.5 was chosen in order to preserve the column life. At this pH no on-column degradation of compounds was observed, even for ERY which is easily degraded in acidic solutions [14].

An acetonitrile content above 35% (v/v) was required to elute JOS in a reasonable analysis time, whereas the percentage must be lower than 25% (v/v) so that the SPI peak (30% ACN, $k'=0.56$) is well separated from the system peak. Thus, a gradient elution was applied and a linear gradient of 20 to 40% ACN in 15 min, followed by a hold of 5 min and a post-time of 10 min was selected. Under these conditions all k' values ranged between 1.5 and 5. In all mobile phases studied there was some overlap between TILM and OLE peaks. Nevertheless, they show absorption maxima at different wavelengths ($<$200 nm for OLE and 287 nm for TILM), which allows their differentiation when LC–multiwavelength UV detection is used (see next section).

Temperature (25–40°C) had no effect on retention times, and although an increase in temperature led to slightly narrower peaks for TYL and KIT, separation was carried out at room temperature for convenience. Flow-rate (1.0–1.5 ml min^{-1}) had no significant effect on peak areas, and thus further chromatograms were obtained at 1.5 ml min^{-1} so the retention time of the last eluted compound was reduced from 24.5 to 18 min.

3.2. Detection system

In order to achieve the maximum response for each macrolide, absorption spectra in water–acetonitrile mixtures at pH 2.5 were recorded. Maximum absorption wavelengths are 285 nm for TYL and TILM and 232 nm for KIT, SPI and JOS. No maxima were observed for ERY or OLE above 200 nm and therefore detection of these analytes must be performed at non-selective short wavelengths, i.e., 204 nm. Two alternatives based on UV absorption were assayed: wavelength-programming and multiwavelength detection using DAD. The use of wavelength-programming detection involves four wavelength changes (see Experimental section) and the poor resolution between OLE and TILM prevents the detection of both compounds in a single chromatographic run. Multiwavelength detection, at 210, 232 and 287 nm, allows spectral resolution of OLE and TILM, in spite of the overlap of their chromatographic peaks. Moreover, DAD has an additional advantage, as the spectra of the eluting peaks can be used to confirm the identity of the analytes. Fig. 2 shows a typical chromatogram of a mixture of the seven macrolides obtained using DAD.

3.3. Analytical performance characteristics

Quality parameters for LC–DAD are summarised in Table 2. Calibration graphs were obtained from both peak height and peak area, and since no significant differences were found the former was selected for quantification. The limits of detection (LODs) and quantification (LOQs) were determined at signal-to-noise ratios of 3 and 10, respectively.

Detection limits for standard solutions were in the range 10–50 $\mu g\ l^{-1}$ for SPI, TILM, TYL, KIT and JOS and about 400 and 900 $\mu g\ l^{-1}$ for JOS and ERY, respectively. Although lower limits were obtained (about half of DAD values) with programming wavelength detection, the use of DAD is preferred due to the spectral information provided. The de-

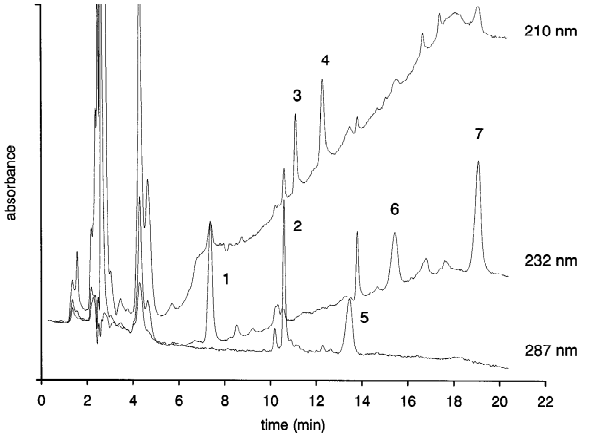


Fig. 2. Chromatogram of a standard mixture of macrolides using LC–DAD. Peaks: 1=SPI (170 μg l^{-1}), 2=TILM (166 μg l^{-1}), 3=OLE (1451 μg l^{-1}), 4=ERY (3078 μg l^{-1}), 5=TYL (138 μg l^{-1}), 6=KIT (167 μg l^{-1}), 7=JOS (168 μg l^{-1}). For conditions see Experimental section.

tection limits attained with DAD are suitable for residue analysis of five of the studied macrolides below their MRLs. For ERY and OLE, although the method is also suitable for some tissues, a more sensitive detection method, such as mass spectrometry, is required to cover the whole residue analysis.

