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Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713597273>

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To cite this Article Mierzwa, Ron , Truumees, Imbi , Patel, Mahesh , Marquez, J. and Gullo, V.(1985) 'High-Performance Preparative Isolation and Purification of Several Mycinamicins', *Journal of Liquid Chromatography & Related Technologies*, 8: 9, 1697 – 1709

To link to this Article: DOI: 10.1080/01483918508074088

URL: <http://dx.doi.org/10.1080/01483918508074088>

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HIGH-PERFORMANCE PREPARATIVE ISOLATION AND PURIFICATION OF SEVERAL MYCINAMICINS

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ABSTRACT

High performance preparative chromatographic systems are described which facilitate the isolation and purification of some naturally occurring, novel mycinamicins. Facile separation and excellent recovery of these macrolide antibiotics was obtained with a C-18 radially compressed Prep 500 cartridge using methanol/trifluoroacetic acid mobile phases. Semi-preparative chromatography on a C-8 reversed-phase sorbent incorporating a volatile ammonium acetate buffer was utilized in the final purification of an enriched mixture of intermediate components. Monitoring of isolated fractions with analytical HPLC provided direct support for the effectiveness of the preparative isolation.

INTRODUCTION

Mycinamicins, a family of 16-membered macrolide antibiotics that exhibit strong antibacterial activity against gram-positive bacteria have recently been discovered and characterized.^{1,2,3,4,5} Almost invariably, isolation of the macrolides has followed conventional approaches using silica gel column chromatography. High performance liquid chromatography developed and utilized over the past decade has been an invaluable tool for the analysis and purification of natural products.^{6,7} Separation of closely related components, difficult to

achieve by classical approaches, has been successfully resolved with microparticulate high-performance chromatography techniques.

Reverse phase chromatography has been applied in the analysis of macrolide antibiotics. Tsuji⁸ analyzed at elevated temperature, erythromycin, erythromycin ethylsuccinate and its degradation products. Tylosin analogs were detected and quantitated from fermentation broths on a u-Bondapak C-18 sorbent with an acetonitrile:2% aqueous monoethanolamine mobile phase.⁹ Turimycin-H-complex was resolved on RSil-C18-HL column with an aqueous acetonitrile mobile phase that incorporated a small percentage of diethylamine.¹⁰ Despite these approaches, preparative reversed-phase chromatography of macrolide antibiotics that involved direct transfer of analytical methodologies has not been fully exploited. As this paper will describe, macrolide antibiotics derived from fermentation sources, after some simple purification steps, can rapidly be purified in excellent yield when combined with reversed-phase HPLC.

MATERIALS

Reagents

Methanol and acetonitrile were HPLC grade (Burdick and Jackson Muskegon, Michigan). All aqueous solutions were prepared using distilled water further purified thru a Milli-Q system (Millipore Corp., Bedford, Mass.). Ammonium acetate and glacial acetic acid were ACS grade (Fisher Scientific, Fairlawn, NJ). Trifluoroacetic acid (TFA) (99%) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). All other solvents and chemicals were of reagent grade.

HPLC Columns and Mobile Phases

u-Bondapak C-18 (10 μ), 30cm x 3.9mm (Waters Associates, Milford, MA) and Chromegabond C-8 (10 μ), 25cm x 4.6mm (E.S. Industries, Marlton, NJ) reversed-phase columns were employed for analytical evaluations. Semi-preparative chromatography was performed on Chromegabond C-8 (10 μ), 30cm x 23mm columns. Preparative

isolation utilized a Preppak-500/C-18 ($\sim 75\mu$), 30cm x 5.7cm radially compressed cartridge (Waters).

The mobile phase for the u-Bondapak and C-18 Prep-500 columns consisted of varying proportions of methanol in 0.05% trifluoroacetic acid. A stock solution (0.5% w/v) of TFA was prepared by dissolving 5.0 gram in one liter of de-ionized water. Stock solution (100 ml) was diluted to one liter and filtered thru a Millex HA (0.45 μ) membrane filter (Millipore). After adding the appropriate volume of organic modifier, the mobile phase was sparged under nitrogen for several minutes. Both analytical and semi-preparative C-8 columns were eluted with acetonitrile: 0.01M ammonium acetate pH 4.0 buffer (40:60 v/v).

