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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION AND QUANTIFICATION OF A DILACTONIC ANTIBIOTIC MIXTURE (ANTIMYCIN A)*

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SUMMARY

High-performance liquid chromatographic (HPLC) conditions are presented for the separation and quantitative determination of a homologous antibiotic complex (antimycin A). Combined HPLC and chemical ionization mass spectrometry proved to be exceptionally useful for the structural identification of chromatographic components. Using electrochemical, fluorescence, and ultraviolet detectors, the minimum detectable amounts of the antibiotics were found to be in the ranges 0.10–1.12, 0.31–1.69, and 4.10–28.2 ng, respectively. Advantages of the preparation of Dns derivatives for use in fluorescence detection are discussed. Application of the HPLC technique to the analysis of the antibiotic mixture in organic tissues is demonstrated.

INTRODUCTION

A cluster of closely related antibiotic substances produced from Steptomyces by fermentation is known as antimycin A and has been used for many years as a general piscicide in fishery management. It consists of a group of chemically labile nine-membered ring dilactonic compounds that differ merely in the homologous alkyl substituents on the 7- and/or 8-side-chains (Fig. 1). In recent years interest in the chemistry, synthesis, and biological activities of the antimycin A complex has stimulated much chemical and biochemical research[1–5]. Despite earlier extensive studies on analytical methodology utilizing pyrolysis gas chromatography[6], semi-quantitative mass spectrometry[7], and gas–liquid chromatography of derivatives[8] for the determination of antimycins in samples from various sources, there existed severe limitations due to lack of selectivity, sensitivity, and specificity in association with separation as well as detection techniques. The published pyrolytic gas chromatographic and mass spectral procedures possess one disadvantage in common that involves sample decomposition prior to assays. A few reported methods of separation by fractional crystallization, paper chromatography, and countercurrent distribution[9–11] are too

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crude to be quantifiable, and yield only partial resolution of the antimycin constituents. We now describe a new method based on the use of octadecylsilica bonded reversed-phase high-performance liquid chromatography (HPLC) in the speciation of the complex titel mixture, and simultaneous quantitative measurement of the series of resolved component homologues. Some applications of various commercial detectors coupled with an HPLC system are presented, and the detection sensitivity and linearity are evaluated. In addition, a method for prior sample cleanup of organic tissue extracts has been developed which embodies an adsorption and thin-layer chromatographic (TLC) procedure.

EXPERIMENTAL

Materials

Solvents and buffer salts for HPLC were of high purity spectral grade purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Other chromatographic solvents were acquired from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) in "distilled-in-glass" quality. Mixtures of antimycin standards were gifts from Aquabiotics Corp. (North Brook, IL, U.S.A.). A sample of the radioactive antibiotics (Ayerst Research Laboratories, Montreal, Canada) that contains mixed homologues of [^{14}C]antimycin A uniformly labeled at the benzene ring was first recrystallized from hexane–methylene chloride and then purified by TLC (silica gel plate, $20 \times 20 \times 0.025$ cm; benzene–chloroform–methanol–acetic acid, 7:5:1:0.7). The pure ^{14}C-labeled antimycin A complex obtained in this manner had a specific activity of 2.82 μCi/mg. 1-Dimethylamino-naphthalene-5-sulfonyl chloride (Dns chloride) and pyridine were products of Aldrich (Milwaukee, WI, U.S.A.) and used as purchased. Silica gel (40–140 mesh) for column adsorption chromatography was of "Baker analyzed reagent" grade. Precoated TLC uniplates were supplied by Analtech (Newark, DE, U.S.A.). All other chemicals used were of "analytical reagent" purity.

Equipment

The general procedure for this study, unless otherwise stated, employed a Varian Model LC-5000 liquid chromatograph with an ultraviolet detector (Varian Varichrom) set at 254 nm, and a reversed-phase, high efficiency, ultrasphere ODS column (5 μm) (25 cm $\times$ 4.6 mm I.D.) (Altex, Berkeley, CA, U.S.A.). The mobile phase was composed of 75:25 methanol–acetate buffer (0.25 M, pH 5) at a flow-rate of 2 ml min. The injection system consisted of a Valco CV-6-UHPa-N60 injection valve and a 10-μl loop (Valco, Houston, TX, U.S.A.). Samples to be analyzed were introduced into the HPLC column via a guard column (5 cm $\times$ 4 mm I.D.) packed with Varian Vydac reversed-phase hydrocarbon (40 μm) as the stationary phase.

The electrochemical detector used in this study was a Model LC-3 detector controller attached to a Model TL-5 glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The electrode potential was maintained at $+1.00$ V *versus* a silver–silver chloride reference electrode. The HPLC-fluorescence measurements were performed with a Varian Fluorichrom detector equipped with a tungsten halide light source, a flow cell, and selected optical filters. The HPLC column effluent was monitored at an excitation wavelength of 365 nm and an emission wavelength of 418 nm.

In all analyses, the output signal was fed into a Varian Model 9176 strip chart recorder and the peak area and retention time were automatically computed by a Varian Model CDS-111L data system.