3.4. Analysis of chicken tissues

The LC–DAD method was applied to spiked macrolide-free samples of chicken muscle. Spiking was performed by adding a microvolume of an aqueous standard solution containing five macrolides to each portion of the weighed samples. Spiked samples were left to stand at room temperature for 15 min in the dark before analysis. Extraction was carried out using a modification of the procedure described by Horie et al. [4] (see Section 2.4). Fig. 3

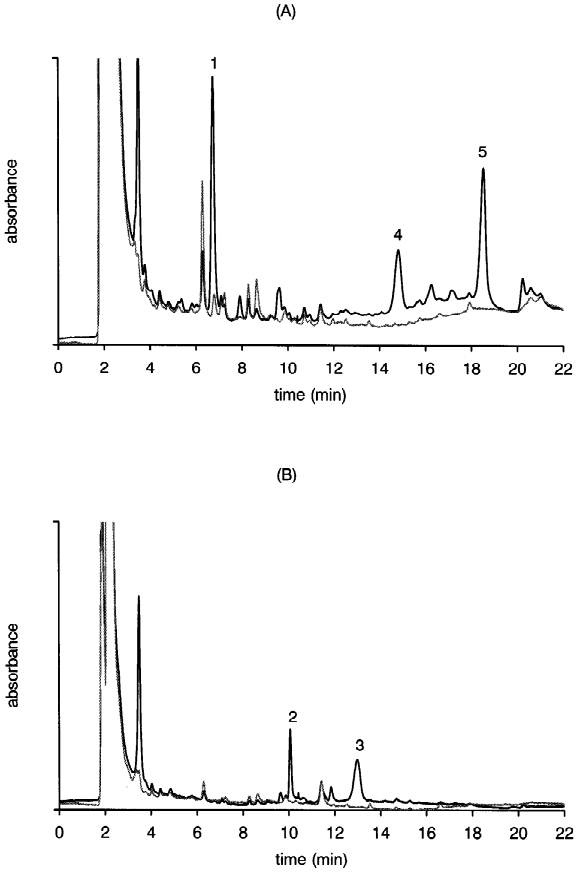


Fig. 3. Chromatograms of a spiked chicken muscle (__) and the blank muscle (. . .) (A) at 232 nm, (B) at 287 nm. Peaks: 1=SPI (450 μg kg^{-1}), 2=TILM (155 μg kg^{-1}), 5=TYL (220 μg kg^{-1}), 6=KIT (390 μg kg^{-1}), 7=JOS (400 μg kg^{-1}). Concentrations correspond to values in the muscle.

Table 2
Figures of merit of the LC–DAD method (all concentrations expressed as μg l^{-1})

Parameter	SPI	TILM	ERY	TYL	OLE	KIT	JOS
Linearity range	50–7700[a]	30–7700[a]	2950–188 000[a]	80–7600[a]	1130–163 000[a]	110–4000[a]	50–7800[a]
RSD (%) (h)[b]	2.1	2.0	0.4	1.7	1.8	1.6	0.9
RSD (%) (rt)[b]	0.3	0.2	0.2	0.1	0.1	0.4	0.1
LOD[c]	20	10	930	30	450	50	20
LOQ[c]	50	30	2950	80	1130	110	50

[a] Maximum concentration assayed.
[b] Five replicates, 500 μg l^{-1} (SPI, TILM, TYL, KIT, JOS) and 7 mg l^{-1} (OLE and ERY).
[c] Calculated as 3 SD (LOD) and 10 SD (LOQ) of the baseline noise.

Table 3
Recovery data for chicken muscle

	MRL ($\mu g\ kg^{-1}$)	Spiking level ($\mu g\ kg^{-1}$)	Recovery (%)±RSD ($n=6$)
SPI	200	100–400	77±8
TILM	75	40–150	65±5
TYL	100	50–200	60±11
KIT	–	100–400	80±10
JOS	200	100–400	70±9

shows the chromatograms of an extract obtained from chicken muscle in the absence of the analytes, and those of an extract of the same tissue spiked with SPI, TYL, TILM, KIT and JOS at twice the MRL values. As can be seen, no interfering peaks are present at the retention times of each analyte, except for SPI. A small peak at SPI retention times was detected in some non-spiked samples of chicken, but the UV spectrum did not match with that of SPI and therefore it was not considered as a false positive.

Recoveries were evaluated for chicken muscles at three spiking levels, around the MRLs. Quantitation was done by using external standards. The recoveries ranged from 60 to 80% (Table 3). The results indicate that the proposed method is suitable for monitoring residues of macrolides in edible animal tissues below their MRLs.

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