HPLC Instrumentation

Analytical HPLC employed Waters M 6000A dual reciprocating pumps with either a 440 dual channel (Waters) or Schoeffel ultraviolet detector (Kratos Inc., Westwood, NJ) set at 254nm. Sample injection was performed with a Wisp 710B (Waters). Data acquisition and reduction for both the analytical and semi-preparative chromatographies were obtained with either a 730 data module (Waters) or a H.P. 3385A automation system (Hewlett Packard, San Diego, CA.) Semi-preparative chromatography was carried out using a Varian 8500 pumping system (Varian Associates, Palo Alto, Calif.) coupled with a Schoeffel 770 variable wavelength uv-vis detector. Samples were injected manually with a U-6K injector (Waters). Preparative chromatography was performed on a Prep-500 liquid chromatographic unit (Waters) with a built-in differential refractometer (R-404).

EXPERIMENTAL

Isolation of Biologically Active Macrolide Complex

Fermentation broth (420 liters) was adjusted to pH 8.5 with sodium hydroxide and extracted twice with ethyl acetate (2 x 425 liters).

The ethyl acetate was concentrated in vacuo at 50°C to a dark oily residue (123.5 g) and agitated with an excess of hexane. The resultant residue (89g) was dissolved in 95% ethanol (65 ml), placed on a Sephadex LH-20 column (1500 ml) and eluted with the same solvent. The biologically active fractions were pooled, evaporated to dryness (72g), redissolved in ethyl acetate (720 ml) and extracted twice with equal volumes of 5% acetic acid. The acidic aqueous layers were combined, adjusted to pH 8.8 with sodium carbonate and back-extracted with ethyl acetate (2 x 750 ml). The ethyl acetate extracts were dried over magnesium sulfate and concentrated in vacuo to a yellow viscous residue. Crude macrolide complex (35g) was obtained by precipitation from methylene chloride into petroleum ether.

The biological activity of the individual components was established by injecting 30 ug of the macrolide complex on a u-Bondapak C-18 column and separately collecting the major 254 nm ultraviolet absorbing peaks (Figure 1). Each fraction was rotary evaporated to remove methanol, lyophilized, reconstituted in methanol (100 ul) and spotted (10 ul) on a silica gel TLC plate. The plates were developed with chloroform:methanol: 7% NH₄OH (40:12:10) and bioautographed against Sarcina lutea. TLC of the complex (Figure 1, inset) reveals three zones of inhibition, with HPLC fractions 2 and 4 possessing the same relative R_f and clear inhibition zones.

Preparative Separation of Components

The antibiotic mixture was chromatographed on a u-Bondapak C-18 (10u) analytical column for optimization of scale-up conditions to the Prep-500. Chromatographic data under varying mobile phase conditions are summarized in Table I. Selectivity values for adjacent peak pairs suggested a 45:55 v/v methanol: 0.05% trifluoroacetic acid mobile phase that would provide for superior resolving power ($K' < 10$) under preparative mass load conditions.

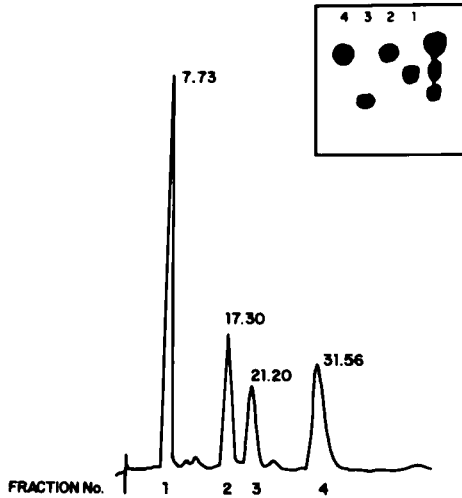


Figure 1: HPLC separation profile of macrolide complex on a μ -Bondapak C-18 (10 μ) column; eluent, MeOH: 0.05% TFA (40:60 v/v); flow rate, 2 ml/min; detection at 254 nm (0.04 AUFS); pressure (2400 psi); chart speed 0.20 cm/min; retention time (min.).

