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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF SUBCOMPONENTS OF ANTIMYCIN A

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SUMMARY

Using a reversed-phase high-performance liquid chromatographic (HPLC) technique, a mixture of antimycins A was separated into eight hitherto unreported subcomponents, A_{1a}, A_{1b}, A_{2a}, A_{2b}, A_{3a}, A_{3b}, A_{4a}, and A_{4b}. Although a base-line resolution of the known four major antimycins A_1, A_2, A_3, and A_4 was readily achieved with mobile phases containing acetate buffers, the separation of the new antibiotic subcomponents was highly sensitive to variation in mobile phase conditions. The type and composition of organic modifiers, the nature of buffer salts, and the concentration of added electrolytes had profound effects on capacity factors, separation factors, and peak resolution values. Of the numerous chromatographic systems examined, a mobile phase consisting of methanol–water (70:30) and 0.005 *M* tetrabutylammonium phosphate at pH 3.0 yielded the most satisfactory results for the separation of the subcomponents. Reversed-phase gradient HPLC separation of the dansylated or methylated antibiotic compounds produced superior chromatographic characteristics and the presence of added electrolytes was not a critical factor for achieving separation. Differences in the chromatographic outcome between homologous and structural isomers were interpretated based on a differential solvophobic interaction rationale. Preparative reversed-phase HPLC under optimal conditions enabled isolation of pure samples of the methylated antimycin subcomponents for use in structural studies.

INTRODUCTION

A few years ago, we reported the high-performance liquid chromatographic (HPLC) resolution of a mixture of dilactonic antibiotics (antimycin A complex)[1]. Since then, because of the reported ready decomposition of these substances in alkaline media[2–6], we have been interested in pursuing studies on structural modifications and on the chemical reactivity of individual antimycin A_1, A_2, A_3, and A_4 components. After a number of unsuccessful attempts at conducting product analyses by spectroscopic methods, it was necessary to examine the reactant antimycin A by nuclear magnetic resonance (NMR) spectroscopy. Results of a subsequent intensive

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NMR study[7] revealed that each of the known major components (A_1, A_2, A_3, and A_4) of the antimycin A complex appeared to comprise two subcomponents isomeric at the 8-acyloxy side chain (Fig. 1). Prompted by the spectroscopic findings, HPLC experiments were performed on the individual antimycins A_1, A_2, A_3, and A_4 with a high-efficiency octadecylsilica (ODS) column (80 000 plates/m). A preliminary study showed that each of the four major components of antimycin A was, in fact, a

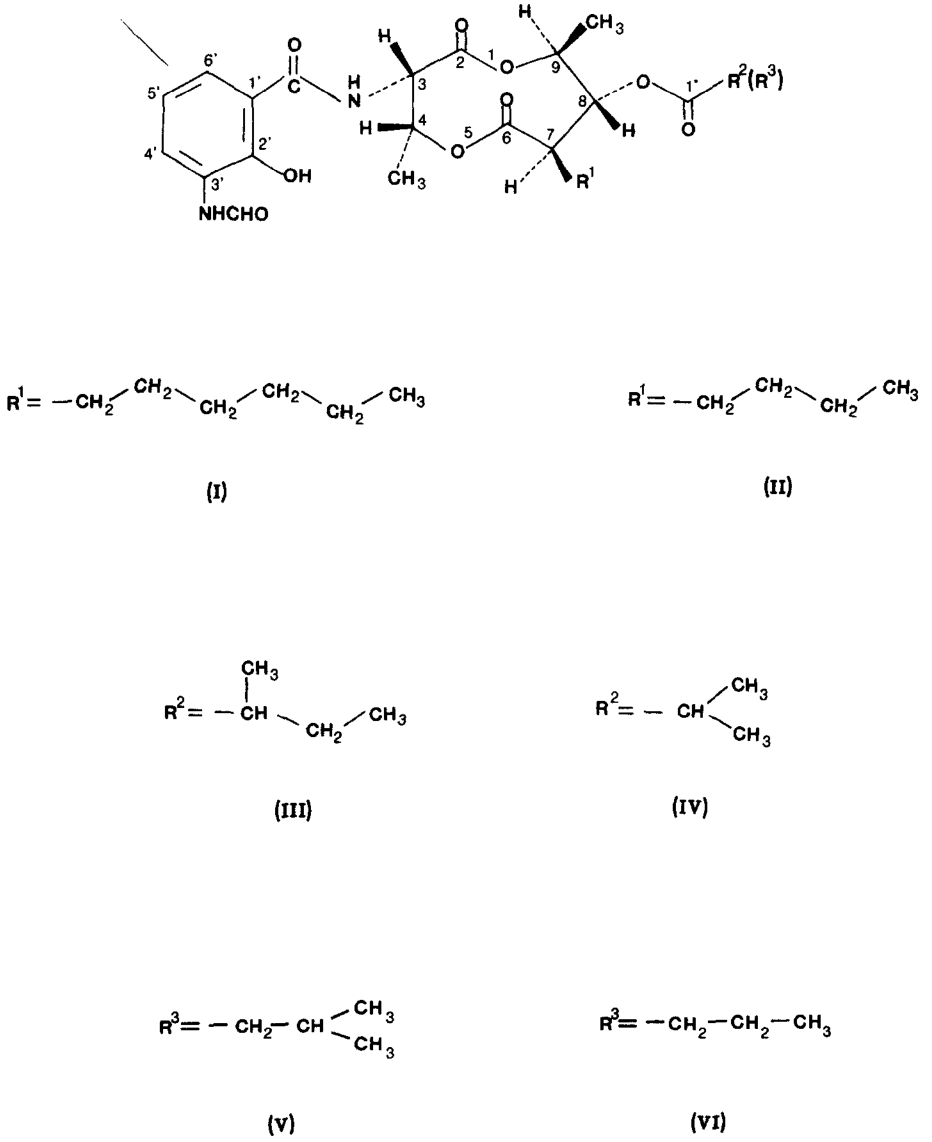

Fig. 1. Structures of subcomponents of antimycin A complex. A_{1a}, R^1 = I, R^2 = III; A_{1b}, R^1 = I, R^3 = V; A_{2a}, R^1 = I, R^2 = IV; A_{2b}, R^1 = I, R^3 = VI; A_{3a}, R^1 = II, R^2 = III; A_{3b}, R^1 = II, R^3 = V; A_{4a}, R^1 = II, R^2 = IV; A_{4b}, R^1 = II, R^3 = VI.