Mass spectra characterization of the antimycin components isolated from the preparative reversed-phase HPLC column (Varian MicroPak-MCH, 10 μm, 30 cm × 8 mm I.D.) was done on a Finnigan Model 4021-T quadrupole mass spectrometer interfaced to a direct probe. The combined HPLC–mass spectrometry (LC–MS) system used was a Finnigan Model 3200 quadrupole mass spectrometer coupled with the Varian Model LC-5000 instrument through a moving belt transport device and a continuous extraction interface. The mass spectrometers were operated in the chemical ionization mode using methane as the reagent gas. The data were collected and processed by an INCOS data system operating on a Data General Nova 3/12 computer.

Isolation of antibiotic components by preparative HPLC

Depending on the amount of the sample (10–50 mg) employed in each injection, preparative HPLC was run on either a Varian MicroPak-MCH reversed-phase column (10 μm, 30 cm × 8 mm I.D.) or a Whatman Partisil Mag 9 ODS column (10 μm, 50 cm × 10 mm I.D.). In a typical run, a 50-mg sample was chromatographed on the latter column with a mobile phase consisting of 70:30 acetonitrile–phosphate buffer (0.2 M, pH 3.5) at a flow-rate of 0.5 ml/min. The eluent was monitored with a Varian refractive index detector, and the separated antimycin components were collected into 0.5-ml fractions utilizing a Buchler linear automatic fraction collector. Twenty injections, which constituted a total of 1.0 g of the sample of the antimycin A complex, were made. Prior to their extraction workup, analytical HPLC–UV chromatograms were obtained for all the fractions collected to check purity of each fraction. The homogeneous fractions that contained identical single antibiotic components were combined. The above process generally yielded highly pure antimycins resolved into ten fractions. The organic matter from the HPLC fractions was isolated by careful removal of acetonitrile by evaporation on a rotary evaporator under a reduced pressure followed by extraction of the remaining aqueous layer with methylene chloride. Upon addition of hexane to the chilled concentrate of the methylene chloride extract, there was obtained a pure antimycin component as white crystals. All specimens were estimated to be greater than 99 % pure by analytical HPLC–UV. The amounts of the individual antimycins isolated from a sample of the antimycin A complex were as follows: A_6, 6.3 mg; A_5, 13.0 mg; A_4, 75.7 mg; A_3, 60.9 mg; A_2, 103.4 mg; A_1, 136.6 mg; A_{0b}, 8.2 mg; A_{0a}, 4.5 mg; A_{0d}, 5.7 mg; A_{0c}, 11.8 mg (see Fig. 1 for structures). As the composition of components varied among the antibiotic samples originated from dissimilar fermentation batches, some of the pure compounds isolted above were added to the analytical samples to serve as internal standards for the HPLC quantitation of different lots of the antimycin samples in which a component having the same structural identity as the internal standard was absent, or present in negligible amount.

Dns derivatives

The preparation of Dns antimycins by the conventional dansylation technique was initially attempted, but the yield was too low to be of analytical signifi-
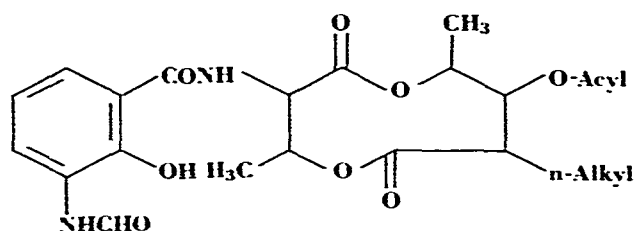
Component	n-Alkyl	Acyl
A_0	(a) n-Hexyl	Hexanoyl
	(b) n-Butyl	Heptanoyl
	(c) Octyl	Butyryl
	(d) Heptyl	Isovaleryl
A_1	n-Hexyl	Isovaleryl
A_2	n-Hexyl	Butyryl
A_3	n-Butyl	Isovaleryl
A_4	n-Butyl	Butyryl
A_5	Ethyl	Isovaleryl
A_6	Ethyl	Butyryl

Fig. 1. Structural formulas of the antimycins isolated by HPLC.

cance. However, a better alternative method was developed at this laboratory. A sample (100 mg) of the antimycin A complex in 100 ml of acetone was treated with 40 ml of 1 "₀ Dns chloride in acetone and 5 ml of pyridine. This mixture was stirred at room temperature for 30 min. The excess solvent was evaporated on a Buchi rotary evaporator (Flavil, Switzerland). Then 50 ml each of water and ethyl acetate were added to the residue. After shaking for a few minutes, the layers were separated. Extraction of the aqueous solution was repeated twice with 50 ml of ethyl acetate. The combined organic extract was dried over anhydrous sodium sulfate, decanted, and evaporated to leave a light yellow solid. The residual material was purified by silica gel dry column chromatography (hexane–methylene chloride–methanol, 7:6:2) followed by recrystallization from cold methanol to give ultrapure leaf crystals (m.p. 147–151 C; overall yield: 90"₀). These dansylated antimycins were suitable for use as calibration standards. Their structures were ascertained by LC–MS. The micro-scale technique for the derivatization of tissue samples spiked with the antibiotics is described in the following section.

