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Note

Separation of tunicamycin homologues by reversed-phase high-performance liquid chromatography

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Tunicamycin is a naturally occurring antibiotic which inhibits N-acetyl-glucosamine-1-phosphate transferase and thereby blocks the production of N-acetyl-glucosamine-pyrophosphoryldolichol[1]. This event culminates in the synthesis of underglycosylated glycoproteins by preventing the formation of intermediates necessary for the synthesis of N-glycosidic linkages[2,3]. As such, tunicamycin has become a valuable experimental tool for the study of the biological role of the carbo-hydrate moiety (of the N-glycosidic type) of glycoproteins and the pathway leading to the formation of lipid-linked oligosaccharides[3,4].

It has been suggested that tunicamycin acts as a substrate analogue of UDP-N-acetylglucosamine and functions as either a competitive inhibitor[5,6] or a non-competitive inhibitor of N-acetylglucosamine-1-phosphate transferase[7]. An appealing argument has also been put forth suggesting that tunicamycin may act as a multi-substrate analogue at the transition state[6]. In addition, tunicamycin has been reported to inhibit protein synthesis to a variable degree depending upon the system of study[8-11]. At times this duality has caused difficulty in the interpretation of results[8].

Recently, we reported that tunicamycin was not a single compound but consists of at least 10 homologues which can be separated by reversed-phase high-performance liquid chromatography (HPLC)[12]. When two of these homologues were tested for biological activity one exhibited very little influence on protein synthesis while the other inhibited protein synthesis by 50% when both displayed their maximum degree of inhibition of protein glycosylation. This difference in biological activities, although not found universally with each cell line or type examined[13,14], has demonstrated the desirability of obtaining pure homologues of tunicamycin.

This paper describes the separation of the tunicamycin homologues by reversed-phase HPLC using volztile solvents to simplify the recovery of individual components. We also propose a simple system to designate the homologues based upon the combination of their molecular weights and the order of their elution from a reversed-phase resin.

MATERIALS AND METHODS

Chemicals

Glass-distilled methanol was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Purified tunicamycin was a generous gift from Dr. R. Hamill, Eli Lilly, Indianapolis, IN, U.S.A. (Lot 361-26E-79A).

High-performance liquid chromatography

Separation of tunicamycin components was carried out using reversed-phase HPLC[12]. HPLC was performed using a Varian Model 5000 high-performance liquid chromatograph equipped with a Rheodyne Model 7125 sample injector with a 100-μl sample loop, a Vari-Chrom detector operating at a wavelength of 260 nm, a Linear Model 260/MM chart recorder, and a 25 × 0.46 cm I.D. LiChrosorb C-5-μm column (Brownlee Labs., Santa Clara, CA, U.S.A.). The HPLC separation was achieved using an isocratic solvent system of 68 or 80% methanol in twice glass-distilled water. Tunicamycin components were quantitated using a Hewlett-Packard 3380 S recording integrator.

Thin-layer chromatography

Analytical C_{18} reversed-phase thin-layer chromatography (TLC) plates ($MKC_{18}F$) containing a fluorescent label (F-254) were purchased from Whatman (Clifton, NJ, U.S.A.; Lot 000444). Chromatograms were developed at room temperature using an eluent consisting of methanol–water (3:1).

RESULTS

The separation of the naturally occurring homologues of tunicamycin was achieved by reversed-phase chromatography on either TLC plates or by HPLC. On TLC plates, using 10 μg of tunicamycin, four spots could be identified by absorbance of fluorescence (chromatogram not shown). The four factors corresponding to the four major molecular weight classes of tunicamycin were designated by the letters A, B, C, and D, with D being the closest to the origin. Table I presents the R_F values and the molecular weights and formulae for the tunicamycin factors.

TABLE I

PHYSICAL CHEMISTRY AND TLC OF TUNICAMYCIN FACTORS

Molecular weights and formulae are provided for the unsaturated form of each tunicamycin factor.

Factor	R_F*	Molecular formulae**	Molecular weight**
A_0	—	$C_{36}H_{59}N_4O_{16}$	804
A	0.31	$C_{37}H_{61}N_4O_{16}$	818
B	0.24	$C_{38}H_{63}N_4O_{16}$	832
C	0.16	$C_{39}H_{65}N_4O_{16}$	846
D	0.10	$C_{40}H_{67}N_4O_{16}$	860

* R_F values were determined on C_{18} thin-layer plates developed using methanol–water (3:1).
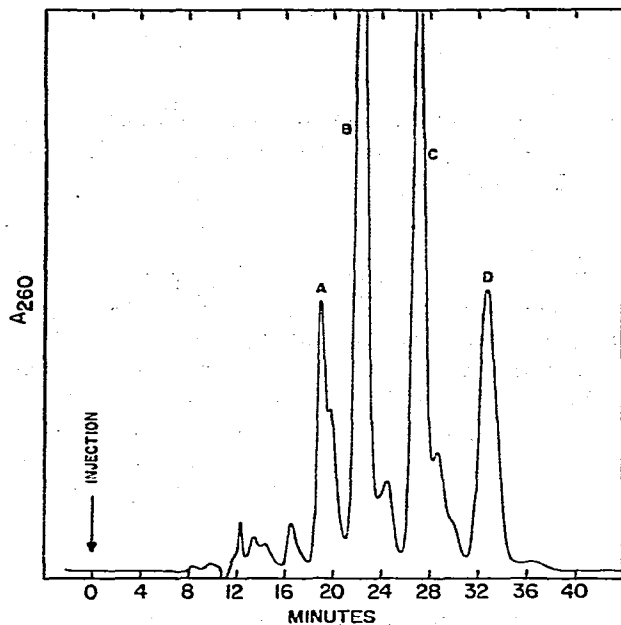** Physical data provided by Dr. R. Hamill and subsequently confirmed (data not shown).