two-component mixture as disclosed by the splitting of individual HPLC peaks. Further elaborate experiments on optimization of the HPLC conditions led to the adequate separation of the antimycin subcomponents. The results are presented in this paper.

EXPERIMENTAL

Chemicals and reagents

Samples of antimycin A complex and individual antimycins A_1, A_2, A_3, and A_4 were purchased from Sigma (St. Louis, MO, U.S.A.) or prepared from the crude antibiotics (Aquabiotics Corp., North Brook, IL, U.S.A.) at the National Fishery Research Center (La Crosse, WI, U.S.A.) according to the published procedure[1]. HPLC-grade sodium acetate, 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride), pyridine, and "Diazald" (diazomethane precursor) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Diazomethane was generated from Diazald following instructions supplied with the chemical by the manufacturer. Antimycins were dansylated as described previously[1]. Methylated derivatives of antimycins were prepared by treating a solution of antimycins in diethyl ether with an ethereal solution of diazomethane (25% in excess of the theoretical amount) followed by stirring the resultant solution at room temperature overnight. Pure methylated antibiotics were obtained from the crude materials by thin-layer chromatography (TLC). This TLC purification experiment was performed on a preparative silica gel plate (20 × 20 cm taper plate, Analtech, Newark, DE, U.S.A.) using a mixed solvent [benzene–chloroform–methanol–acetic acid (15:5:1:1)] for development of the chromatogram. A *ca.* 50-mg sample was streaked onto the TLC plate (impregnated with a fluorescence indicator by Analtech) and, after development, visualized with a mineralight lamp (Model UVSL-58 multiband UV-254/366 nm, Ultraviolet Products, San Gabriel, CA, U.S.A.). The dark purple band as seen under the short-wavelength UV light (or the fluorescence band as observed under the long-wavelength UV light) was scraped off, extracted three times with 10 ml methanol–methylene chloride (1:1), and evaporated at a reduced pressure to give pure methylated antimycins. Tetrabutylammonium salts were products of Eastman Kodak (Rochester, NY, U.S.A.) and were used as received. All HPLC solvents and other buffer salts were of chromatography-quality and purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.).

High-performance liquid chromatography

A Varian Model LC-5020 liquid chromatograph was used in all HPLC experiments. The chromatograph was equipped with a multiple-wavelength UV–VIS detector (Varian Model 100), a Varian Model 9176 strip chart recorder, and a high-efficiency reversed-phase column [Ultrasphere ODS column, 5 μm, 25 cm × 4.6 mm I.D. (Altex, Berkeley, CA, U.S.A.)]. The analytical column was connected to a Valco (Houston, TX, U.S.A.) CV-6-UHPa-N60 injector. In a typical analysis, samples (20–50 ppm) were injected via a Valco 10-μl loop and a mobile phase eluent was pumped through the column at a flow-rate of 2 ml/min. For analyses of underivatized antimycin A complex, it was necessary in all cases to use mobile phases that consisted of methanol and water containing different concentrations of various buffer salts (or counter ion electrolytes). On the other hand, HPLC separations of both methylated and dansylated antimycins were carried out with mobile phases of methanol–water in various proportions. No electrolyte was required in the mobile phase for analyses of either type of derivatives.

The detector output signals were fed into the strip chart recorder and the chromatographic data [peak areas, retention times, isomeric ratios, retention (k'), selectivity (α), and resolution (R)] were automatically processed by a Varian Model 1427 integrator.

Subcomponents of methylated antimycin A complex were simultaneously resolved by gradient elution with an initial mobile phase composition of methanol–water (60:40) to a final composition of 70:30 in the same solvent system over a period of 3 h at a flow-rate of 1.5 ml/min. The analytical procedure was applied to the isolation of individual subcomponents by preparative HPLC. An Altex reversed-phase column (Ultrasphere ODS, 5 μm, 25 cm × 10 mm I.D.) was used for this purpose. In a routine preparative HPLC experiment, a 50–100 mg sample was chromatographed and the column effluents, while monitored with a Varian refractive index detector, were collected in 0.5-ml fractions using a Buchler linear automatic fraction collector. Fractions containing pure subcomponents as revealed by analytical HPLC–UV detection were combined and evaporated under reduced pressure. The aqueous residue was extracted three times with methylene chloride. The combined organic extract was dried over anhydrous sodium sulfate and evaporated to yield pure subcomponents. The structures of these materials were determined by ^{1}H and ^{13}C NMR[7] and confirmed by fast atom bombardment mass spectrometry.

In an alternative method for the isolation of subcomponents, HPLC experiments were performed individually with samples of methylated antimycin components A_1, A_2, A_3, and A_4 obtained by our published preparative HPLC method[1]. Thus, under isocratic conditions with a mobile phase of methanol–water (70:30), pure subcomponents of antimycins A_{1a}, A_{1b}, A_{2a}, and A_{2b} were obtained in milligram quantities. Likewise, preparative HPLC under isocratic conditions using a mobile phase consisting of methanol–water (60:40) afforded pure subcomponents of antimycins A_{3a}, A_{3b}, A_{4a}, and A_{4b}.

