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Macrolide and ketolide antibiotic separation by reversed phase high performance liquid chromatography

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Abstract

Twenty different macrolide and ketolide antibiotics were analyzed by reversed phase high performance liquid chromatography on an ODS-2 cartridge column. Each of these compounds was uniquely separated and purified by varying the flow rate. Retention times of the individual drugs were proportional to the flow rate of the mobile phase. Recovery of antimicrobial activity for most of the drugs was greater than 90% based on a microbiological assay of material recovered from the column. Retention times were related to structural differences between these antimicrobial agents. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Reversed phase HPLC; Macrolide antibiotics; Ketolide antibiotics

1. Introduction

The macrolide antibiotics are a class of antimicrobial compounds widely used against infectious diseases caused by a number of different microorganisms [1]. Erythromycin has been the most commonly used compound derived from natural sources and has been an important drug for over 30 years. It binds to the larger 50S ribosomal subunit and prevents the translocation step in protein synthesis [2]. Erythromycin and other macrolide antibiotics have recently been shown to have a second inhibitory activity, preventing of the formation of the 50S subunit from its component RNAs and proteins [3–7].

An increasing number of semi-synthetic derivatives of the macrolides have been made [8]. The more clinically important of these include azithromycin, clarithromycin and roxithromycin [9–12]. These and other novel drugs are now increasingly used as antimicrobial agents and many are more stable and persist longer in the body compared with erythromycin [13–15]. These compounds also inhibit both translation and 50S subunit formation in cells [3,5,7].

The ketolide compounds represent a new generation of semi-synthetic derivatives of the macrolides [16,17]. These compounds have the 3 cladinose sugar replaced with a keto group and most have additional aromatic substitutions on

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the 11 and 12 carbons [18,19]. Two of these have recently been shown to be particularly effective against a number of different types of bacterial species [20–23]. In addition, the ketolides do not induce resistance by stimulation of ribosomal RNA methylase gene expression, unlike the macrolides [24,25].

A comprehensive review describing the analysis of macrolides has been recently published by Kanfer et al. [26]. These antibiotics have been separated and characterized previously by TLC, HPLC and capillary electrophoresis methods. Reversed phase HPLC analysis of erythromycin has been extensively documented [27–31]. Other investigators have described the separation of compounds like oleandomycin [32], clarithromycin [33,34], azithromycin [35–38] and roxithromycin [34,39–41]. However, no method has systematically examined the separation of these and other related macrolides under a common set of reversed phase HPLC conditions. Furthermore the ketolides have not been described by any HPLC separation method to date. This study describes a rapid, specific and sensitive method for the examination of 20 of these compounds, using a volatile mobile phase, allowing for the easy recovery and subsequent analysis of these antibiotics.

2. Materials and methods

2.1. Materials and reagents

Erythromycin and oleandomycin were from Sigma Chemical Co. (St. Louis, MO, USA), azithromycin was a gift from Pfizer (Groton, CT, USA) and the remaining macrolides used were gifts from Abbott Labs (Abbott Park, IL, USA). ^{14}C-erythromycin was from New England Nuclear (Boston, MA, USA) with a specific activity of 55.1 mCi/mmol. Nine of the ketolides used were gifts from Abbott Labs; two others (HMR 3004 and HMR 3647) were gifts from Hoechst Marion Roussel (Romainville, FR) Stock solutions of all compounds were made at 1 mg/ml in methanol. The antibiotics were stable for at least 6 months at 4°C.

2.2. Microbiological methods

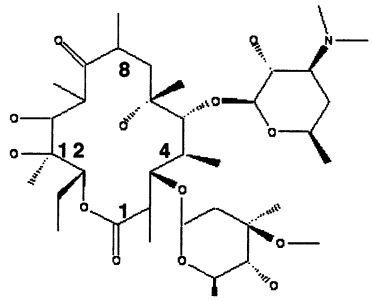
Antibiotic activity was measured by a zone of inhibition (ZOI) assay, conducted on *S. aureus* strain RN 1786, provided by J. Sutcliffe of Pfizer. A volume of 4 ml of 0.8% tryptic soy broth (TSB) soft agar was inoculated with 50 μl of an overnight culture of strain RN 1786 and poured onto a square TSB plate. Filter paper disks received 1–5 μl of each compound to be tested. The diameters of the zones of inhibition were measured after 12 h at 37°C.

