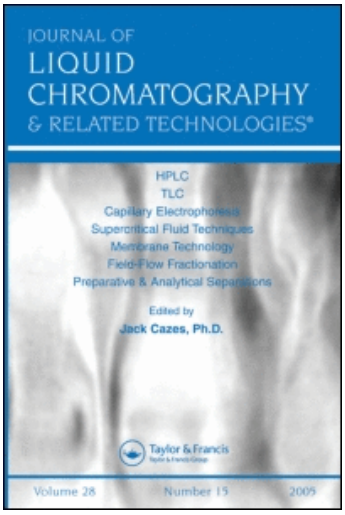

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Determination of Dirithromycin, LY281389 and Other Macrolide Antibiotics by HPLC with Electrochemical Detection

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DETERMINATION OF DIRITHROMYCIN,LY281389 AND OTHER MACROLIDE ANTIBIOTICS BY HPLC WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

Sensitive and rapid assays have been developed for the determination of the macrolide antibotics dirithromycin, erythromycylamine, and LY281389 in plasma. The methods utilize dichloromethane extraction of alkalinized plasma and isocratic reverse phase HPLC with electrochemical detection. The lower limit of detection is 10 ng/ml. Calibration curves are linear and highly reproducible over the range of 20-500 ng/ml. Precision of the calibration curves is very good having relative standard deviations of 5% or less over the dynamic range. These methods can be used for other macrolide antibiotics with minor modifications to the mobile phase. The electrochemical response of various macrolides was found to be dependent upon the functionality at C-9 of the macrolide ring.

INTRODUCTION

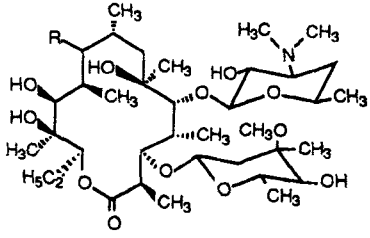
High performance liquid chromatography coupled with electrochemical detection has been used for the assay of a number of 14-member ring macrolide antibiotics (1,2,3) owing to the method's selectivity and sensitivity. Other HPLC methods have been reported which utilize ultraviolet detection (4) requiring complicated extraction procedures, and fluorometric detection (5) requiring post column derivatization and extraction. Additionally, both methods

lack the selectivity and sensitivity of the electrochemical detection method. Dirithromycin and LY281389 are new gram positive, 14-member ring macrolide antibiotics which are undergoing preclinical evaluation. We report herein the development of rapid, selective and sensitive assays for dirithromycin, it's metabolite erythromycylamine and LY281389 in plasma utilizing a dichloromethane extraction of alkaline plasma and HPLC with electrochemical detection. The assays have been utilized for the analysis of macrolides in human, dog and rat plasma samples.

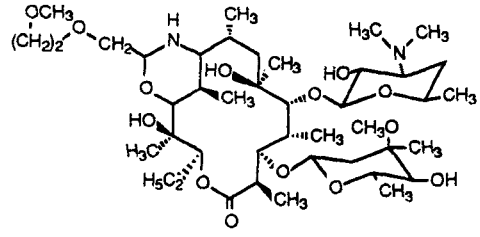
MATERIALS AND METHODS

Reagents

All reagents were analytical grade or higher quality. Acetonitrile, methanol and dichloromethane (ChromAr HPLC grade) were obtained from Mallinckrodt. Water was purified with a Milli-Q water purification system (Millipore). Ammonium acetate (A.C.S.), Certified Buffer Solution, 0.5M, pH 10 (Fisher SO-B-116) and sodium perchlorate (A.C.S.) were purchased from Fisher Scientific Co. Sodium heparin (USP Lilly) was derived from porcine intestinal mucosa. Erythromycylamine (EMA), LY023907, LY281389 and LY090189 were synthesized at the Lilly Research Laboratories. Dirithromycin was obtained from Dr. Karl Thomae, GmbH (Biberach, FRG). The structures of the macrolides are shown below.