Procedure for cleanup and analysis of organic tissue samples

Each of the frozen (− 70 C) ground fish tissue samples (10–50 g) fortified with the antimycin mixtures at various levels and the ^{14}C-labeled analogues (10 nCi) was placed in an erlenmeyer flask containing 200 ml of ethyl acetate–methanol–acetic acid (3:4:1). The suspension was shaken in a Lab-Line 3535 shaker for 40 min at 25 C. The mixture was then centrifuged at 34,575 g for 5 min in an IEC 3401 centrifuge. After the complete withdrawal of the supernatant, the tissual matter was quantitatively transferred into a soxhlet thimble and extracted continuously with 200 ml of ether–methylene chloride (1:1) for 60 min and subsequently with 150 ml of

acetone–methanol (1:1) for 30 min. These tissue extracts, including the foresaid supernatant, were combined. Removal of the solvents at room temperature in the usual manner afforded an oily residue which was taken up in 100 ml of methylene chloride. The latter solution was extracted with three 50-ml portions of 0.5 M pH 5.5 phosphate buffer. The pooled aqueous solution was back-washed with 60 ml of benzene. Then the concentrate (*ca.* 3 ml) of the combined methylene chloride solution and the benzene washing was chromatographed onto an adsorption column prepared by packing in sequence 50 g of silica gel and 10 g of anhydrous sodium sulfate (as the top layer) in a glass tube (50 × 2.5 cm I.D.). The column was eluted sequentially with 200 ml each of hexane, benzene, methylene chloride–methanol (1:1) and methanol. The eluate was dripped into 20-ml test-tubes at a flow-rate of 2.5 ml/min. Most of the radioactivity was found in the methylene chloride–methanol (1:1) fractions, as determined by liquid scintillation counting with a Beckman LS-7500 liquid scintillation counter. The bulk of the pooled radioactive eluate was reduced to 2 ml, which was efficiently pipetted into a 15-ml test-tube with a PTFE-lined screw cap. The contents of the tube were brought to dryness under a stream of nitrogen. The residue was redissolved in 0.5 ml of acetone and 0.2 ml of 1 % Dns chloride in acetone and 0.1 ml of pyridine was added to it. The tube was sealed and the mixture was thoroughly agitated on a vortex mixer for 10 min. Acetone was driven off by purging with nitrogen. The remaining liquor was mixed with 3 ml of water and 5 ml of ether. After separation of the ether phase, the aqueous layer was extracted twice with 3 ml of methylene chloride. The combined organic solution was concentrated to *ca.* 0.5 ml and purified on a preparative TLC plate (silica gel, 20 × 20 × 0.05 cm) using hexane–ethyl acetate–acetic acid (2:2:0.2) as the developing solvent. The TLC band whose R_F value (0.53) corresponded to that of the Dns antimycin standards was scraped from the plate and extracted successively with 25 ml of ether–methanol (2:1) and 15 ml of methylene chloride–methanol (3:2). To prepare analytical samples ready for HPLC measurements in the reversed-phase system with a mobile phase of 63:37 acetonitrile–water at a flow-rate of 2 ml/min, the extract collected from TLC cleanup was evaporated to *ca.* 0.2 ml. This was diluted with methanol to exact volume (1 ml). Aliquot samples (10 μl) were then injected into the HPLC column.

RESULTS AND DISCUSSION

Earlier attempts to derivatize antimycins through mild alkylation with an intention to analyze the intact molecule by gas chromatography resulted in sample decomposition. In consideration of the chemical and thermal instability inherent with the structures of these dilactonic antibiotics, HPLC would, therefore, appear to be the most suitable method for maintaining structural integrity and permitting adequate differentiation of the components on account of its proven record of merits in this regard. Under optimal HPLC conditions as specified in the Experimental sections, all antimycin components including four major and six minor compounds were well resolved. The structure of each component was examined and identified by direct chemical ionization mass spectrometry of the isolated material from preparative HPLC and by LC–MS (chemical ionization). The two separate mass spectral analyses gave identical fragmentation patterns exhibiting pseudo molecular ions at $(M_1 + 1)$ m/e 480, $(M_2 + 1)$ m/e 494, $(M_3 + 1)$ m/e 508, $(M_4 + 1)$ m/e 522, $(M_5 + 1)$ m/e 536,