2.3. Absorption spectra

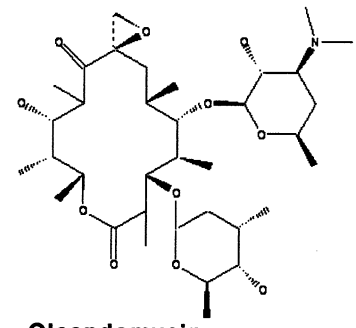
Absorbance measurements were made on a Hewlett Packard (Palo Alto, CA, USA) Model 4552A diode array spectrophotometer. Scans were made between 200 and 400 nm for each compound. Samples were examined at 50 μg/ml in 1 ml of 50 mM NH_4 acetate buffer, pH 7.0, in a 1 cm cuvette. Extinction coefficients were calculated for 1 mg of each drug at the wavelength of maximum UV absorbance.

2.4. Reversed phase HPLC

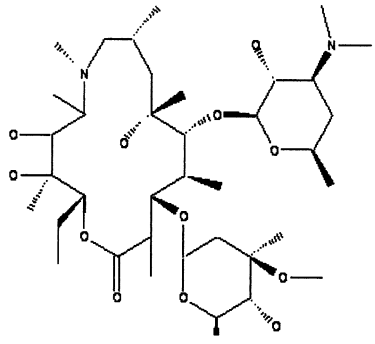
The HPLC system consisted of a Perkin Elmer (Norwalk, CT, USA) Series 410 Bio LC pump and a Model LC135 diode array detector. The Rheodyne injector had a 250 μl loop. Sample volumes were 10–50 μl. Separation was performed on a Pharamcia LKB Spherisorb 3 μm ODS-2 cartridge column, 4 × 125 mm. The flow rates were either 0.3 ml/min or 0.5 ml/min. The mobile phase consisted of acetonitrile/methanol/NH_4 acetate (pH 7.0; 0.1 M) (65:20:15, v/v/v). Data were collected on a Fisher Recordall strip chart recorder (Series 5000) at 0.5 inch/min and on a Macintosh LCII computer using Dynamax software from Rainin (Woburn, MA, USA). Fractions were collected at 1 min intervals for antibiotic analysis or for liquid scintillation counting. Column fractions were dried in a SpeedVac (Savant Instruments, Farmingdale, NY, USA) centrifuge and redissolved in methanol for further analysis. ^{14}C-erythromycin radioactivity in each fraction was measured in 3 ml of Scintisafe count-