Inset: Bioautographic results (*Sarcina lutea*) sorbent, Quantum silica gel LK6DF (20 x 20 cm; Whatman, Clifton, NJ) development system; chloroform:methanol: 7% ammonium hydroxide (40:12:10) (right to left) macrolide complex (5 ug); fractions 1-4; RF's 0.58, 0.71, 0.47, 0.71 respectively.

Table I

Effect of Mobile Phase Composition on Chromatographic Parameters^{a, b}

	Component	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
I 50:50 ^a	3.03 (1.08)	4.93 (2.38)	5.56 (2.81)	7.40 (4.07)	
	$\alpha_{2,1} = 2.20$	$\alpha_{3,2} = 1.18$	$\alpha_{4,3} = 1.45$		
II 45:55	4.70 (2.07)	8.90 (4.82)	10.40 (5.80)	14.66 (8.58)	
	$\alpha_{2,1} = 2.33$	$\alpha_{3,2} = 1.20$	$\alpha_{4,3} = 1.48$		
III 40:60	7.73 (3.83)	17.30 (9.81)	21.20 (12.25)	31.56 (18.73)	
	$\alpha_{2,1} = 2.56$	$\alpha_{3,2} = 1.25$	$\alpha_{4,3} = 1.53$		

a - Mobile phase: MEOH: 0.05% TFA; flow rate 2ml/min.
Column: μ -Bondapak C-18 (10 μ) 30cm x 3.9mm;

b - Format of Data: Retention time in minutes (capacity factor K')
 $\alpha_{y,x} = K'_y/K'_x$ (t_r or void volume is taken as the retention time of the first detected peak; I (1.46 min), II (1.53 min), III (1.60 min))

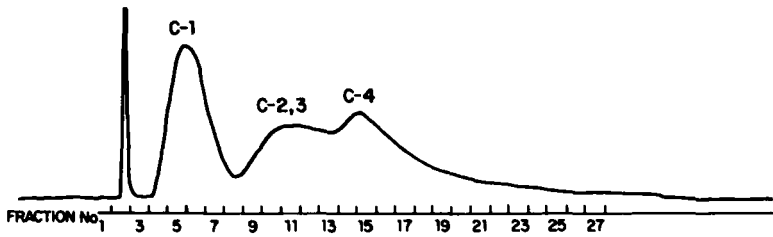


Figure 2: Refractive index recording (10 x) from Prep 500 run showing separation of components; chart speed 5 min/cm, flow rate 100 ml/min, individual fractions 0.50 c.v. = 250 ml.

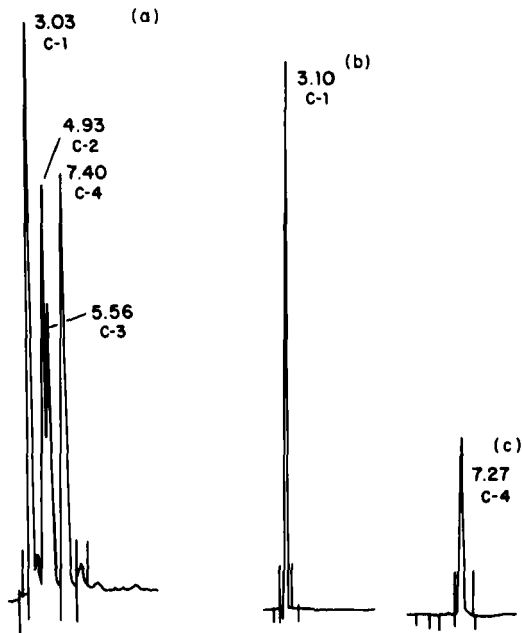


Figure 3: Analytical HPLC of (a) macrolide complex (10 ug) and purified Prep-500 pooled fractions (b) component 1 (6 ug) and (c) component 4 (6 ug); retention time (min).