RESULTS AND DISCUSSION

As demonstrated in our earlier publication[1], a homologous mixture of antimycin A complex can be efficiently resolved into its four major component homologues A_1, A_2, A_3, and A_4 by reversed-phase HPLC with a chemically bonded hydrocarbonaceous silica column. The only structural differences among the component antibiotics are in the alkyl substituents at the 7 and 8 side-chains that differ by at least one methylene (CH_2) unit (Fig. 1). Considering the homologous structures of the antimycin A complex in the context of the basic principles of reversed-phase HPLC on an alkyl-bonded silica, optimization of reversed-phase HPLC conditions for achieving an excellent separation of the homologous mixture should be straightforward. This has been experimentally corroborated[1]. However, the situation in reversed-phase HPLC of subcomponents of this antibiotic complex would be more complicated because the structural isomers of these subcomponents are no longer homologously interrelated. As depicted in Fig. 1, the 8-acyloxy ester moieties of the eight subcomponents are described as follows: A_{1a} *vs.* A_{1b}, isopentanoyl *vs.* isovaleryl; A_{2a} *vs.* A_{2b}, isobutyryl *vs.* *n*-butyryl; A_{3a} *vs.* A_{3b}, isopentanoyl *vs.* isovaleryl; A_{4a} *vs.* A_{4b}, isobutyryl *vs.* *n*-butyryl. The differential solvophobic interactions of the a–b subcomponents with a given straight-chain alkyl bonded silica stationary phase during separation processes would be more specific and more susceptible to changes in mobile phase conditions than those would be expected in the separation of homologous components A_1, A_2, A_3, and A_4. Ideally, HPLC on a branched-chain alkylsilica would facilitate resolution of the subcomponents because of increased degrees of specific hydrophobic interactions.

Fig. 2 presents two representative chromatograms showing separations of subcomponents of antimycin A complex with mobile phases of methanol–water (70:30) containing (Fig. 2A) 0.2 *M* sodium acetate at pH 5 and (Fig. 2B) 0.005 *M* tetrabutylammonium phosphate at pH 3.0. An eight-subcomponent constitution of antimycin A complex is immediately discernible from each of the chromatograms, even though, in both instances, only partially resolved peaks are seen for the earlier eluting antimycins that have shorter alkyl side-chains at the 7- and 8-alkyl substituents. In spite of the required longer retention times, incorporation of tetrabutylammonium phosphate into the mobile phase appeared to be more advantageous than that of sodium acetate for the separation of a–b subcomponents. In the light of the straight-chain nature of the alkyl bonded phase used throughout this study, the later-eluting components (the more strongly retained components) (Fig. 2) corresponded to the minor components (b-isomers) in which the α-carbons contain no methyl substituent (Fig. 1). We presume that hydrophobic interactions between the major components (a-isomers) and the stationary phase were weaker and less efficient than for the minor counterparts as a result of branching of the hydrocarbon chain at the α-carbons (a-isomer, Fig. 1).

Fig. 3 shows the effect of mobile phase composition on the separation of the eight antimycin constituents and their dansylated and methylated derivatives. Using a 0.2 *M* acetate buffer at pH 5, the a–b components were not at all resolved with a mobile phase of methanol–water (80:20), though the four antimycin homologues A_1, A_2, A_3, and A_4 were well resolved under these conditions (Fig. 3A). Similarly, with a mobile phase of methanol–water (80:20), HPLC of derivatized antimycin A complex yielded no separation of the subcomponents of the dansylated (Fig. 3B) and methylated (Fig. 3C) antimycin subcomponents, notwithstanding excellent resolution of the homologous components. We found that the presence of buffer salts and other counter ion electrolytes in mobile phases was not necessary for separating either type of subconstituent derivatives. Variations in separation factors (α) of a homologous pair with mobile phase solvent compositions were much greater than those of an a–b subcomponent pair as predicted. Solvophobic interactions between homologous solutes and the reversed-phase bonded stationary phase would be much stronger than those derived from structural isomers of equal carbon contents. As seen in Fig. 3, resolution of the latter isomers (a–b pairs), as well as of homologous isomers, increases as the percent methanol in the mobile phase decreases. This compositional effect of the mobile phase seemed to follow conventional trends normally observed in reversed-phase HPLC of isomeric compounds[8]. A maximum methanol content of 70% was found for obtaining a quantifiable simultaneous resolution of all eight subcomponents of both parent and methylated antimycins (Fig. 3A and C). For the separation of dansylated subcomponents, a mobile phase of methanol–water (75:25) was required to be analytically useful in practical application.