Fig. 1. HPLC chromatogram illustrating the resolution of the homologues of tunicamycin. Sample, 100 μg of tunicamycin dissolved in methanol–water (7:3); chart speed, 1 cm/min; pressure, 80 atm; temperature, 40°C; flow-rate, 1 ml/min. The chromatogram was developed isocratically using methanol–water (4:1).
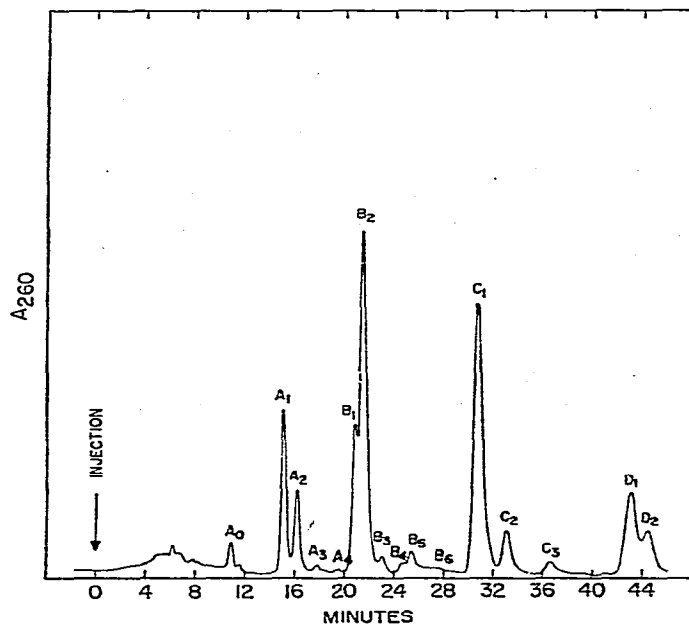

Fig. 2. HPLC chromatogram illustrating the typical resolution of the homologues of tunicamycin using methanol–water (68:32). Sample, 100 μg of tunicamycin dissolved in methanol–water (7:3). Pressure, 68 atm; chart speed, 1 cm/min, temperature, 40°C; flow-rate, 1 ml/min.

Upon analysis by HPLC using a solvent system consisting of methanol–water (4:1), four major factors could again be identified. However, there was clear additional heterogeneity within each factor (Fig. 1). When this analysis was repeated using only 68% methanol several components within each factor were identified. Fig. 2 shows a typical chromatogram. Table II presents the chromatographic data and the yield of each homologue. Increasing the temperature above 40°C decreases the separation of A_1 from A_2 and B_1 from B_2 although it speeds the analysis. By collecting B_1, B_2, D_1, and D_2 and resubjecting this mixture to HPLC using 55–60% methanol these individual components can be isolated in pure form.

TABLE II

CHROMATOGRAOHIC AND YIELD DATA FOR TUNICAMYCIN HOMOLOGUES ON C_8 REVERSED-PHASE HPLC

Flow-rate, 1 ml/min at 40°C.

Homologue	Relative movement	Capacity factor (k')*	Yield (μg)**	Previous nomenclature[12]
A_0***	1.00	2.6	0.20	–
A_1	1.36	4.0	0.95	I
A_2	1.43	4.4	0.56	II
A_3	1.54	4.9	0.08	
A_4	1.65	5.5	0.05	
B_1	1.74	5.9	11.22	
B_2	1.78	6.1	33.09	IV
B_3	1.89	6.7	2.53	
B_4	2.00	7.2	1.76	
B_5	2.05	7.4	3.63	V
B_6	2.18	8.2	0.01	
C_1	2.39	9.2	33.28	VI
C_2	2.56	10.0	0.59	VII
C_3	2.80	11.2	0.24	VIII
D_1	3.21	13.3	11.26	IX
D_2	3.30	13.8	0.65	X

* Capacity factor: $k' = (t_R - t_0)/t_0$, where t_R is the sample retention time and t_0 is the time required for non-retained material to pass through the column.
** Yield of individual homologues using a starting injection of 100 μg of purified tunicamycin.
*** See Discussion for details of nomenclature.

DISCUSSION

We previously identified ten homologues within the tunicamycin family by HPLC and found each to be active in inhibiting N-acetylglucosamine-1-phosphate transferase, although differences were found when the biological activities of A_1 and A_2 were compared[12]. The method presented here provides higher yields of each homologue and allows easy recovery from volatile solvents using lyophilization, with no loss of biological activity. The relative amounts of the individual factors and their respective components appear to vary with lot number and thus apparently with the growth conditions of the producing strains of Streptomyces. So rather than having two major components as previously reported[12], in most preparations of tunicamycin

there are four major molecular weight classes consisting of between two and six minor components of variable amount.

Since the tunicamycin factors elute from a reversed-phase column in the order of increasing molecular weight, we propose that each factor (molecular weight class) be identified with the letters A, B, C, and D in the order of elution. We also suggest that components within the same general molecular weight class be identified by the subscript 1, 2, 3, etc., also in the order of elution from reversed phase (Table II).

The differences between factors A, B, C, and D have been explained by the presence of four different carbon chain lengths within the lipid moiety of the antibiotic (Table I)[15]. Recently, Takatsuki et al.[16] have shown that more than ten different fatty acids can be identified as components of tunicamycin. Although it is not yet known what the structure of the lipid moiety is from each of the homologues isolated by HPLC, we are currently working toward this goal in addition to further investigating the biological differences between tunicamycin homologues.

ACKNOWLEDGEMENTS

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