$(M_6 + 1)$ *m/e* 550, $(M_7 + 1)$ *m/e* 550, $(M_8 + 1)$ *m/e* 564, $(M_9 + 1)$ *m/e* 564, and $(M_{10} + 1)$ *m/e* 564, respectively, for the HPLC peaks from 1 to 10 in sequence (Fig. 2). The latter peaks corresponded unambiguously to the antimycin components A_6, A_5, A_4, A_3, A_2, A_1, A_{0b}, A_{0a}, A_{0d}, and A_{0c} (Fig. 2) on the basis of mass spectral information.
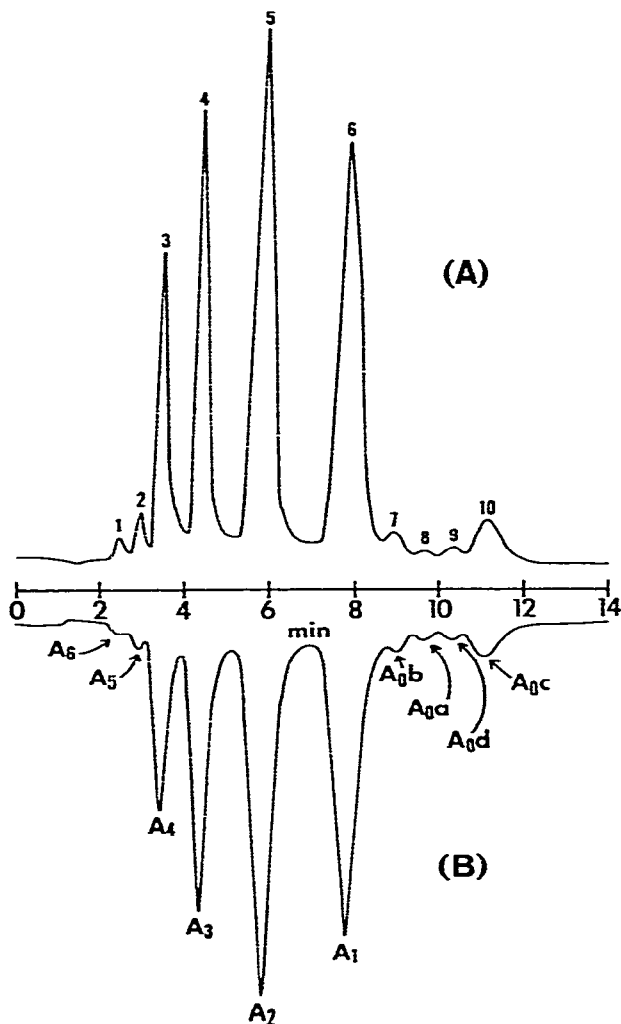
Fig. 2. HPLC separation of antimycin components from a recrystallized commercial sample: (A) UV detector at 254 nm; (B) electrochemical detector at 1.00 V (flow-rate 2 ml/min).

Results of the comparative evaluation of minimum detection limits of the ten isolated antimycins using electrochemical, UV, and fluorescence detectors interfaced independently with the HPLC instrument are shown in Table I. Since a phenolic function group contributes to the primary structural features of an antimycin, it is not surprising to learn from the data that the antibiotic compounds of fishery interest are electroactive. Their detectability in the electrochemical system is comparable with (or

even better than) that in the fluorescence detection system. Inspection of the voltammograms as depicted in Fig. 3 for the smallest and largest peaks disclosed that the optimum voltage on the electrochemical detector is 1.00 V on the glassy carbon electrode *versus* the silver chloride reference electrode. At this voltage, the normalized peaks showed the least changes in reproducibility. With a fluorescence detector, the sample was chromatographed on the same reversed-phase column as the one employed in the electrochemical and UV detection except that the mobile phase was modified to contain 0.05 M acetate buffer at pH 6 in 75% methanol (Fig. 4). Under these conditions, the sample fluoresced with five times greater intensity than in the lower pH buffers. A maximum fluorescence detector response was observed upon subjection of the sample to an excitation wavelength of 365 nm and an emission cut-off filter wavelength of 418 nm. Calibration curves for all three detectors studied were linear from *ca.* 1 mg/ml to the minimum detection limit values. Some typical examples are presented in Fig. 5 to illustrate the linear relationship between the detector (electrochemical and UV) response and the concentration of samples injected.

TABLE I

MINIMUM DETECTION LIMITS OF ANTIMYCINS DETERMINED BY HPLC INTERFACED WITH VARIOUS DETECTORS

A standard solution containing equal molar concentrations of antimycin components was used. Values were obtained for the injected amount at a signal-to-noise ratio of 2:1. Coefficient of variation for three replicate injections ranged from 3.8 to 7.1%.

Antimycin	Minimum detection limit (ng)		
	Electro-chemical	UV	Fluorescence
A_o	0.10	4.1	0.31
A_5	0.10	4.3	0.35
A_4	0.13	5.5	0.42
A_3	0.17	7.9	0.50
A_2	0.23	10.4	0.58
A_1	0.41	15.3	0.65
A_{0b}	0.67	18.6	0.77
A_{0a}	0.83	21.0	0.91
A_{0d}	0.90	25.1	1.19
A_{0c}	1.12	28.2	1.69

If the mobile phase consists of identical proportions of methanol and water instead of buffers (pH 3–7) or, synonymously, if it is devoid of any modifying organic and inorganic salts (Table II), the component peaks tend to be poorly resolved and display peak broadening accompanied by loss of peak symmetry. Evidently, small variations in mobile phase conditions have a large effect on peak resolution. Table II shows the retention (capacity factor k') and resolution data obtained with a number of chosen mobile phase conditions. There was distinct improvement in resolution by the addition of either acetate buffer or tetrabutylammonium phosphate to the straight methanol–water system, as the chromatographic separation was preferably controlled by the ionic suppression and ion-pair formation processes. The effect of pH on