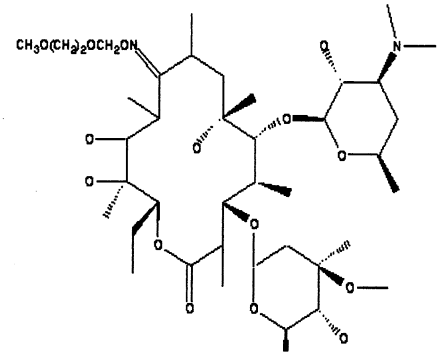
Erythromycin



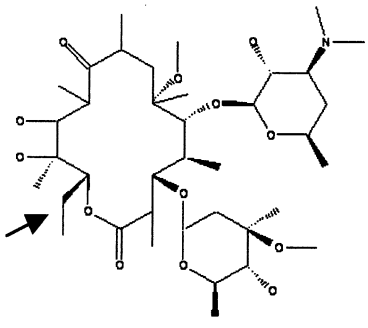
Oleandomycin



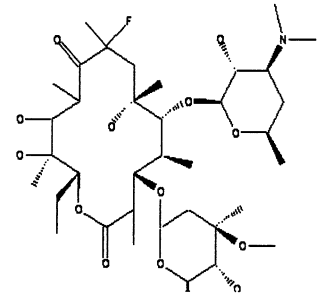
Azithromycin



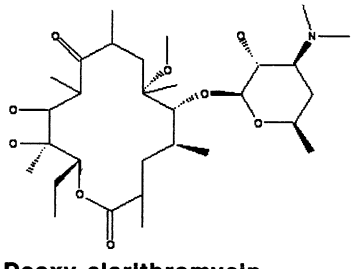
Roxithromycin



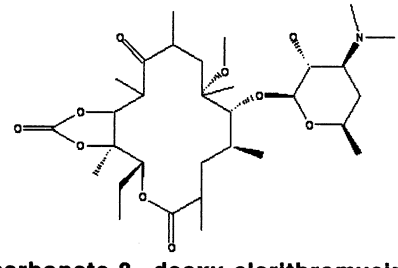
Clarithromycin



Flurithromycin



3-Deoxy-clarithromycin



11,12-carbonate-3 deoxy-clarithromycin

Fig. 1. Structures of the nine macrolide antibiotics studied. The position of the 14- hydroxy group in 14-hydroxy clarithromycin is indicated by an arrow.

ing fluid in a Beckman (Fullerton, CA, USA) Model LS3801 liquid scintillation counter.

3. Results

Nine structurally similar macrolide antibiotics were examined for their ability to be separated and purified by reversed phase HPLC. The structures of these nine compounds are shown in Fig. 1. Erythromycin and oleandomycin are naturally occurring antibiotics produced by Streptomyces bacteria [42]. They are 14-membered macrolactone compounds with attached neutral and amino sugars. Azithromycin, flurithromycin and roxithromycin are newer semi-synthetic compounds chemically derived from erythromycin [8]. Clarithromycin is 6-methoxyerythromycin and 14-hydroxy clarithromycin is a metabolic derivative of clarithromycin [12]. The neutral cladinose