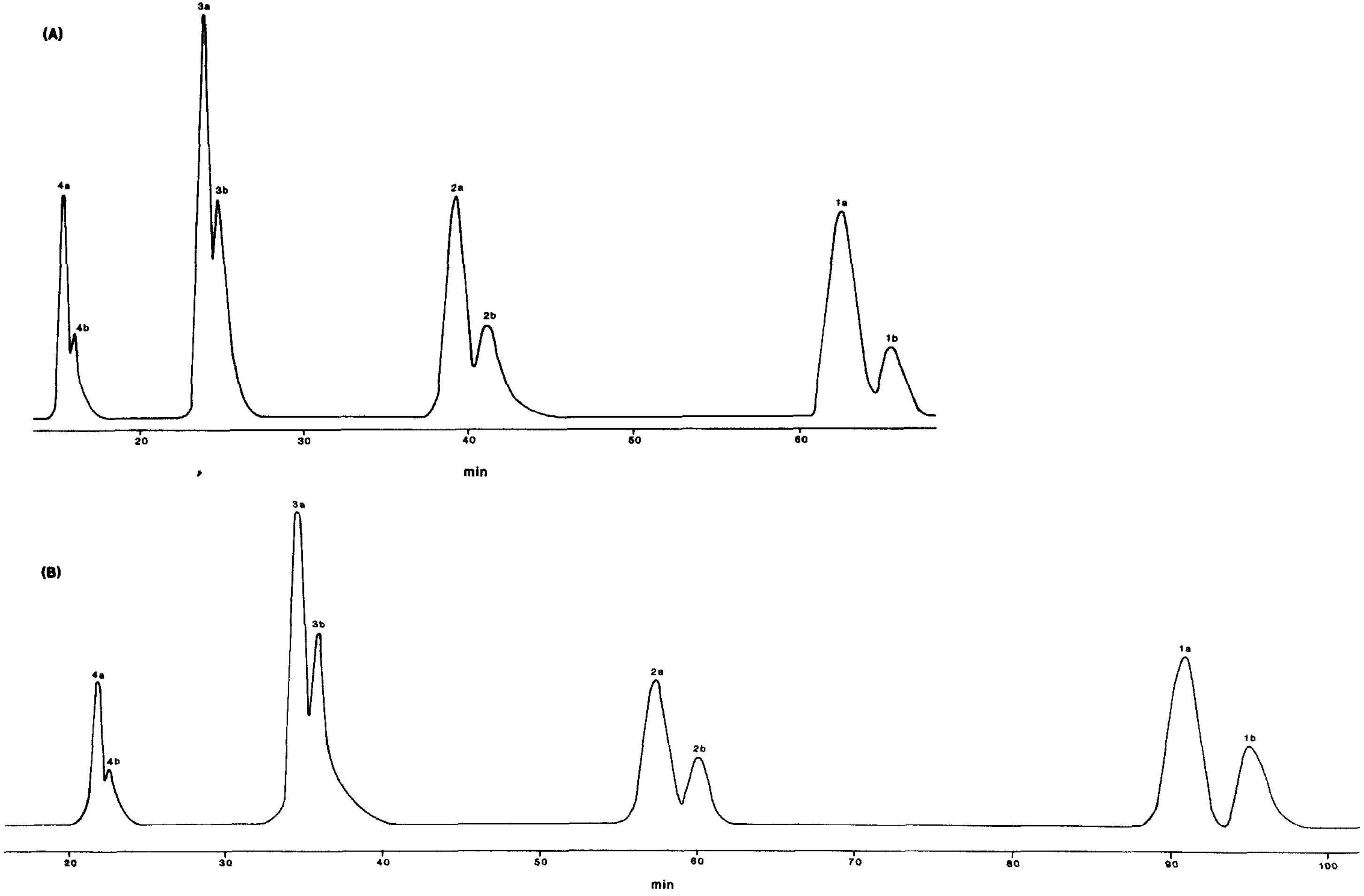

Fig. 2. Isocratic HPLC separation of antimycin A subcomponents. Peak designations same as in Fig. 1. (A) Mobile phase, methanol–water (70:30) containing 0.2 *M* sodium acetate at pH 5.0. (B) Mobile phase, methanol–water (70:30) containing 0.005 *M* tetrabutylammonium phosphate (TBAP) at pH 3.0.