Fig. 3. Voltammograms (normalized peak height *versus* applied voltage) for the smallest and largest antimycin component peaks as represented respectively by the lower and upper curves.
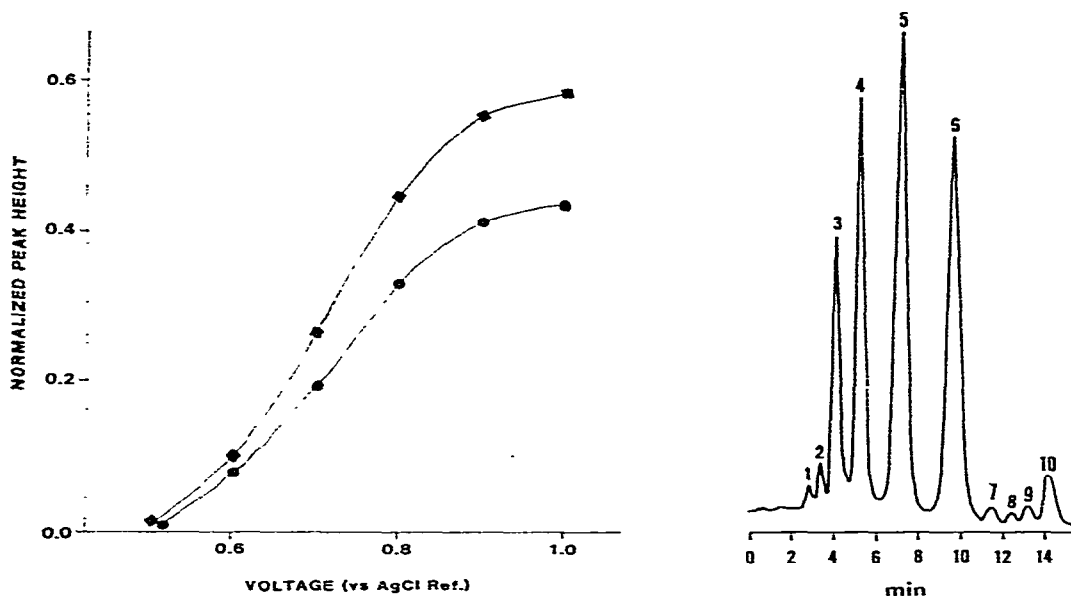
Fig. 4. HPLC separation of antimycin components from a recrystallized commercial sample: fluorescence detector at 365 nm (ex.) and 418 nm (em.) (flow-rate 1.5 ml min).

retention behavior and selectivity (α) (Table III) follows the general trend that both capacity factor (k') and selectivity (α) values decrease with the increase in pH. This is expected with reversed-phase, because the weakly acidic analyte solutes have higher affinity for the non-polar hydrocarbonaceous silica stationary phase at lower pH by virtue of ionic suppression. Such influence of pH on the retention and selectivity parameters seems to be remarkably effective in the methanol–acetate buffer system in comparison to that in the acetonitrile–phosphate buffer system. Table IV shows the results of retention measurements at various buffer concentrations. While the UV absorption detector response gradually inclines to a maximum value with the change in buffer concentration from 0.05 M to 0.3 M (not shown here), no significant change of the capacity factors was noted to occur as a result of increasing the buffer concentration at constant pH 5.

Attempted separations of the antimycin mixture on two normal-phase cyano- and amino-bonded silica columns by use of a wide variety of solvent pairs including added modifiers were unsuccessful. In all instances, only one ill-defined peak, presumably representing all unresolved components, appeared early on the liquid chromatogram with little retention.

A summary of the HPLC analysis of four different batches of the antimycin A complex as supplied from commercial sources is given in Table V to demonstrate the compositional diversity of the antibiotics concerned, which is apparently governed by the fermentation origin.

Our experience with the derivatization of the piscicidal antibiotics under study suggests that the rapid alkaline degradation of these compounds precludes forma-
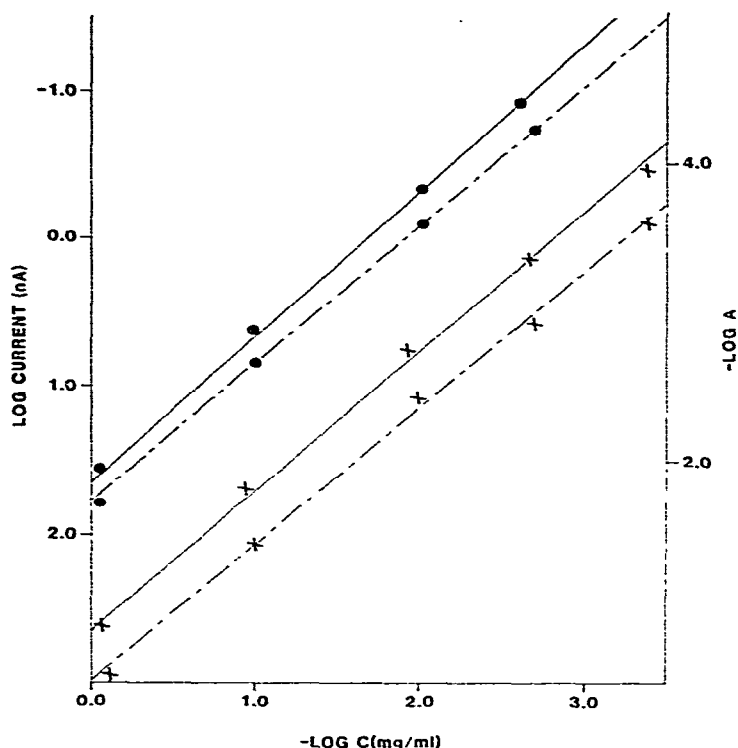
Fig. 5. Plot of detector response as a function of concentration for antimycin analytes. Electrochemical detection, lower two lines; UV detection, upper two lines: smallest component peak, solid line; largest component peak, broken line.