Conditions as outlined except eluent ratio 50:50 (MeOH: 0.05% TFA) UV (254 nm) .02 AUFS.

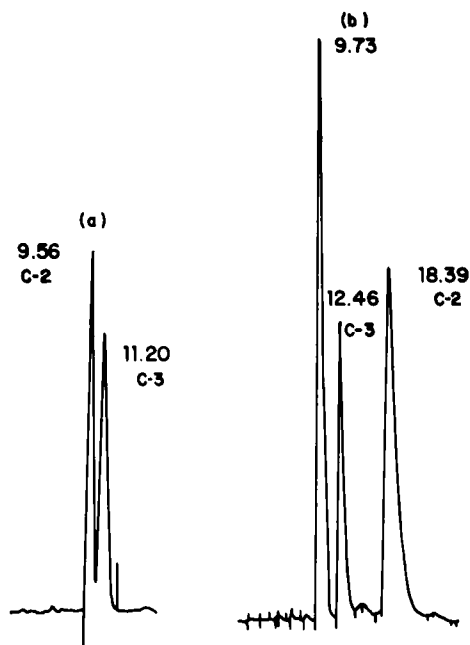


Figure 4: (a) Analytical elution profile of the Prep-500 enriched mixture (components 2 and 3) on a μ -Bondapak C-18 column with MeOH: 0.05% TFA (45:55 v/v), flow rate 2 ml/min; retention time (min).

(b) Analytical HPLC of macrolide complex (10 μ g); column; C-8 (10 μ), 30 cm x 4.6 mm, eluent; CH₃OH: 0.01M NH₄OAc pH 4.0 (40:60 v/v); flow rate 1.3 ml/min; UV (254 nm) .02 AUFS, peaks 12.46 min (component 3) and 18.39 min (component 2).

A single C-18 Prep-500 cartridge was conditioned with two liters of 90% methanol and then equilibrated with the eluent mobile phase (MEOH: 0.05% TFA, 45:55 v/v) until a stable refractive index baseline was obtained. Macrolide complex (3g) was dissolved in 12 ml of MEOH: 0.05% TFA (80:20 v/v) and manually injected. Fractions (250 ml) were collected at a flow rate of 100 ml/min (Figure 2). Individual fractions were assayed by analytical HPLC and after pooling, the methanol was removed in vacuo. The aqueous solutions were

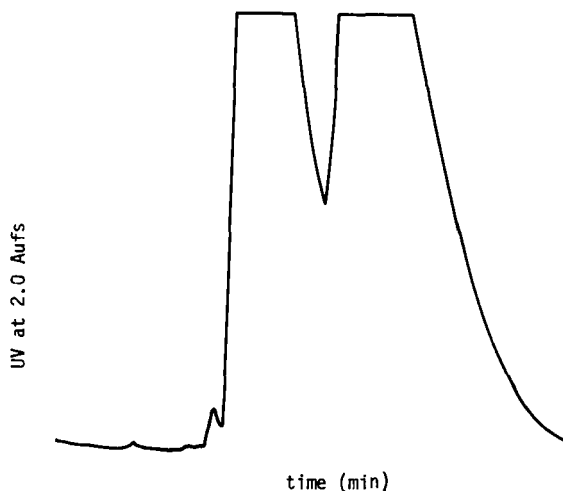


Figure 5: Semi-preparative chromatographic UV profile from a 100 mg injection of enriched components 2,3.

Conditions: as described in text.

lyophilized and 750 mg of component 1 \geq 98% and 250 mg of component 4 \geq 95% as white solids were obtained (Figure 3). Fractions enriched in components 2 and 3 (\sim 800 mg) were further purified as outlined under semi-preparative isolation.