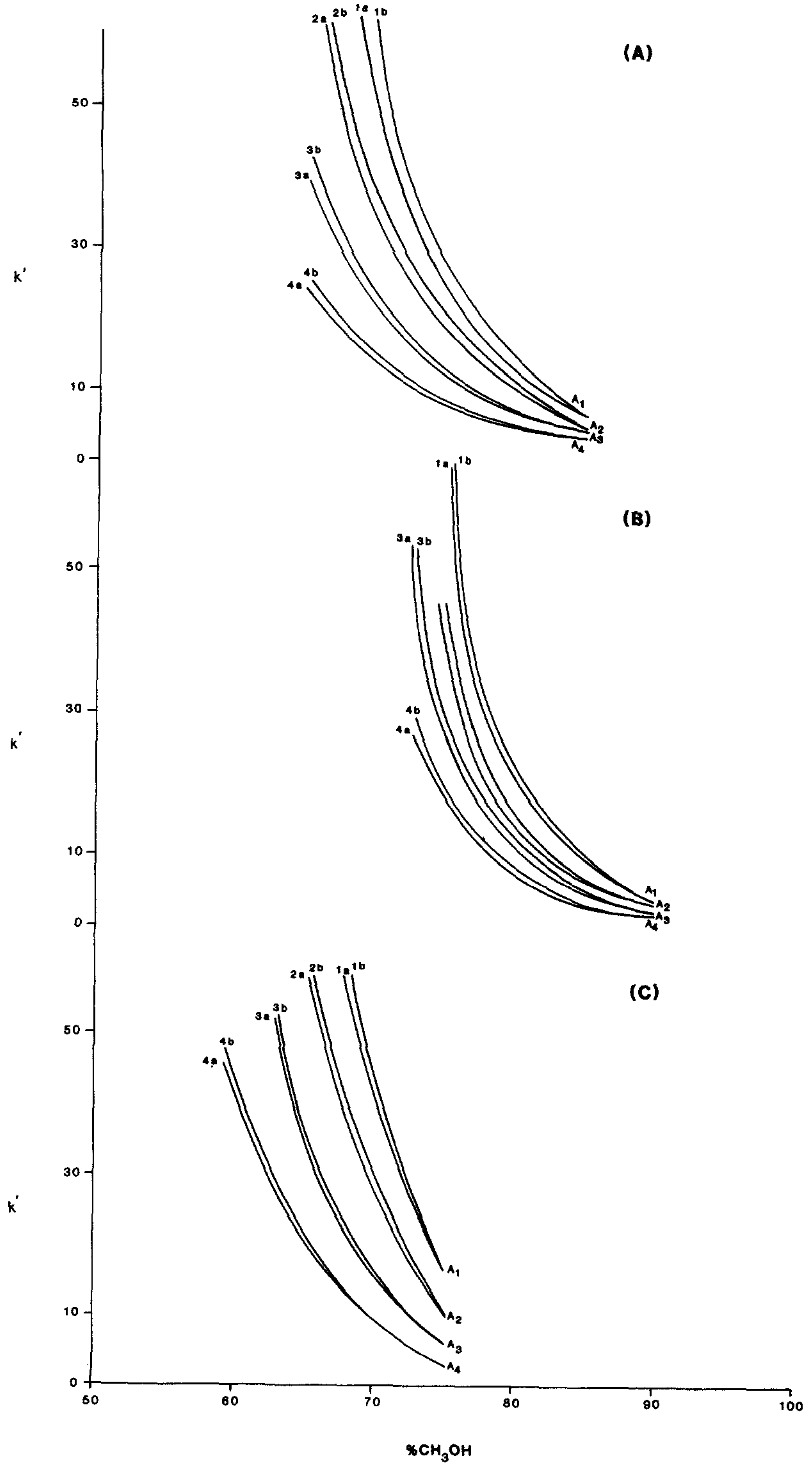

Fig. 3. Mobile phase (methanol–water) effects on the separation of subcomponents of (A) antimycin A, (B) dansylated derivatives and (C) methylated derivatives. Mobile phase in (A) contained 0.2 *M* sodium acetate at pH 5.

TABLE I

MOBILE PHASE EFFECTS ON CHROMATOGRAPHIC CHARACTERISTICS OF ANTIMYCIN A SUBCOMPONENTS (flow-rate = 2 ml/min)

*Mobile phase pH**	A_{1a}		A_{1b}	A_{2a}		A_{2b}	A_{3a}		A_{3b}	A_{4a}		A_{4b}
	k'	(α)	k'	k'	(α)	k'	k'	(α)	k'	k'	(α)	k'
(I) Methanol–water (70:30)												
(A) 6.6	19.4	(1.06)	20.5	12.3	(1.04)	12.8	7.24	(1.00)	7.24	4.52	(1.00)	4.52
(B) 5.4	42.4	(1.06)	44.8	26.9	(1.04)	28.1	16.1	(1.04)	16.8	10.1	(1.00)	10.1
(C) 3.0	71.6	(1.05)	75.2	41.9	(1.06)	44.4	24.9	(1.06)	26.4	15.4	(1.05)	16.1
(D) 3.0	62.4	(1.04)	64.6	39.0	(1.03)	40.2	15.8	(1.04)	16.4	14.5	(1.02)	14.8
(E) 5.0	49.0	(1.05)	51.4	30.4	(1.05)	31.8	18.1	(1.04)	18.8	11.2	(1.05)	11.8
(F) 5.0	42.4	(1.03)	43.7	25.9	(1.03)	26.7	15.2	(1.02)	15.5	9.32	(1.00)	9.32
(II) Acetonitrile–water (60:40)												
(G) 3.0	28.7	(1.00)	28.7	18.8	(1.00)	18.8	12.0	(1.00)	12.0	7.80	(1.00)	7.80
(H) 6.6	8.28	(1.00)	8.28	5.64	(1.00)	5.64	3.72	(1.00)	3.72	2.44	(1.00)	2.44
(I) 4.5	20.6	(1.00)	20.6	13.5	(1.00)	13.5	9.24	(1.00)	9.24	6.20	(1.00)	6.20
(J) 4.5**	44.8	(1.00)	44.8	29.2	(1.00)	29.2	18.4	(1.00)	18.4	11.9	(1.00)	11.9

* Mobile phases contained 0.005 *M* tetrabutylammonium phosphate (A, B, C, G, H), 0.005 *M* tetrabutylammonium hydrogensulfate (D), 0.2 *M* sodium acetate (E, I, J), and 0.2 *M* ammonium acetate (F).

** Acetonitrile–water (55:45).

Results in Table I (all flow-rates were 2 ml/min) indicate that the chromatographic behavior of both homologous and structural isomers of antimycin A complex was heavily dependent on the mobile phase constituents (the nature of the organic modifiers, the type of added electrolytes, pH, and the solvent composition). In particular, the nature of the organic modifier in the mobile phase had a dramatic influence on the separation of antimycin subcomponents (the structural isomers). When methanol was used in the mobile phase [as in mobile phases A, B, C, D, E, and F (Table I)], most of the subcomponent a–b pairs [(A_{1a}–A_{1b}), (A_{2a}–A_{2b}), (A_{3a}–A_{3b}), and (A_{4a}–A_{4b})] were resolved with α values in the range 1.00–1.08. However, no separation (α = 1.00) of any of these subcomponent pairs was observed in all cases where acetonitrile was used (as in mobile phases G, H, I, and J). It should be pointed out that, with all mobile phases listed in Table I, undoubtedly the homologous components A_1, A_2, A_3, and A_4 were all very well resolved. The slightly stronger solvent strength of acetonitrile (3.1) relative to methanol (3.0)[9,10] might be attributable to the apparent reduction in the hydrophobic interactions that are critical for the specific differentiation of the subcomponent isomers.