tion of any useful derivatives without undergoing rupture of the dilactone rings by the bases present and eventually giving the decomposition products. Following a carefully controlled procedure developed in this laboratory, we were able to carry out fluorescence labeling of antimycins with 1-dimethylaminonaphthalene-5-sulfonyl chloride (Dns chloride) in a non-aqueous solution using pyridine in place of the conventional alkaline salts. The crude derivatized material was purified by silica gel dry column chromatography and recrystallization. Confirmatory examination of the pure derivatives by LC–MS (chemical ionization) established their structural identities as the fluorigenic dansylated antimycins. In the individual mass spectra of the HPLC components, there were relatively intense signals assignable to the pseudo molecular ions as predicted on the basis of structural formulas. The detected pseudo molecular ions recorded herein are listed according to the order of increasing retention time on the liquid chromatogram: $(M_1 + 1)$ m/e 713, $(M_2 + 1)$ m/e 727, $(M_3 + 1)$ m/e 741, $(M_4 + 1)$ m/e 755, $(M_5 + 1)$ m/e 767, $(M_6 + 1)$ m/e 783, $(M_7 + 1)$ m/e 783, $(M_8 + 1)$ m/e 797, $(M_9 + 1)$ m/e 797, $(M_{10} + 1)$ m/e 797. The compositional distribution of parent antimycins in the mixture remained unaffected by the derivatization process. This is indicative of the non-discriminative reactivity among the homologous components toward the derivatizing agents. The chromatographic conditions described so far for the separation of the underivatized antimycins were found to be equally applicable to the separation of the dansylated derivatives. Excellent baseline resolu-

TABLE II

THE EFFECT OF MOBILE PHASE CONDITIONS ON CHROMATOGRAPHIC RETENTION (k')
AND RESOLUTION (R) CHARACTERISTICS (HPLC-UV)

Component peak	Methanol–water				Methanol–acetate buffer*				Methanol (TBA)–Water (TBA)**			
	63:37 (%)		65:35 (%)		63:37(%)		65:35(%)		63:37(%)		65:35(%)	
	k'	R	k'	R	k'	R	k'	R	k'	R	k'	R
1	0.36		0.29		0.44		0.38		0.42		0.20	
		0.00		0.00		1.20		1.11		1.60		1.18
2	0.36		0.29		0.68		0.58		0.67		0.31	
		0.00		0.00		1.14		1.00		1.52		1.29
3	0.36		0.29		0.96		0.77		1.00		0.56	
		0.53		0.49		1.16		1.13		1.57		1.23
4	0.64		0.48		1.40		1.15		1.27		1.00	
		0.95		0.78		1.53		1.36		1.98		1.75
5	1.21		1.15		2.16		1.69		2.60		1.71	
		0.89		0.83		1.33		1.26		1.93		1.57
6	1.86		1.74		3.04		2.35		3.93		2.56	
		0.00		0.00		0.75		0.72		1.06		1.01
7	1.86		1.74		3.55		2.68		4.54		2.95	
		0.00		0.00		0.69		0.66		1.00		0.97
8	1.86		1.74		3.82		2.96		4.99		3.29	
		0.00		0.00		0.68		0.65		1.03		0.99
9	1.86		1.74		4.23		3.32		5.58		3.42	
		0.00		0.00		0.81		0.77		1.13		1.07
10	1.86		1.74		4.56		3.59		5.93		3.87	

* Acetate buffer: 0.05 M, pH 7.
** TBA, tetrabutylammonium phosphate: 0.05 M, pH 7 (adjusted with NaH_2PO_4).

tion of all the components was easily achieved. In a mobile phase of acetonitrile–
water (70:30), the derivatized antimycins showed superior chromatographic charac-
teristics over the underivatized counterparts. The use of buffer salts in the mobile
phase was not essential and could be omitted in most cases. The benefits of derivatiza-
tion were fully realized, as the observed one-hundred-fold increase in sensitivity by
fluorescence detection was undoubtedly accredited to the incorporation of the dansyl
fluorotag into the antimycins. By this method of detecting the antibiotics as the
Dns derivatives, the lowest detectable amounts were estimated to be 5–10 pg. The
fluorescence detector was found to respond linearly with the injected amounts of
analytes (Dns antimycins) over the range from 10 μg to lower picograms (minimum
detection limit). The latter observation has a crucial bearing on the success of ac-
curate and reliable analyses of traces of antimycins, which often coexist with a host of
degradation products.

