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Note

Reversed-phase thin-layer chromatography of homologues of antimycin A and related derivatives

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During the course of recent studies[1–3] of the chemical analysis of antimycin A, a fish toxicant, we routinely employed thin-layer chromatographic (TLC) techniques for product isolation and sample purification. Since antimycin A was produced commercially from Streptomyces by fermentation, in every initial phase of the studies it was necessary to separate the pure antimycin A complex from other fermentation contaminants invariably present in the crude antibiotic products. The enrichment and further purification of these materials were readily accomplished by normal-phase TLC on silica gel plates. The methods utilizing conventional unmodified silica gel were found to be equally applicable in the isolation and purification of pure methylated and dansylated antimycins from the corresponding crude derivatization products.

However, numerous attempts to resolve antimycin A complex into its major homologous components A_1, A_2, A_3, and A_4 (Fig. 1) by normal-phase TLC have not been successful. In light of the general procedural simplicity of TLC techniques, adequate TLC resolution of the antibiotic homologues would provide a convenient means for studies dealing with subcomponents[2,3] of antimycin A or for product analysis entailing compositional determination of antibiotic mixtures derived from different batches of fermentation processes. We have explored the analytical potential of reversed-phase (RP) TLC methods for the quantification of antimycin A homologues and related methyl and dansyl derivatives. The first TLC separation of homologues of antimycin A is reported in this paper.

EXPERIMENTAL

Chemicals and reagents

Antimycin A mixtures and individual antimycin components A_1, A_2, A_3 and A_4 were either obtained from Sigma (St. Louis, MO, U.S.A.) or purified from the crude industrial materials[1,3]. Derivatives of antimycin A used in this study were synthesized at the National Fishery Research Center (La Crosse, WI, U.S.A.) following our published methods: methyl antimycins[3] were prepared by treating the parent antimycins with diazomethane in ethereal solutions; dansyl antimycins[1,3] were prepared from the corresponding antimycins by reactions with dansyl chloride in

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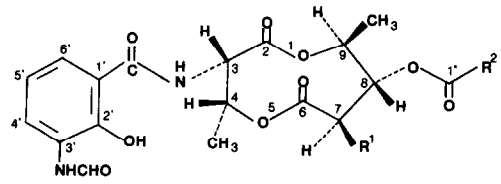
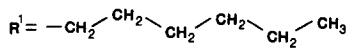
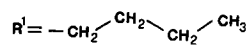




(I)



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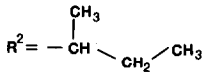

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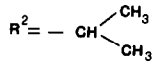

(IV)

Fig. 1. Structures of the homologues of antimycin A. A_1, $R^1 = I$, $R^2 = III$; A_2, $R^1 = I$, $R^2 = IV$; A_3, $R^1 = II$, $R^2 = III$; A_4, $R^1 = II$, $R^2 = IV$.

acetone in the presence of pyridine. TLC solvents and buffer reagents were acquired from J. T. Baker (Phillipsburgh, NJ, U.S.A.).

TLC plates were purchased from various commercial suppliers: (i) C_{18} RP-TLC and normal-phase TLC plates, 5 μm, 10 cm (length) × 20 cm (width) (with preadsorbent zone), E. M. Science (Cherry Hill, NJ, U.S.A.); (ii) C_{18} RP-TLC (bonded) plates, 10 μm, 20 × 20 cm, Whatman Chemical Separation (Clifton, NJ, U.S.A.); C_{18} RP-TLC (impregnated and not bonded), 10 μm, 20 × 20 cm, Analtech (Newark, DE, U.S.A.).

Thin-layer chromatography

In all experiments, plates with fluorescence indicators were used for visualization. Prior to sample applications, development chambers of suitable dimensions were saturated with solvents with the aid of saturation pads (Analtech) for 2 h. For qualitative analysis, 1-μl aliquot solutions (0.2%) of the antibiotic analytes in methylene chloride were spotted on a plate by means of Hamilton microliter syringes. After development of the plate, the solvent front was immediately measured when it reached a distance exactly 5 mm from the top edge of the plate. The chromatogram was then analyzed for the component spots. The unknown samples were identified by using known reference standards spotted alongside with the unknowns. Except for the methylated compounds, the antibiotics of interest were visible under short-wavelength or long-wavelength UV light generated from a mineralight lamp (Model UVSL-58 multiband UV-254/366 nm, Ultraviolet Products, San Gabriel, CA, U.S.A.). TLC spots of methyl antimycins could be seen only under 254 nm light.

Quantitative TLC analyses of antimycin samples in ethanol solutions were performed with a TLC scanning densitometer (Camag, Wrightsville Beach, NC, U.S.A.) equipped with a programmable integrator (Spectra Physics Model 4290). Calibration curves were constructed by plotting observed densities against the corresponding known amounts (0.1–10 μg) of samples spotted. The percent compositions of antimycin mixtures were determined automatically with the instruments. Minimum detection limits of the three types of antibiotic analytes were also determined at a signal-to-noise ratio of 4:1.