HPLC experiments with mobile phases containing 0.005 *M* tetrabutylammonium phosphate (TBAP) as in A, B, and C (Table I) showed remarkable pH effects on the retention and separation characteristics of antimycin subcomponents. The pK_a value of antimycin A in water has been estimated to be 5.1 (ref. 5). Of the three mobile phases, C (pH 3) provided the most satisfactory results yielding α values of 1.04–1.05 for the four a–b pairs. Experiments A and B, both conducted at higher pH values, afforded incomplete resolution of the structural isomers with α values of 1.00–1.06 and with some of the early-eluting pairs remained unresolved. The higher k' values obtained at lower pH values may presumably reflect the less ionic character of the 2′-OH group of the aromatic side-chain of antimycin A. Comparisons of the data (Table I) between C and D and between E and F indicate that the k' and α values of antimycin subcomponents were somewhat affected by changes in the nature of added ion species, including cations and anions of closely related electrolytes. TBAP and sodium acetate seemed to be somewhat better mobile phase additives than the respective tetrabutylammonium hydrogensulfate and ammonium acetate for effecting separation of antimycin subcomponents.

The chromatographic data in Table II were obtained under the same mobile phase conditions as in Table I except for the flow-rate which were at 1 ml/min in all cases. There was no appreciable improvement in selectivity values for the separation of subcomponents as the flow-rates of mobile phases were reduced from 2 ml/min (Table I) to 1 ml/min (Table II). In some cases at lower flow-rates, the retention times of the analytes became too long to be of analytical significance.

Table III shows the concentration effect of sodium acetate on the separation of subcomponents. It is clearly demonstrated that the presence of sodium acetate at higher concentrations (0.2–0.25 *M*) in the mobile phases consisting of methanol–water (70:30) tended to favor the separation of the isomers of interest. In these cases, the peak resolution (*R*) (0.51–1.01) and α (1.04–1.05) values were greater than those found at lower concentrations (0.05–0.10 *M*) (R = 0.00, α = 1.00). At an intermediate concentration 0.15 *M*, the respective *R* and α values ranged from 0.00 to 0.67 and from 1.00 to 1.05. The only inseparable peak (A_4) at this concentration was for the A_{4a}–A_{4b} pair that had a k' value of 12.6.

TABLE II

MOBILE PHASE EFFECTS ON CHROMATOGRAPHIC CHARACTERISTICS OF ANTIMYCIN A SUBCOMPONENTS (flow-rate = 1 ml/min)

*Mobile phase pH**	A_{1a}		A_{1b}	A_{2a}		A_{2b}	A_{3a}		A_{3b}	A_{4a}		A_{4b}
	k'	*(α)*	*k'*	*k'*	*(α)*	*k'*	*k'*	*(α)*	*k'*	*k'*	*(α)*	*k'*
(I) Methanol–water (70:30)												
(A) 6.6	20.0	(1.06)	21.2	12.5	(1.05)	13.1	7.28	(1.04)	7.57	4.54	(1.00)	4.54
(B) 5.4	44.0	(1.06)	46.6	28.1	(1.05)	29.5	16.9	(1.04)	17.6	10.6	(1.00)	10.6
(C) 3.0	72.9	(1.06)	77.3	43.0	(1.06)	45.6	25.8	(1.06)	27.3	15.8	(1.05)	16.6
(D) 3.0	63.5	(1.04)	66.0	40.1	(1.03)	41.3	16.5	(1.04)	17.2	14.9	(1.02)	15.2
(E) 5.0	50.4	(1.05)	52.9	31.2	(1.05)	33.4	18.9	(1.04)	19.7	11.6	(1.05)	12.2
(F) 5.0	43.9	(1.04)	45.7	26.6	(1.03)	27.4	16.1	(1.02)	16.4	9.35	(1.00)	9.35
(II) Acetonitrile–water (60:40)												
(G) 3.0	28.9	(1.00)	28.9	19.0	(1.00)	19.0	12.2	(1.00)	12.2	7.84	(1.00)	7.84
(H) 6.6	8.40	(1.00)	8.40	5.69	(1.00)	5.69	3.75	(1.00)	3.75	2.46	(1.00)	2.46
(I) 4.5	20.8	(1.00)	20.8	13.7	(1.00)	13.7	9.40	(1.00)	9.40	6.25	(1.00)	6.25
(J) 4.5	45.1	(1.00)	45.1	29.4	(1.00)	29.4	18.5	(1.00)	18.5	12.0	(1.00)	12.0

* For mobile phase designations, see footnote to Table I.

Table IV shows the concentration effect of TBAP on the separation of antimycin subcomponents. The observed decrease in k' values with increasing concentrations of TBAP may be interpreted as a competition for adsorption sites by the neutral solutes and the mobile phase additives. In the concentration range studied (0.005–0.10 *M*), resolution of a–b pairs was achieved with various degrees of separations (R = 0.30–1.25, α = 1.02–1.06) despite the obvious failure in resolving the A_{4a}–A_{4b} pair (R = 0.00, α = 1.00) with 0.10 *M* TBAP. In general, the concentration effect on the R and α values seemed to be small, even though the R values declined gradually as the TBAP concentration increased. Evidently, the α values of the early-eluting pairs (A_{3a}–A_{3b} and A_{4a}–A_{4b}) were adversely affected by the higher TBAP concentrations of 0.05–0.10 *M*. In addition, some stationary phase effect was observed when R values obtained with two octadecylsilica (ODS) columns (Altex Ultrasphere ODS and Ultrasphere ODS-IP) were compared. Significantly higher R values were attained with the latter column (experiments B and C, Table IV) than with the former (experiment A, Table IV).