Semi-Preparative Isolation of Components 2 and 3

The HPLC analysis of the enriched mixture of components from the preparative run is illustrated in Figure 4a. In order to establish greater separation between these components, additional chromatographic systems were investigated. Changes in elution order and enhanced resolution ($\alpha=1.52$) resulted when a C-8 (10 μ) column was employed under different eluent conditions (Figure 4b). For scale up to semi-prep, two C-8 (10 μ), 30cm x 23mm, columns were attached in series using zero dead volume connectors. The columns were conditioned with 50% aqueous acetonitrile followed by a mixture of

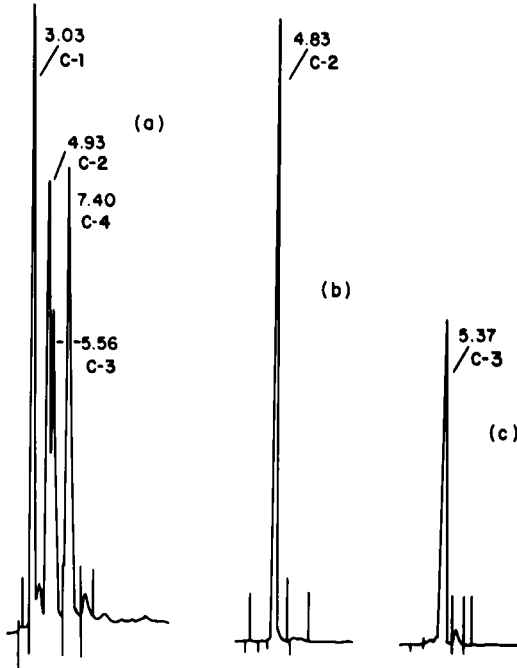


Figure 6: Analytical HPLC of macrolide complex (10 ug); and purified semi-preparative fractions (b) component 2 (6 ug) and (c) component 3 (6 ug); retention time (min).

Conditions: μ -Bondapak C-18 (10 u); MeOH: 0.05% TFA (50:50 v/v), flow rate 2 ml/min; UV (254 nm) .02 AUFS.

acetonitrile and 0.01 M ammonium acetate buffer pH 4.0 (9:1 v/v) and finally equilibrated with an acetonitrile/0.01 M ammonium acetate pH 4.0 eluent (40:60 v/v) at a flow rate of 10 ml/min. The enriched mixture (100 mg) was dissolved in 1 ml of acetonitrile containing 100 μ l of pH 4.0 ammonium acetate buffer. After sample injection, the separation was monitored at 254 nm (2.0 auFS). Individual fractions were concentrated in vacuo, lyophilized and yielded 23 mg of component 2 > 97% and 34 mg of component 3 \geq 94% (Figures 5,6).

RESULTS AND DISCUSSION

The biologically active samples isolated as a result of the preparative and semi-preparative chromatographies were subjected to spectroscopic evaluations. Proton, C-13 NMR, mass spectral and ultraviolet data were found to be consistent with several known and two new macrolide antibiotics (HPLC components #1,2) belonging to the mycinamicin class.^{11,12} Figure 7 summarizes the chemical structures and co-identities established. In addition, an HPLC comparison of an authentic sample of mycinamicin III with the macrolide complex confirmed its elution similarities with component 4. (Figure 8)