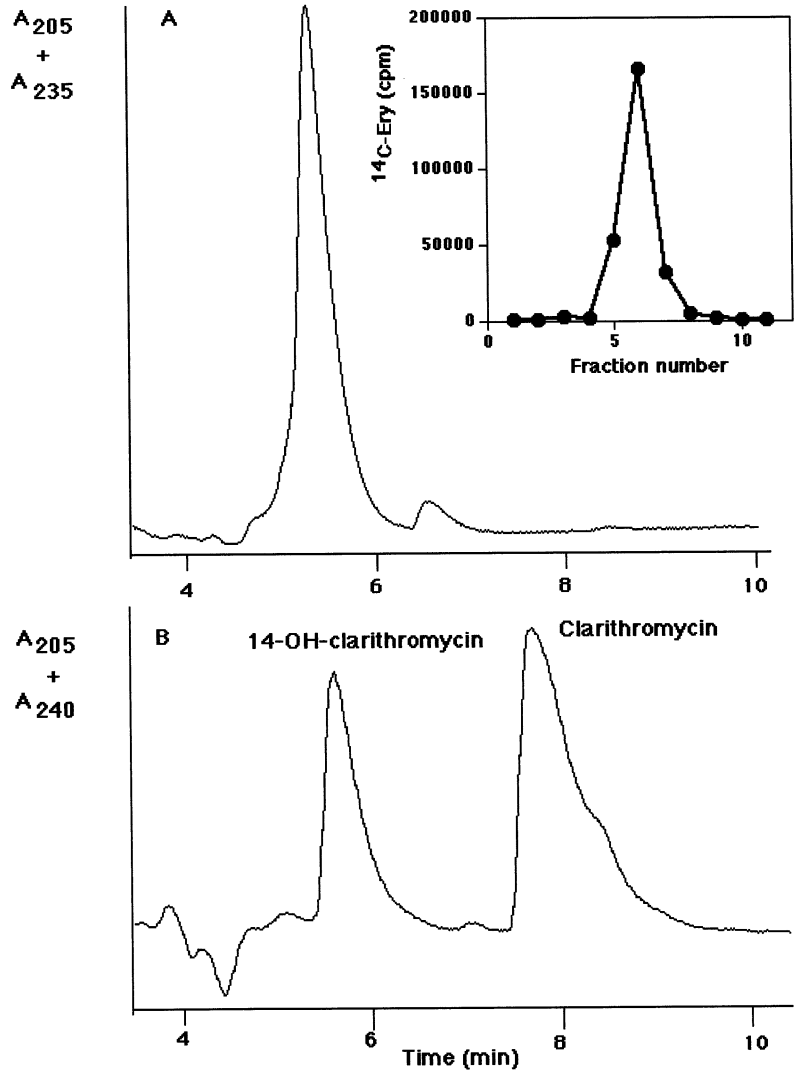


Fig. 2. Reversed phase chromatography of (A) erythromycin (75 μg) and (B) clarithromycin (25 μg) with 14-hydroxy clarithromycin (25 μg). The inset shows the co-chromatography of ^{14}C-erythromycin (1.5 μg; 0.1 μCi) with the sample in (A). Flow rate of 0.5 ml/min.

Table 1
Characteristics of nine macrolides separated by reversed phase HPLC

Antibiotic	Extinction (λ)[b]	Detector Wavelengths (nm)	Retention times (min)[a] Flow rate 0.3	Flow rate 0.5	Percent Recovered[c]
Oleandomycin	0.64 (244)	230+245	7.0	4.4	79
Azithromycin	0.54 (236)	205+235	7.2	4.5	57
Flurithromycin	0.20 (238)	205+235	8.7	5.1	93
Erythromycin	0.50 (236)	205+235	9.3	5.4	71
14-Hydroxy clarith.	0.33 (238)	205+240	9.6	5.8	100
3-Deoxy clarith.	0.34 (235)	205+240	10.9	6.7	100
11,12-carbonate-3-deoxy clarith.	1.36 (236)	230+240	11.2	7.0	100
Clarithromycin	0.43 (235)	205+235	11.9	7.6	95
Roxithromycin	0.20 (226)	225+250	13.0	7.9	93

[a] Retention times are the means of 3–7 determinations with an average S.D. of ± 0.41 min at flow rates of 0.3 and 0.5 ml/min.
[b] Extinction for 1 mg in 1 cm light path at wavelength indicated.
[c] Ratio of diameter of ZOI for column sample/ZOI for standard sample at same concentration $\times 100$.

sugar is missing from 3-deoxy clarithromycin and a cyclic carbonate ring has been added to the 11, 12 hydroxyl groups to give 3-deoxy-11,12-carbonate clarithromycin.

Chromatograms of erythromycin, clarithromycin and 14-hydroxyclarithromycin are shown in Fig. 2 (A and B). Erythromycin eluted with a retention time of 5.4 min at a flow rate of 0.5 ml/min. Authentic ^{14}C-erythromycin eluted with the identical profile (inset, Fig. 2A). Clarithromycin and its metabolic derivative 14-hydroxyclarithromycin, were readily separated from each other under these conditions, with retention times of 7.6 and 5.8 mins (Fig. 2B). Six other macrolides examined were also separated under these conditions and their elution characteristics are listed in Table 1. Dual UV wavelengths were used to identify different compounds during the same run, based on the λ_{max} measured for each drug. This is illustrated in Fig. 3 which shows the good separation of the four most commonly used macrolide antibiotics, azithromycin, erythromycin, clarithromycin and roxithromycin. At a flow rate of 0.3 ml/min, each of these compounds was well resolved from the others (Table 1). Each of these drugs was recovered in a volume of less than 0.5 ml.

Antibiotics were collected after column chromatography and were tested for recovery and activity. The nine macrolides collected all showed better than 60% recovery and excellent inhibitory activity (Table 1). Fig. 4 shows the ZOI test for inhibitory activity by six different macrolides.

Fig. 5 shows the structures of the 11 ketolide antibiotics which were examined. Each of these lacks the 3-cladinose sugar and is substituted with a 3-keto group. In addition each is modified in the 11,12 ring position with different aliphatic or aromatic substitutients, as the figure indicates. Compounds HMR3647 and HMR3004 are currently being examined for use as novel antimicrobial agents [20–23]. A good separation of these drugs was obtained under these chromatography conditions with HMR3647 eluting at 6.6 min and HMR3004 at 8.2 min. (Fig. 6A). Fig. 4 shows the recovery of inhibitory activity for column separated HMR3647 and HMR3004. The most most highly retained compounds examined, A24 and A11 could also be separated with retention times of 10.6 and 11.9 min. at a rate of 0.5 ml/min (Fig. 6B).