R GROUP	COMPOUND
	LY090189
	LY281389
NH2	EMA
OH	LY023907



DIRITHROMYCIN

Plasma Extraction

Plasma samples (0.5 ml) containing 1.0 ml of pH 10 buffer were placed in 15 ml glass centrifuge tubes, 250 ng of internal standard (LY023907 for System I or LY090189 for System II) was added in 10 µl of methanol and the solution was mixed for 10 seconds. Dichloromethane (5.0 ml) was added to the tubes and the samples were mixed for 10-15 seconds followed by centrifugation for 10 minutes at 2000 rpm. The dichloromethane layer was removed and an additional 5.0 ml of dichloromethane was added to the sample and the extraction was repeated. The two dichloromethane extracts were combined and reduced to dryness under nitrogen. Samples were dissolved in 100 µl of acetonitrile and placed in limited volume WISP auto injector vials and capped.

HPLC Chromatography

Chromatography System I was used for the assay of plasma samples containing dirithromycin and EMA and was accomplished with an Analytichem Sepralyte 2DP (5 µm, 25 cm X 4.6 mm, i.d.) chromatography column. The mobile phase was 60% acetonitrile/10% methanol/30% water with a final ammonium acetate buffer concentration of 50 mM at an apparent pH of 7.5. Chromatography System II was used for the assay of plasma samples containing LY281389 and was accomplished with a Supelco 2DP (5 µm, 25 cm X 4.6 mm, i.d.) chromatography column. The mobile phase was 25% acetonitrile/35% methanol/40% water with a final ammonium acetate buffer concentration of 150 mM at an apparent pH of 7.0. Columns were maintained at 40 °C using a BAS block heater controlled by a LC-22A controller (Bioanalytical Systems, Inc.). Solvent flow was maintained at 1 ml/min with a Waters Model 6000A pump and the mobile phase was recirculated. Samples (40-60 µl) were applied to the system by a Waters model 710B WISP autosampler. Electrochemical detection was accomplished utilizing an ESA Coulochem Model 5100A electrochemical detector in screen mode, equipped with a model 5020 guard cell maintained at a potential of 1 volt placed in the solvent flow between the pump and the autosampler. The HPLC column eluate was monitored with a model 5011 analytical cell with the first electrode screening at a potential of 0.7 volts and the second electrode at a potential of 0.9 volts. Detector output was captured by a Hewlett Packard HP1000 series computer for peak analysis.

Calibration curves were generated over a concentration range of 20-500 ng/ml by admixing control plasma with known concentrations of dirithromycin, EMA, LY281389 and internal standard. Drug concentrations of unknowns were calculated by comparing the peak height ratios of macrolide standard/internal standard to known standard drug concentrations using least squares linear regression. For plasma samples containing drug concentrations greater than 500 ng/ml, samples were diluted with control plasma, processed and analyzed again.

Dosing and Plasma Collection

Dirithromycin in enteric coated gelatin capsules was administered orally to female hounds (18-21 kg) at 30 mg/kg. LY281389 in gelatin capsules was administered orally to female hounds (18-21 kg) at 10 mg/kg. Blood samples were collected in sodium heparinized vacutainer tubes (Becton/Dickinson) and plasma was obtained by centrifugation at 2000 rpm for 10 minutes in an I.E.C. Model CL centrifuge. Aliquots of plasma (0.5 ml) were removed and admixed with 1.0 ml of pH 10 buffer (Fisher SO-B-116) and stored at -70 °C until analyzed.

RESULTS AND DISCUSSION

The efficiency of extraction of dirithromycin and EMA from plasma ranged from 91 to 111% recovery (Table 1). The efficiency of LY281389 extraction from plasma was somewhat less, however, ranging from 78 to 83% recovery.