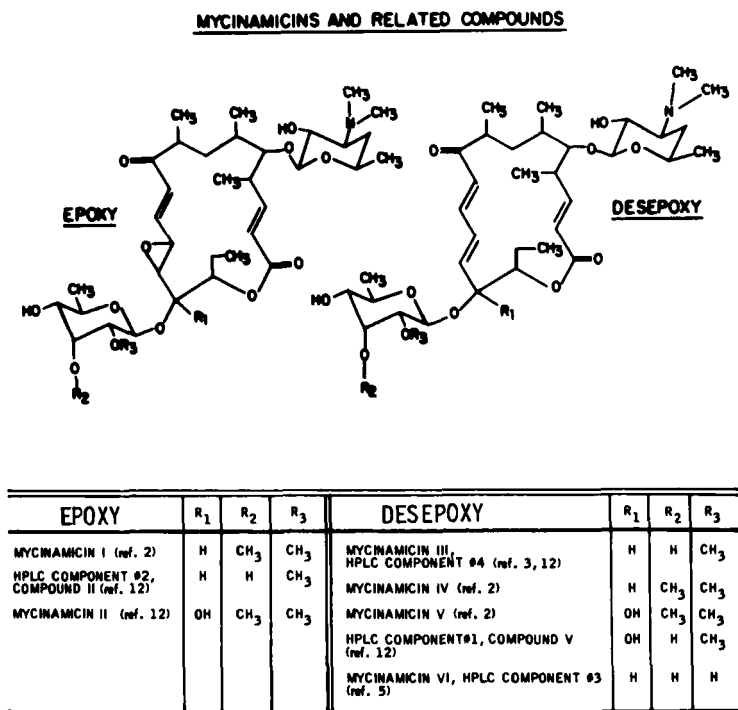


Figure 7: Structures of known and novel Mycinamicins

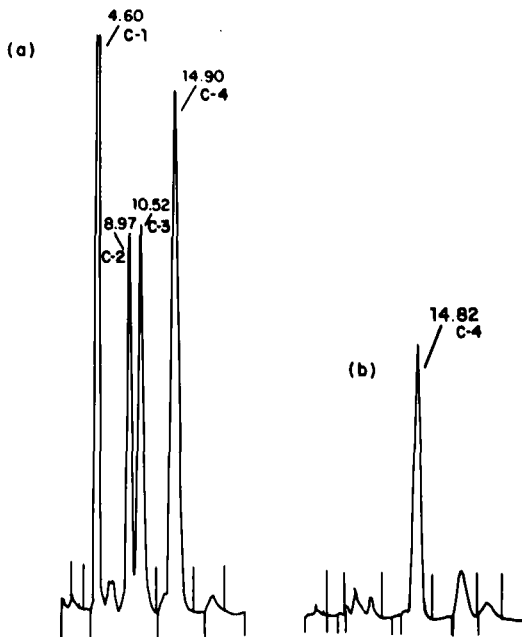


Figure 8: Analytical HPLC of (a) macrolide complex (15 ug) and (b) mycinamicin III (3 ug); retention time (min).
 Conditions: μ -Bondapak C-18 (10 μ) MeOH: 0.05% TFA (45:55 v/v), flow rate 2 ml/min; UV (254 nm) .02 AUFS.

Reverse phase preparative chromatography has been shown to be an effective and useful technique for the large scale purification of mycinamicins. Direct transfer of methods from analytical to the preparative mode was clearly established. Choice of C-18 and C-8 columns when combined with the appropriate eluent composition provided significant changes in solute selectivity thereby assisting purification of closely related components.

Perfluorinated carboxylic acid containing mobile phases, common to peptide isolation and analysis¹³ appear not to have been fully exploited in macrolide purification schemes. The major advantages of trifluoroacetic acid mobile phases was its general usefulness in

differentiating a diversified range of macrolide antibiotics while sample recovery was facilitated owing to eluent volatility. A major consideration for its choice as an eluent, however, should be the stability of the compounds under strongly acidic conditions.

Conventional silica gel chromatography has been routinely employed for the isolation of macrolides. In this case, the mycinamicin components 2 and 4 were not resolved with all the normal phase systems investigated but were easily resolved using reverse phase systems. The methods described in this paper represent a successful alternative to normal phase silica gel chromatography for the isolation of macrolides.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. M.S. Puar for spectroscopic evaluations; Dr. Shuzo Sato (Toyo Jozo Co. Ltd.) for graciously supplying mycinamicin III and Darcelle Ragin for preparation of this manuscript.

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