The seven other ketolides examined were also readily separated and identified by this procedure. This data is summarized in Table 2. Nine of the

11 ketolides were recovered with over 80% activity. In addition, these compounds could be identified and purified based their unique retention times and UV absorption maxima (Table 2).

4. Discussion

This work is the first attempt to systematically examine the chromatographic behavior of 20 related antibiotic compounds by reversed phase HPLC. Certain of these macrolide compounds have been described previously by their separation on reversed phase columns with mobile phases of different types (reviewed by Kanfer et al. [26]). The present description of the chromatographic behavior of these related compounds under a common set of conditions allows some predictions to be made regarding their elution behavior.

Column retention was related to the mobile phase flow rate and a decrease in rate from 0.5 to 0.3 ml/min resulted in a proportional increase in retention time for each compound examined (Tables 1 and 2). The mobile phase was designed to elute these compounds efficiently, to be UV transparent and to be completely volatile to allow for efficient sample recovery. Extremes of pH, used in some other studies, were avoided in order to protect the structural integrity of these drugs. These conditions were met in this study.

The extinction coefficients measured for the macrolides were in the range of 0.2–0.65 for eight of the nine compound examined. These values are similar to those published by Omura and Tanaka [42]. The 11,12 carbonate ring structure of the 3-deoxy derivative increased the absorbance of this drug compared with the others. For the ketolides, the monocyclic compound A22 had a significantly lower absorbance compared with the remaining compounds. The heterocyclic and aromatic rings of the remaining compounds resulted in extinction coefficients which were 10–30 times those of the macrolides.

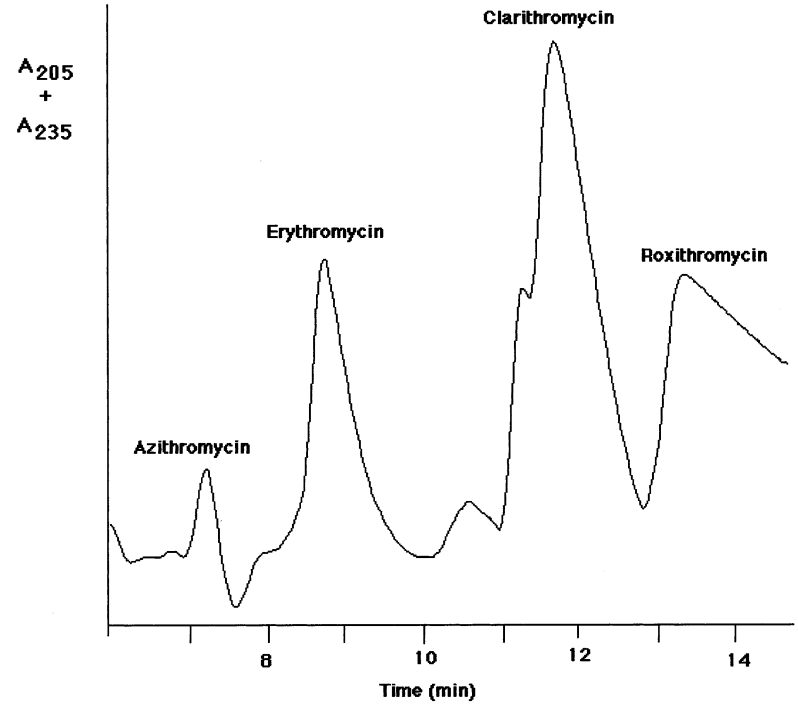


Fig. 3. Cochromatography of azithromycin (75 μg), erythromycin (100 μg), clarithromycin (25 μg) and roxithromycin (25 μg). Flow rate of 0.3 ml/min.