Since there are several polar groups in an antimycin structure (Fig. 1), the observed mobile phase effects, as well as stationary phase effects, on the separation of antimycin subcomponents are indicative of strong solute-silanol interactions in the HPLC systems.

As noted earlier from Fig. 3, substitution of 2'-phenolic proton with methyl and

TABLE III

EFFECT OF SODIUM ACETATE CONCENTRATIONS (pH 5) ON THE SEPARATION OF ANTIMYCIN A SUBCOMPONENTS

Mobile phase, methanol–water (70:30).

Subcomponent		*Acetate concentration (M)*				
		0.05	*0.10*	*0.15*	*0.20*	*0.25*
A_{1a}	k'	66.2	60.8	55.5	49.0	46.0
	(α)	(1.00)	(1.00)	(1.05)	(1.05)	(1.05)
	R	0.00	0.00	0.67	1.00	1.01
A_{1b}	k'	66.2	60.8	58.0	51.4	48.1
A_{2a}	k'	41.0	37.7	34.9	30.4	28.8
	(α)	(1.00)	(1.00)	(1.04)	(1.05)	(1.05)
	R	0.00	0.00	0.56	0.65	0.67
A_{2b}	k'	41.0	37.7	36.4	31.8	30.3
A_{3a}	k'	24.3	22.3	20.6	18.1	17.2
	(α)	(1.00)	(1.00)	(1.04)	(1.04)	(1.04)
	R	0.00	0.00	0.28	0.51	0.54
A_{3b}	k'	24.3	22.3	21.4	18.8	17.9
A_{4a}	k'	14.1	13.1	12.6	11.2	10.6
	(α)	(1.00)	(1.00)	(1.00)	(1.05)	(1.04)
	R	0.00	0.00	0.00	0.53	0.55
A_{4b}	k'	14.1	13.1	12.6	11.8	11.0

TABLE IV

EFFECT OF TBAP CONCENTRATIONS ON THE SEPARATION OF ANTIMYCIN A SUBCOMPONENTS

Mobile phases consisted of methanol–water (70:30) at pH values of 5.0 (A, B), and 5.2 (C). Stationary phases used in A, and B and C were Altex Ultrasphere-ODS and Ultrasphere-ODS-IP, respectively.

Subcomponent		*Mobile phase TBAP concentration (M)*						
		0.005	*0.01*		*0.05*		*0.10*	
		A	*A*	*B*	*A*	*C*	*A*	*C*
A_{1a}	k'	65.4	53.8	64.6	44.5	38.0	35.4	28.9
	(α)	(1.04)	(1.05)	(1.06)	(1.05)	(1.05)	(1.05)	(1.05)
	R	1.02	0.94	1.25	0.92	1.02	0.88	0.97
A_{1b}	k'	68.2	56.3	68.6	46.8	39.9	37.2	30.3
A_{2a}	k'	40.2	34.0	39.2	27.9	24.2	22.4	18.8
	(α)	(1.05)	(1.02)	(1.06)	(1.05)	(1.05)	(1.05)	(1.04)
	R	0.96	0.93	1.05	0.93	0.95	0.78	0.88
A_{2b}	k'	42.2	34.6	41.5	29.1	25.3	23.4	19.6
A_{3a}	k'	23.6	19.3	22.4	16.4	14.4	13.6	11.6
	(α)	(1.04)	(1.04)	(1.04)	(1.03)	(1.04)	(1.02)	(1.03)
	R	0.59	0.53	0.70	0.51	0.70	0.30	0.40
A_{3b}	k'	24.5	20.0	23.2	16.9	15.0	13.9	12.0
A_{4a}	k'	14.3	11.6	13.6	10.1	9.08	8.47	7.32
	(α)	(1.04)	(1.03)	(1.03)	(1.04)	(1.03)	(1.00)	(1.00)
	R	0.70	0.58	0.62	0.54	0.55	0.00	0.00
A_{4b}	k'	14.8	12.0	14.0	10.5	9.32	8.47	7.32

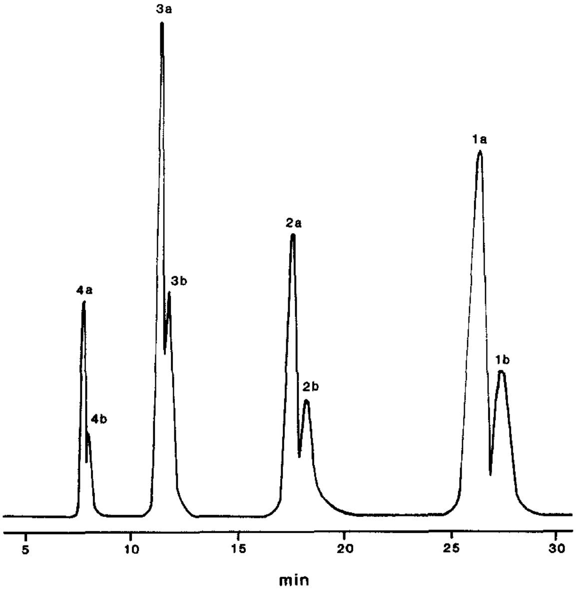

Fig. 4. Isocratic HPLC separation of methylated antimycin A subcomponents. Mobile phase: methanol–water (75:25).