TABLE 1
Plasma Extraction and Recovery

Macrolide	Plasma Extraction Efficiency (%)		
(ng/ml)	Dirithromycin	EMA	LY281389
50	102 (4)	103 (8)	83 (3)
200	106 (11)	91 (7)	78 (3)
350	111 (6)	106 (7)	78 (3)

50-350 ng/ml (relative standard deviation for n=3 at each concentration) were prepared in 1.0 ml of dog plasma and 0.5 ml was subjected to the extraction procedure described in Methods.

Nevertheless, the extraction efficiency of the compounds was consistent, with the relative standard deviations ranging from 3 to 11%. The results are comparable with those reported by other investigators (1,2).

An HPLC chromatogram of extracts of control dog plasma and control dog plasma admixed with 250 ng/ml of dirithromycin, EMA, decladinose-EMA and internal standard which were subjected to chromatography System I is shown in fig.1. Also shown is an HPLC chromatogram of extracts of control dog plasma and control dog plasma admixed with 250 ng/ml LY281389, decladinose-LY281389, EMA and internal standard which were subjected to chromatography System II. Peak resolution of dirithromycin, EMA, decladinose-EMA and internal standard LY023907 was very good in System I. However, these four compounds were not well resolved in System II as indicated by their K' values shown in Table 2. There was significantly lower retention of EMA and decladinose-EMA in System II than in System I. Chromatography of LY281389, decladinose-LY281389, EMA and internal standard LY090189 in System I offered very good peak resolution as indicated by their K' values (Table 2), however, the assay time was very long. System II offered good resolution of these four components with a much reduced assay time (Fig. 1 panel B).

TABLE 2
K' Values and Peak Areas of Various Macrolides

Macrolide	System I K'	System II K'	Peak Area nAmps/sec/μmole
Erythromycin	1.7	1.5	182
EMA	7.3	1.1	132
Decladinose-EMA	8.1	0.8	100
Dirithromycin	2.4	1.9	101
Roxithromycin	2.6	2.6	147
LY281389	12.6	2.1	146
Decladinose-LY281389	8.6	1.3	156
LY023907	1.5	1.4	197
LY090189	14.1	3.3	151

K' = retention time of macrolide minus retention time of void volume divided by *retention time* of void volume.