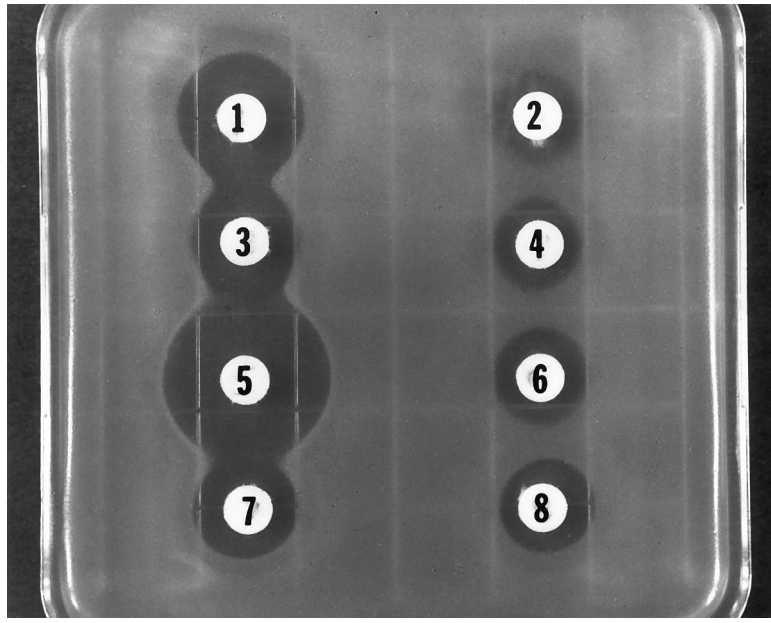


Fig. 4. Zone of inhibition assay for eight antibiotics purified by reversed phase HPLC. Dried samples were spotted on filter paper disks on a lawn of *S. aureus* cells and incubated at 37°C. Samples are: 1, erythromycin (5 μg); 2, oleandomycin (5 μg); 3, azithromycin (10 μg); 4, roxithromycin (1 μg); 5, clarithromycin (1 μg); 6, 14 hydroxy-clarithromycin (1 μg); 7, HMR3004 (0.1 μg); 8, HMR3647 (0.1 μg).

The relative retention times of the macrolide compounds were reflective of their structural differences. Compared with erythromycin, oleandomycin was retained least well, consistent with its difference of two fewer methyl groups. Azithromycin eluted next from the column, due to the extra lactone ring N atom and missing carbonyl group. The negative fluorine atom of flurithromycin caused it to elute slightly sooner than erythromycin. Clarithromycin and its derivatives containing an extra *O*-methyl group, were retained on the column longer than erythromycin. 14-Hydroxy clarithromycin, more hydrophilic than clarithromycin, eluted earlier than clarithromycin itself. The lack of the 3-cladinose sugar promoted increased retention of the two 3-deoxy compounds. The last compound to elute from the column was roxithromycin which contains the unique extended aliphatic side chain allowing for additional interactions with the C18 column. The same relative order of elution of azithromycin, erythromycin, clarithromycin and roxithromycin from a C18 column was described by Croteau et al. [43].

The ketolide retention times were also consistent with the differences in molecular structure of these compounds [18,19]. The monocyclic compound A22 was retained least well on the C18 column. The tricyclic compounds A51 and A53 were similar in their retention times. Interestingly, the 11,12 carbonate ketolide A55, with a 3-keto group, was more hydrophilic and was retained less well than the 11,12 carbonate macrolide (3-deoxyclarithromycin) lacking a 3-keto function (compare Tables 1 and 2). The heterocyclic rings of A23 and HMR3647 caused them to elute with similar retention times. A54 was retained less well than A24, in spite of their very similar structures, presumably due to the ether linkage in the aliphatic arm of A54. The conjugated ring system of HMR3004 and the bi-phenyl rings of A52 gave these drugs similar retention features. Finally, A11, with sterically separated phenyl groups was retained most strongly, longer than both A52 and A24.