RESULTS AND DISCUSSION

Table I shows the results of the RP-TLC separation of homologous of the antimycin A complex under various chromatographic conditions. All the experiments listed in the table were performed with C_{18} RP-TLC plates of 5-μm particle size (10 × 20 cm with preadsorbent zone, E.M. Science). Examination of the R_F values indicated that the major antimycin components A_1, A_2, A_3 and A_4 were resolved only in solvent systems (A–H, and N) in which either acetonitrile or methanol was used as the organic modifier. The solvent pH and the presence of a buffer salt in a solvent system bore little significance on the resolution of the homologues. In contrast, mobile

TABLE I

RP-TLC HOMOLOGUES OF ANTIMYCIN A

All experiments were performed with E.M. Science RP-TLC plates, 5 μm, 10 × 20 cm with preadsorbent zone. Solvent systems: (A) methanol–water (9:1), 0.2 *M* sodium acetate, pH 2.5; (B) methanol–water (4:1), 0.2 *M* sodium acetate, pH 2.5, developed twice; (C) first with methanol–water (7:3), 0.2 *M* sodium acetate, pH 5, then with methanol–water (4:1), 0.2 *M* sodium acetate, pH 5; (D) methanol; (E) methanol–water (9:1); (F) methanol–water–acetic acid (18:1:1); (G) methanol–acetonitrile–water (9:9:2); (H) acetonitrile–water (9:1); (I) ethanol–water (9:1); (J) acetone–water (9:1); (K) tetrahydrofuran–water (9:1); (L) dimethyl formamide; (M) dimethyl formamide–water (4:1); (N) methanol–water (9:1), plate length 20 cm. For experiments with solvent systems A–M, plate length 10 cm.

Solvent system	*R_F values*[a]			
	A_1	A_2	A_3	A_4
A	0.286	0.356	0.419	0.481
B	0.031	0.053	0.088	0.129
C	0.181	0.291	0.401	0.527
D	0.601	0.654	0.709	0.709
E	0.253	0.316	0.376	0.437
F	0.459	0.522	0.579	0.662
G	0.347	0.412	0.473	0.545
H	0.396	0.466	0.538	0.592
I	0.586	0.586	0.586	0.586
J	0.698	0.698	0.698	0.698
K	0.932	0.932	0.932	0.932
L	0.716	0.716	0.716	0.716
M	0.152	0.152	0.152	0.152
N	0.329	0.389	0.449	0.546

[a] Mean values of three determinations; coefficients of variation ranged 1.57–2.89%.

phase buffers played crucial roles in the high-performance liquid chromatographic (HPLC) separation of these antimycin components[1,3]. The data in Table I clearly demonstrate that the antibiotic analytes were more strongly retained on the C_{18} RP-TLC phase as the percentage of an organic modifier decreased. Generally, the R_F values tended to increase with solvents of different solvent strength in the following order: methanol < acetonitrile < ethanol < acetone < tetrahydrofuran. The effect of distance travelled by the solvent on the degree of component resolution appeared to be small (experiment E *vs.* experiment N).

Examples of TLC results obtained with other types of silica-based stationary phases are presented in Table II. In the RP-TLC experiments (O–R) where C_{18} RP-bonded or impregnated stationary phases of 10 μm particle size and low carbon content (12.5–14.0%) were used, the homologues of antimycin A complex remained unresolved in all cases investigated. Apparently, the selection of a TLC stationary phase of high carbon content (20%, 5 μm, EM Science plates) was the determining factor for achieving the separation of antimycin homologues. In this regard, it seemed immaterial whether the hydrocarbonaceous phase was bonded or impregnated to silica (O, P *vs.* Q, R, Table II).

Normal-phase TLC on 5-μm silica, 10 × 10 cm (E. M. Science, this material was used in the manufacture of C_{18} RP-TLC plates) failed to separate the antimycin components as shown in experiments S and T, Table II. Apparently, the homologues were not differentiable by the adsorption mechanism that involved separations based on the polarity of antimycin solutes.

Table III compares the TLC data for homologues of antimycin A (AT) and the corresponding methylated (AT-ME) and dansylated (AT-DNS) derivatives. The plates used in these experiments were either C_{18} RP-bonded phases (A–D) or normal-phase silica (E) (all of 5-μm particle size, E.M. Science). These derivatives of

TABLE II

TLC OF ANTIMYCIN A COMPLEX ON VARIOUS SILICA-BASED PLATES

TLC systems: Whatman RP bonded plates with preadsorbent zone, 10 μm, 20 × 20 cm, (O) methanol–water (7:3); (P) methanol–water (1:1), 0.2 *M* sodium acetate, pH 5. Analtech RP impregnated plates, 10 μm, 20 × 20 cm, (Q) methanol–water (9:1); (R) methanol–water (7:3). E.M. Science normal-phase silica plates, 5 μm, 10 × 10 cm, (S) benzene–chloroform–methanol–acetatic acid (30:5:1:1); (T) chloroform–methanol–acetic acid (13:4:1).