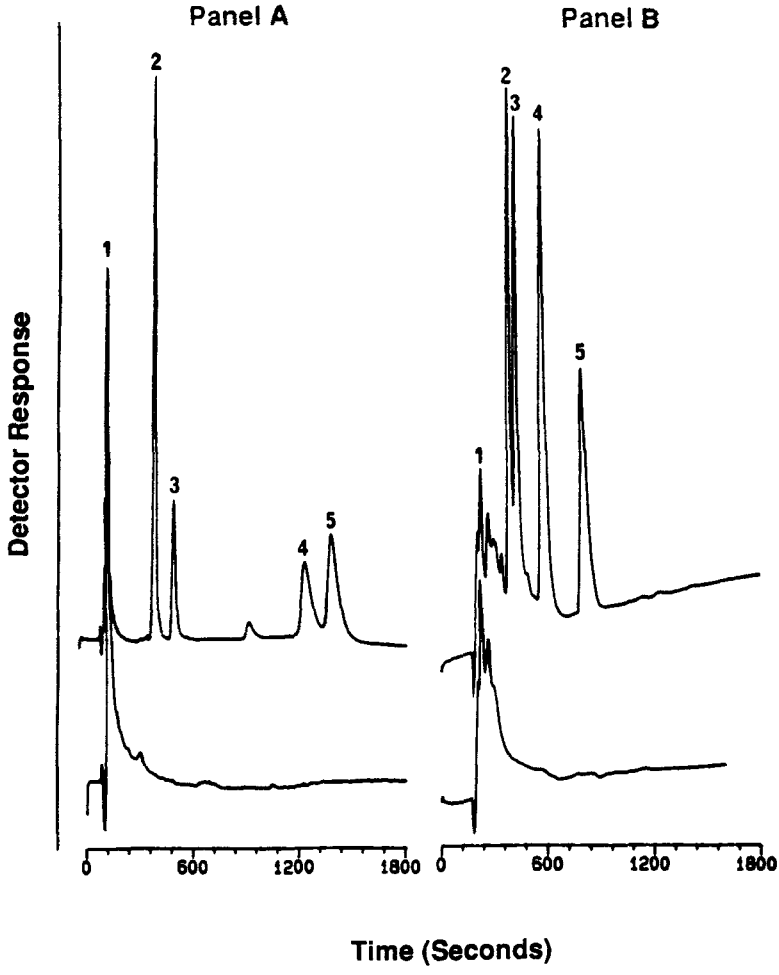


FIGURE 1. Panel A: Chromatogram of an extract of 1 ml of control dog plasma admixed with internal standard LY023907 (2), 250 ng dirithromycin (3), 250 ng EMA (4) and 250 ng decladinose-EMA (5). Lower trace shows a chromatogram of an extract from control dog plasma only and solvent front (1). Chromatography was accomplished in System I as described in Methods. Panel B: Chromatogram of an extract of 1 ml of control dog plasma admixed with 250 ng EMA (2), 250 ng decladinose-LY281389 (3), 250 ng LY281389 (4) and internal standard LY090189 (5). Lower trace shows a chromatogram of an extract from control dog plasma only and solvent front (1). Chromatography was accomplished in System II as described in Methods.

Additionally, the two chromatography systems were found suitable for separating and quantitating other macrolides such as erythromycin and roxithromycin. It is of interest to note the significant changes in K' values of some of the macrolides between the two systems. The K' values of EMA, decladinose-EMA, LY281389, decladinose-LY281389 and internal standard LY090189 changed the most from one system to the other. This indicates a relationship to the functional group at the C-9 position of the macrolide ring since each of these compounds contains an amine function at that position. A major contributing factor is the different ammonium acetate buffer concentrations in the two systems. For example, significantly longer retention times and poor peak symmetry were observed for C-9 amine containing macrolides (EMA, decladinose-EMA, LY281389, decladinose-LY281389 and LY090189) when lower concentrations of ammonium acetate were used in both systems. In addition, although the HPLC columns used in System I and II were both 5 μm diphenyl columns, chromatograhic differences were observed depending upon the column manufacturer. For example, when each column was evaluated using the System I mobile phase, significantly longer retention times and decreased peak symmetry and resolution were observed using the System II column (Supelco 2DP) for the C-9 amine containing macrolides compared with the System I column (Sepralyte 2DP). Other investigators have also noted the importance of column selection for resolution of macrolides (2,6).

Correlation was also found between the electrochemical detector response and the functional group in the C-9 position of the macrolide ring using an oxidation potential of 0.9 volts (Table 2). Erythromycin and LY023907, which both have an oxy-function at the C-9 position, produced greater electrochemical response than the other macrolides shown, many of which have nitrogen containing functional groups at the C-9 position of the macrolide ring. Additionally, Duthu (1) reported the importance of the desosamine sugar in determining the electrochemical response of the macrolide molecule.

Six point calibration curves of dirithromycin, EMA and LY281389 in control dog plasma were highly linear (coefficient of determinations of 0.999, 0.996 and 0.996, respectively) over the nominal range of 20-500 ng/ml (Table 3). Relative standard deviations ranged from 2 to 5% for the three compounds indicating very good calibration curve precision. Determinations were found to have good accuracy and precision when mutiple

TABLE 3
Linearity and Precision of Calibration Curves

Statistics	Dirithromycin	EMA	LY281389
Log-Log Slope	1.010	1.015	1.015
Max.(%) dev.[1]	9	8	13
RSD (%)[2]	2	5	2
CD[3]	0.999	0.996	0.995

Six point calibration curves from 20 to 500 ng/ml dog plasma (n=4) were prepared, processed and analyzed on the same day. Dirithromycin and EMA were analyzed using chromatography System I. LY281389 was analyzed using chromatography System II.