This investigation describes a consistent set of conditions for the separation and identification of

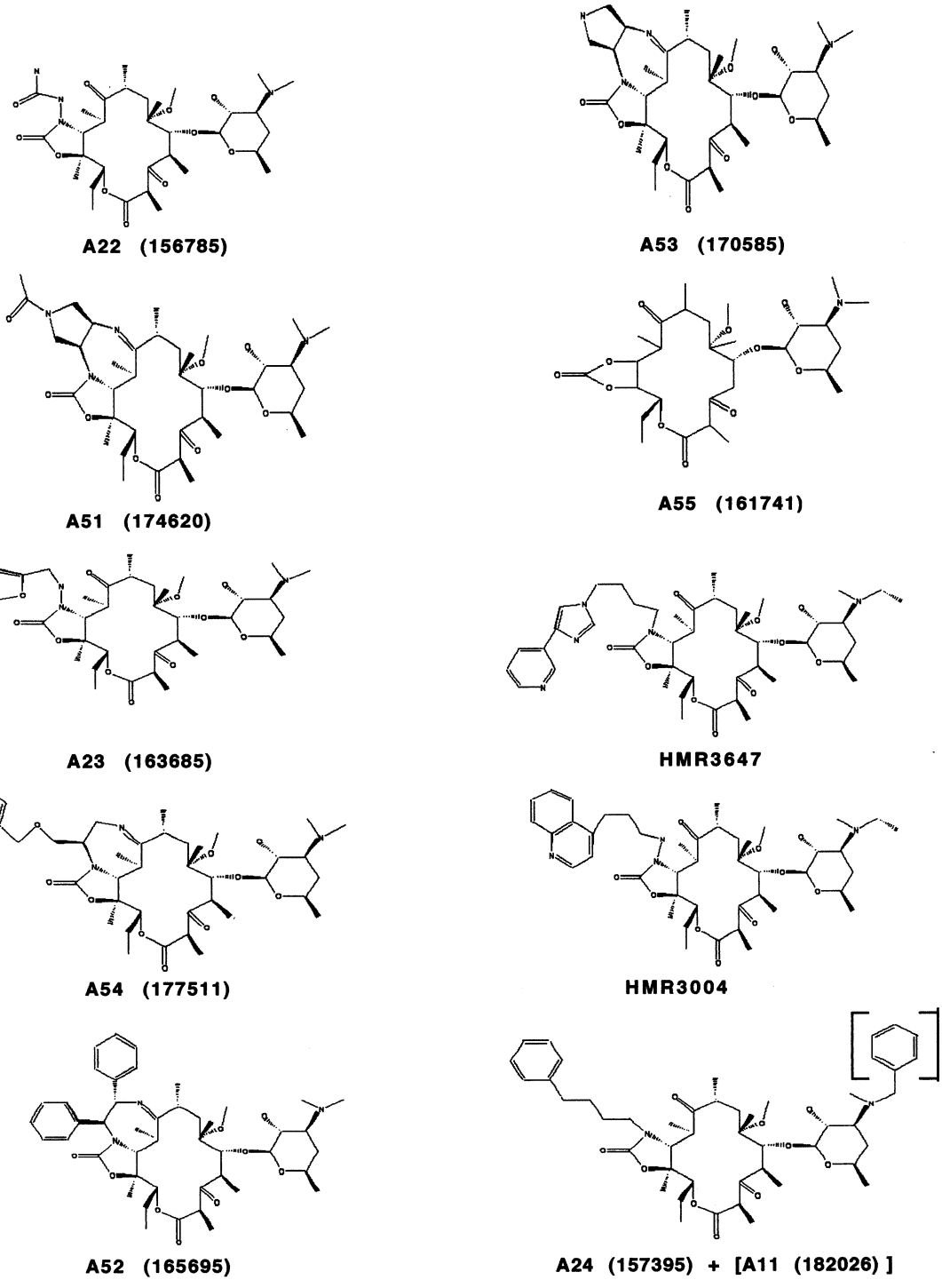


Fig. 5. Structures of the 11 ketolide antibiotics studied. The Abbott drug numbers are indicated in parentheses following the laboratory name. The additional phenyl group characteristic of A11 is indicated by a bracket.