TLC system	R_F *values*[a]			
	A_1	A_2	A_3	A_4
Reversed-phase				
O	0.364	0.364	0.364	0.364
P	0.000	0.000	0.000	0.000
Q	0.893	0.893	0.893	0.893
R	0.742	0.742	0.742	0.742
Normal phase				
S	0.276	0.276	0.276	0.276
T	0.769	0.769	0.769	0.769

[a] Mean values of three determinations; coefficients of variation ranged 2.51–3.32%.

TABLE III

COMPARISONS OF TLC DATA FOR HOMOLOGUES OF ANTIMYCIN A AND DERIVATIVES

Solvent systems: (A) acetonitrile; (B) acetonitrile–water (9:1); (C) methanol–water (9:1); (D) tetrahydrofuran–water (7:3); (E) benzene–chloroform–methanol–acetic acid (8:5:1:1). E.M. Science RP-TLC (5 μm) plates (10 × 20 cm) were used in A–D; E.M. Science normal-phase TLC (5 μm) plates were used in E. AT = Antimycin A; AT-ME = methylated antimycin A; AT-DNS = dansylated antimycin A.

Solvent system	R_F *values*[a]			
	A_1	A_2	A_3	A_4
A				
AT	0.520	0.573	0.607	0.667
AT-ME	0.507	0.560	0.613	0.640
AT-DNS	0.427	0.480	0.527	0.573
B				
AT	0.357	0.429	0.490	0.557
AT-ME	0.414	0.486	0.536	0.586
AT-DNS	0.314	0.386	0.457	0.529
C				
AT	0.233	0.293	0.360	0.407
AT-ME	0.240	0.307	0.373	0.427
AT-DNS	0.147	0.187	0.227	0.280
D				
AT	0.669	0.669	0.669	0.669
AT-ME	0.712	0.712	0.712	0.712
AT-DNS	0.718	0.714	0.718	0.718
E				
AT	0.482	0.482	0.482	0.482
AT-ME	0.439	0.439	0.439	0.439
AT-DNS	0.488	0.488	0.488	0.488

[a] Mean values of three determinations; coefficients of variation ranged 2.67–3.08%.

antimycins were useful in analytical studies[1,3] and in a structural investigation of its subcomponents[4]. In each case (Table III), the three types (the parent and the two derivatives) of antimycin compounds were chromatographed simultaneously on the same plate to eliminate the possible influence of different chromatographic variables. The chromatographic behavior of the derivatives under various TLC conditions was strikingly similar to that of the parent compounds. It was of interest to note that the elution order of the compounds seemed to be solvent-dependent. Thus, in the RP modes, TLC in acetonitrile–water (9:1) [or methanol–water (9:1)] afforded the analyte spots in a decreasing order of R_F values; AT-ME > AT > AT-DNS as found in solvent systems B and C. No definite trend of elution order could be delineated from TLC experiments in other solvents (A and D). On the other hand, normal phase TLC of the same sets of samples resulted in a reversal of the above elution order (B and C) as illustrated in (E) of Table III.

Unknown samples were quantified using calibration curves of all four individual antimycins A_1, A_2, A_3 and A_4. Determination of minimum detection limits by absorption scanning densitometry at a signal-to-noise ratio of 4:1 gave 20, 30 and 55 ng for AT, AT-ME, and AT-DNS, respectively. By virtue of their fluorescence properties,

TABLE IV

RP-TLC DETERMINATION OF THE COMPOSITION OF HOMOLOGUES IN DIFFERENT BATCHES OF ANTIMYCIN A COMPLEX

The HPLC method described in ref. 1 was used. The five samples represent materials derived from different batches of commercial fermentation processes.

Component	*Composition (%)[a] of batch No.*				
	1	*2*	*3*	*4*	*5*
A_1					
TLC	36.10	35.21	33.05	45.93	37.54
HPLC	36.42	34.99	33.26	46.10	37.20
A_2					
TLC	26.96	30.08	17.60	12.01	10.25
HPLC	27.10	29.67	17.42	11.75	10.34
A_3					
TLC	24.31	21.50	37.96	36.44	43.78
HPLC	24.27	21.56	38.09	36.23	43.81
A_4					
TLC	12.27	14.01	10.99	5.82	8.63
HPLC	12.21	13.78	11.23	5.92	8.65

[a] Mean values of three determinations; coefficients of variation for respective TLC and HPLC methods ranged 2.01–2.35% and 1.56–1.88%.

the detection sensitivities were somewhat improved in the cases of AT and AT-DNS. Both of these fluorescent compounds were detectible at a lower limit of 50 pg by fluorescence scanning densitometry. Table IV gives the results of compositional analyses of five antimycin mixtures by RP-TLC–UV and HPLC–UV. The two methods yielded comparable results.

In conclusion, the major components of antimycin A homologues can be separated by RP-TLC. Using the TLC method in conjunction with absorption and fluorescence scanning densitometry, quantification of unknown homologues as well as the composition of antimycin A complex can be achieved with good reproducibility.

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