As frequently is the case encountered with the analysis of a complex sample for
analytes of trace concentration, a somewhat elaborate cleanup procedure is needed to
achieve the analytical goal with high precision. In the method developed for the
determination of the antimycin A complex in fish tissues, an adsorption chromato-
graphic purification step was interposed between the tissue extraction and HPLC
quantification procedures to facilitate sample quantitation with considerable reduc-
tion in interferences from the endogenous substances and consequently with im-

TABLE III

THE EFFECT OF pH ON CAPACITY FACTOR (k') AND SELECTIVITY (α)* (HPLC-UV)

Component peak	Methanol-acetate buffer**								Acetonitrile-phosphate buffer**							
	pH 3		pH 4		pH 5		pH 6		pH 3		pH 4		pH 5		pH 6	
	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'	(α)
1	1.55	(1.00)	1.36	(1.00)	0.91	(1.00)	0.64	(1.00)	2.53	(1.00)	2.49	(1.00)	2.42	(1.00)	2.38	(1.00)
2	2.27	(1.46)	1.94	(1.43)	1.27	(1.40)	0.87	(1.36)	3.57	(1.41)	3.50	(1.41)	3.36	(1.39)	3.25	(1.37)
3	3.00	(1.94)	2.61	(1.92)	1.73	(1.90)	1.09	(1.70)	4.58	(1.81)	4.47	(1.80)	4.35	(1.80)	4.25	(1.79)
4	4.36	(2.81)	3.67	(2.70)	2.45	(2.69)	1.55	(2.42)	6.85	(2.71)	6.61	(2.65)	6.29	(2.60)	6.13	(2.58)
5	6.55	(4.23)	5.73	(4.21)	3.73	(4.10)	2.36	(3.69)	9.79	(3.87)	9.58	(3.85)	9.20	(3.80)	8.88	(3.73)
6	9.36	(6.04)	8.09	(5.95)	5.05	(5.55)	3.36	(5.25)	13.3	(5.25)	12.9	(5.19)	12.5	(5.15)	12.0	(5.03)
7	11.1	(7.16)	9.66	(7.10)	6.30	(6.92)	4.08	(6.38)	15.9	(6.28)	15.5	(6.24)	15.0	(6.18)	14.7	(6.16)
8	12.7	(8.19)	10.2	(7.52)	6.67	(7.33)	4.44	(6.94)	16.8	(6.64)	16.4	(6.58)	15.8	(6.51)	15.3	(6.43)
9	14.4	(9.29)	11.4	(8.35)	7.45	(8.19)	4.82	(7.53)	18.7	(7.40)	18.1	(7.27)	17.5	(7.23)	17.0	(7.14)
10	16.6	(10.7)	12.3	(9.02)	8.18	(8.99)	5.09	(7.95)	20.0	(7.90)	19.5	(7.83)	18.9	(7.82)	18.5	(7.77)

* All selectivity values were determined based on the capacity factors of the least retained component peak 1.

** The buffer concentration was 0.2 M. The mobile phase was composed of 75:25 (%) methanol (or acetonitrile)-acetate (or phosphate) buffer.

TABLE IV

RETENTION MEASUREMENTS OF ANTIMYCIN COMPONENTS WITH VARIOUS ACETATE
BUFFER CONCENTRATIONS IN TWO MOBILE PHASE SYSTEMS (HPLC–UV)

Buffer concentration (M)	Capacity factor k′									
	Component peak									
	1	2	3	4	5	6	7	8	9	10
Methanol–acetate buffer (70:30)*										
0.05	1.08	1.48	2.10	2.79	4.07	5.71	6.64	7.15	7.86	8.93
0.1	1.07	1.47	1.97	2.71	3.93	5.57	6.58	6.99	7.82	8.76
0.2	1.06	1.43	1.93	2.60	3.89	5.50	6.55	6.96	7.74	8.48
0.3	1.08	1.41	1.87	2.58	3.87	5.47	6.43	6.94	7.58	8.31
0.5	1.03	1.40	1.88	2.53	3.83	5.46	6.33	6.90	7.45	8.00
Acetonitrile–acetate buffer (60:40)*										
0.05	2.25	3.22	4.00	5.04	6.84	9.02	10.5	11.4	12.2	13.4
0.1	2.19	3.15	3.93	5.00	6.82	9.00	10.3	11.4	12.0	13.1
0.2	2.20	2.91	3.82	4.90	6.50	8.56	9.94	11.1	11.7	13.0
0.3	2.16	2.90	3.79	5.00	6.46	8.33	9.87	10.6	11.4	12.7
0.5	2.17	2.88	3.72	4.97	6.37	8.30	9.83	10.4	11.1	12.5

* Ratio of the mobile phase solvent composition.

proved sensitivity. Table VI gives the results of recovery studies of fortified tissue
samples using the ^{14}C-labeled radiotracer technique. The data indicate that the ho-
mologous antibiotic compounds added to tissue samples at various concentrations
can be completely extracted from the tissues. They can be quantitatively adsorbed on