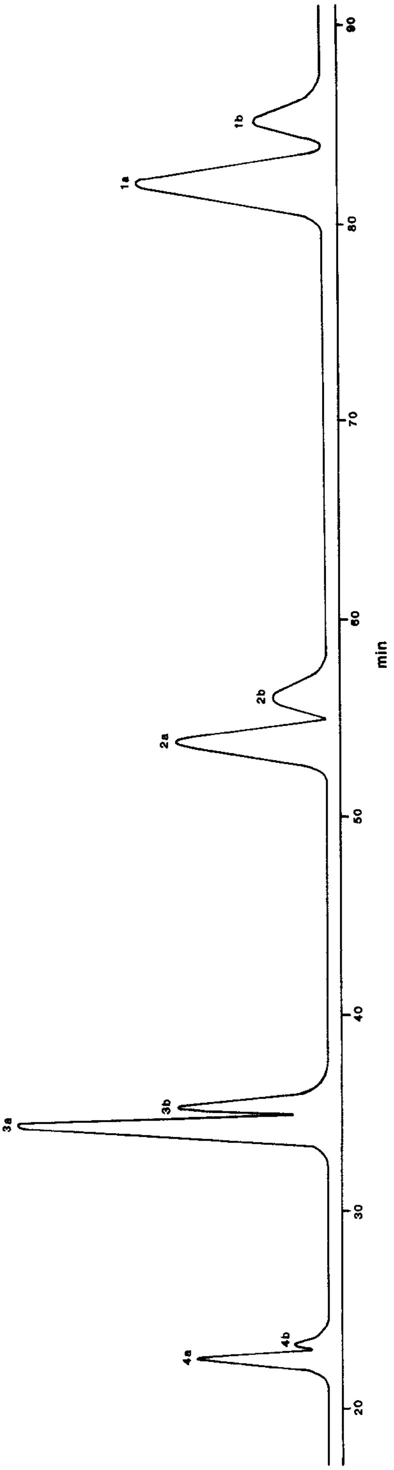

Fig. 5. Isocratic separation of dansylated antimycin A subcomponents. Mobile phase same as in Fig. 4.

TABLE V

EFFECT OF MOBILE PHASE ELUTION MODES (METHANOL–WATER) ON THE SEPARATION OF METHYLATED ANTIMYCIN A SUBCOMPONENTS

Mobile phases were under isocratic conditions, 2 ml/min (A) and gradient conditions (B) methanol–water (60:40) (15 min) → (70:30) for 2 h at 1.5 ml/min; (C) methanol–water (60:40) → (70:30) for 3 h at 2 ml/min; (D, E) methanol–water (60:40) → (70:30) for 3 h at 1.7 ml/min; (F) methanol–water (60:40) → (70:30) for 3 h at 1.5 ml/min. Stationary phases used in (A–D) and (E, F) were Altex Ultrasphere ODS-IP and Ultrasphere ODS, respectively.

Subcomponent		*Mobile phase elution mode*					
		A	*B*	*C*	*D*	*E*	*F*
A_{1a}	*k'*	20.0	123	122	125	125	144
	(α)	(1.04)	(1.03)	(1.02)	(1.03)	(1.03)	(1.03)
	R	0.83	1.09	1.25	1.47	1.29	1.60
A_{1b}	*k'*	20.8	127	125	129	129	148
A_{2a}	*k'*	13.1	97.0	93.2	97.0	95.3	111
	(α)	(1.04)	(1.02)	(1.03)	(1.03)	(1.03)	(1.03)
	R	0.71	0.87	1.05	1.24	1.15	1.44
A_{2b}	*k'*	13.6	99.0	96.0	99.8	98.0	114
A_{3a}	*k'*	8.12	71.3	59.8	65.4	62.6	77.4
	(α)	(1.03)	(1.03)	(1.05)	(1.04)	(1.04)	(1.04)
	R	0.40	0.97	1.17	1.31	1.24	1.79
A_{3b}	*k'*	8.36	73.6	62.6	68.3	65.3	80.5
A_{4a}	*k'*	5.24	50.0	34.5	43.2	37.7	51.2
	(α)	(1.01)	(1.04)	(1.04)	(1.05)	(1.06)	(1.05)
	R	0.33	0.98	1.06	1.24	1.14	1.50
A_{4b}	*k'*	5.32	52.1	36.0	45.2	39.8	53.6

dansyl groups gave rise to the corresponding antimycin derivatives whose subcomponents were separable with mobile phases devoid of added electrolytes. Adding either sodium acetate or TBAP to the mobile phases produced no appreciable change in the separation of subcomponent isomers. Typical chromatograms showing reasonable separations of the methylated and dansylated compounds are given in Figs. 4 and 5, respectively. Each of the HPLC profiles obtained under isocratic elution manifests a logarithmic relationship between the *k'* values and the number of carbons[11–14]. Examination of the isocratic HPLC chromatograms in Figs. 4 and 5 together with those in Fig. 2 indicates that the extent of peak resolution for each a–b pair diminishes with a shortening of the carbon chain length. To resolve the problem of uneven distribution of peak resolution among the four pairs of a–b isomers, gradient elution techniques were employed. Table V summarizes the results obtained with different elution modes of the mobile phases. When the method of elution was switched from isocratic (A, Table V) to gradient systems (B–F, Table V), there was a clear indication of improvement (higher *R* values) in peak resolution for the subcomponents of lower members of antimycin homologues (A_{3a}–A_{3b} and A_{4a}–A_{4b}). Further, HPLC at a lower flow-rate and a longer period of elution under gradient conditions resulted in higher R values (D, E, and F, Table V). The gradient HPLC techniques were scaled up for the preparative isolation of subcomponents in sufficient amounts for use as substrates in chemical and spectroscopic studies.

For analytical application, the underivatized antimycin subcomponents can be simultaneously and sensitively determined by reversed-phase HPLC–electrochemical detection[1] using the optimized HPLC conditions developed in this study.

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