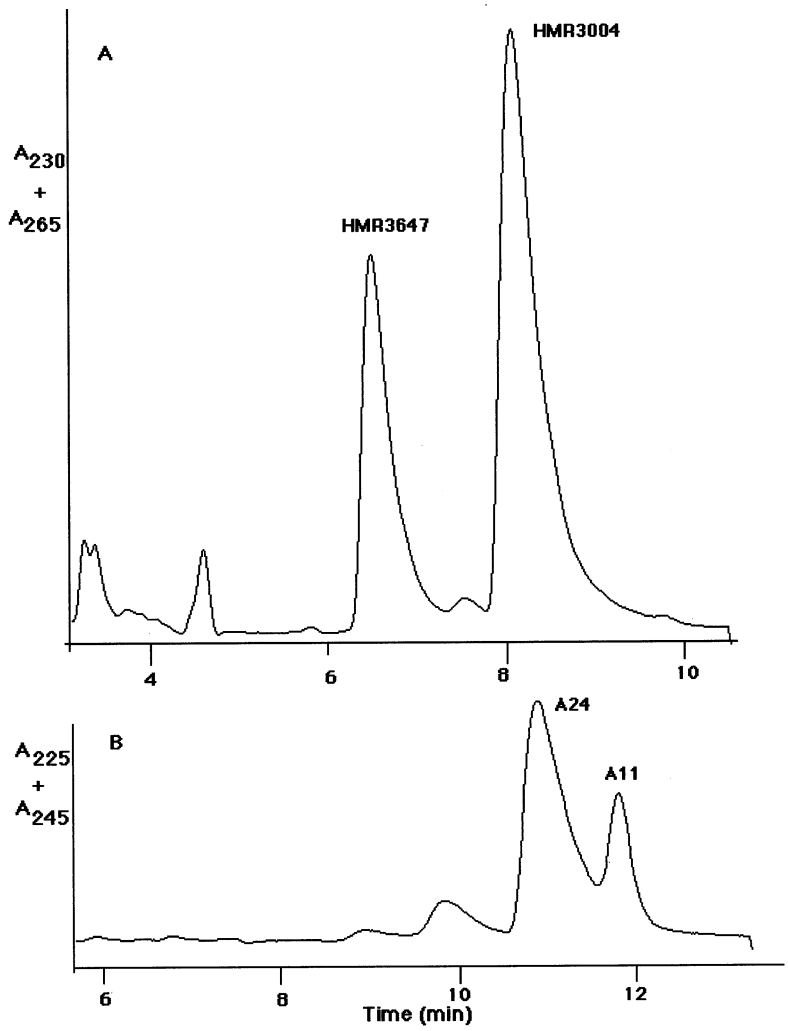


Fig. 6. Cochromatography of (A): HMR3647 (1 μg) and HMR3004 (1 μg) and (B):A24 (1 μg) and A11 (1 μg). Flow rate 0.5 ml/min.

20 related antibiotics. Differences in structure between these compound have been related to differences in antibiotic effectiveness between these drugs [4,5]. We intend to use this method to examine metabolic derivatives of these drugs and to separate and purify certain des-methyl derivatives as substrates for radioisotope labeling [44,45]. These chromatographic differences can also help to relate structural features to functional activities and are predictive for the behavior of new compounds to be tried as antimicrobial agents.

Acknowledgements

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Table 2
Characteristics of 11 ketolides separated by reversed phase HPLC

Antibiotic	Extinction (λ)[b]	Detector Wavelengths (nm)	Retention times[a] Flow rate 0.3	Flow rate 0.5	Percent Recovered[c]
A22	1.9 (220)	220	6.3	3.9	100
A53	9.4 (218)	220	7.6	4.7	85
A51	14.6 (218)	220	7.8	5.1	56
A55	8.32 (220)	220	8.5	5.1	100
A23	8.0 (220)	220	10.1	6.1	100
HMR3647	22.4 (264)	230+265	11.1	6.6	80
A54	10.2 (218)	220	12.5	7.6	73
HMR3004	34.0 (228)	230+260	13.8	8.2	100
A52	36.4 (226)	225	15.4	9.0	100
A24	13.8 (244)	225+245	17.4	10.6	100
A11	21.4 (224)	225+245	19.6	11.9	100

[a] Retention times are the means of three determinations with an average S.D. of ± 0.1 min at flow rates of 0.3 and 0.5 ml/min.
[b] Extinction for 1 mg in 1 cm light path at wavelength indicated.
[c] Ratio of diameter of ZOI for column sample/ZOI for standard sample at same concentration $\times 100$.

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