1 Maximum % deviation

2 (Standard deviation/mean)x100

3 Coefficient of determination

TABLE 4
Accuracy and Precision of Intraassay Determinations

Standard Added (ng/ml)	Determination (ng/ml)	RSD (%)
	Dirithromycin	
50	50	11
200	202	3
350	349	2
	EMA	
50	56	12
250	245	3
400	406	3
	LY281389	
50	51	6
200	198	1
350	351	1

Control dog plasma samples (1 ml) were admixed with macrolide standards at three concentations (n=4 at each concentation). Samples were prepared, processed, analyzed and calculated using six point standard curves that were prepared on the same day. Dirithromycin and EMA were analyzed using chromatography System I. LY281389 was analyzed using chromatography System II.

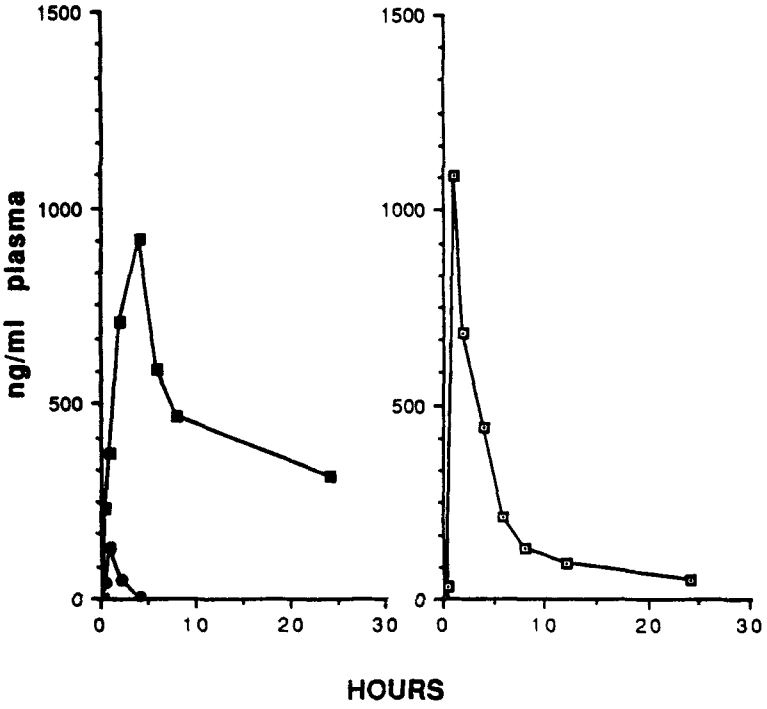


FIGURE 2. Dirithromycin (●) and EMA (■) plasma concentrations in dogs (n=8) dosed once orally with 30 mg/kg of dirithromycin are shown in the left panel. LY281389 (■) plasma concentrations in dogs (n=3) dosed once orally with 10mg/kg of LY281389 are shown in the right panel.

samples were assayed on the same day; relative standard deviations ranged between 1 and 12% (Table 4).

Dogs were given a single oral dose of dirithromycin (30 mg/kg) or LY281389 (10 mg/kg) and the plasma levels of dirithromycin, EMA or LY281389 are shown in figure 2. When dogs were dosed with dirithromycin, the maximum concentration of dirithromycin (130 ng/ml) and its metabolite EMA (920 ng/ml) were observed 1 and 4 hr after dosing, respectively. EMA was the major component observed in plasma when dirithromycin was administered and decreased with a plasma half-life of 4.1 hr. In dogs dosed with LY281389, the maximum plasma concentration of LY281389 (1100 ng/ml) was found 1 hr after dosing. Plasma levels of LY281389 decreased with a half-life of 3.9 hr.

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