TABLE V

HPLC DETERMINATION OF THE COMPOSITION OF FOUR DIFFERENT BATCHES OF THE
ANTIMYCIN A COMPLEX

Component	Composition (%)			
	Batch			
	1	2	3	4
A_6	·0.29	1.37	1.61	TR*
A_5	0.92	8.10	3.05	4.29
A_4	10.84	10.18	12.19	11.30
A_3	19.23	34.51	19.07	22.46
A_2	32.30	15.78	26.25	25.08
A_1	29.57	30.14	30.96	33.71
A_{0b}	2.39	ND**	2.89	TR
A_{0a}	0.68	ND	0.38	TR
A_{0d}	1.12	ND	2.18	0.99
A_{0c}	2.66	ND	1.42	2.07

* TR = trace amount (less than 0.10 %)
** ND = none detected.

TABLE VI

RECOVERY OF AN ANTIMYCIN MIXTURE FROM TISSUE SAMPLES (HPLC–FLUORES-CENCE DETECTION)

Amount added ($\mu g/g$)	Stepwise sample clean-up			Overall		Amount found ($\mu g/g$)
	A*	B**	C***	Recovery (%)	C.V.[§] (%)	
	Recovery (%)	Recovery (%)	Recovery (%)			
0.45	95.3	89.4	90.2	76.7	7.3	0.35
1.10	96.4	90.2	91.2	79.3	6.1	0.87
2.35	96.0	90.4	90.9	78.6	7.3	1.85
6.05	95.8	91.3	91.4	79.9	7.2	4.83
10.25	96.4	89.8	92.5	79.7	5.7	8.17
16.00	95.9	90.5	92.0	79.8	5.4	12.77
20.40	96.6	93.4	91.8	82.8	4.9	16.89
40.30	95.5	92.9	92.3	81.9	4.8	33.01
80.15	94.8	94.1	91.8	81.9	4.2	65.64
100.65	96.1	94.0	93.5	84.5	4.1	85.05

* A = tissue extraction step.
** B = column adsorption chromatography step.
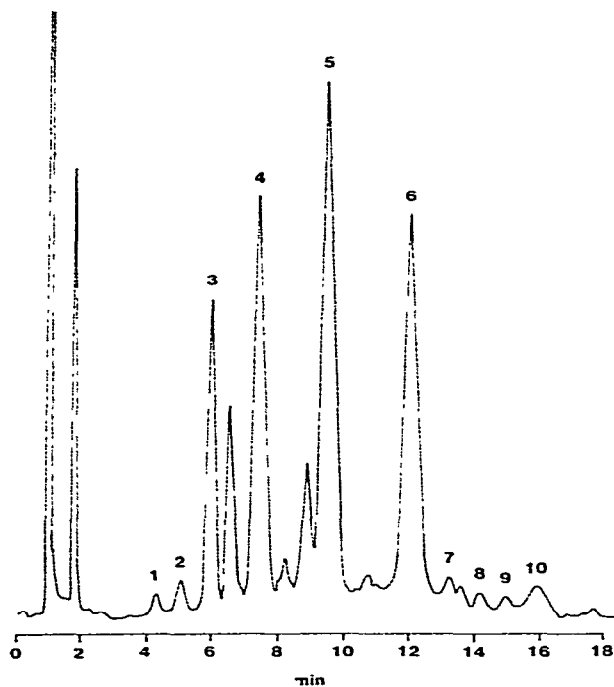*** C = derivatization step (dansylation).
[§] C.V. = coefficient of variation; analytical values are mean values of three determinations.

Fig. 6. HPLC separation of dansylated antimycin components in a purified tissue sample spiked with 1.10 $\mu g/g$ of the antimycin standards. The chromatogram was obtained with a fluorescence detector; mobile phase, acetonitrile–water (70:30); flow-rate, 2 ml/min.

the silica gel column using a non-polar hydrocarbon solvent (hexane or benzene) as the eluent, and quantitatively eluted from the column with methylene chloride–methanol (1:1). Initial attempted use of more polar solvents gave unsatisfactory results, since these solvents expedited column elution at the expense of eluting impurities which interfered with subsequent HPLC measurements. The overall recoveries including the Dns derivatization step are as shown within the acceptable range (77–85 %). In the low level antimycin calculations, it was necessary to make a correction for the amount of [^{14}C]antimycin tracer used as the internal standard in the individual tissue sample analysis. An HPLC chromatogram of a purified tissue sample spiked with antimycin standards is provided in Fig. 6 to show efficient separation of the dansylated antimycin components. The chromatogram exhibits the characteristic homologous components similar to that of the underivatized antimycin homologues.

CONCLUSIONS

The findings in this study demonstrate the potential utility of HPLC for the effective resolution and sensitive detection of a mixture of compositional complexity that has hitherto remained unexplored by this chromatographic technique. The method developed here facilitates for the first time the isolation of each individual antibiotic in high purity and provides a non-destructive means of separation and quantitative measurement. Inclusion of a sample purification procedure to the HPLC quantification technique presents a viable method for the practical analysis of the antimycin antibiotics in organic tissues.

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