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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COMPARISON OF THE TUNICAMINYLURACIL-BASED ANTIBIOTICS CORYNETOXIN, TUNICAMYCIN, STREPTOVIRUDIN AND MM 19290

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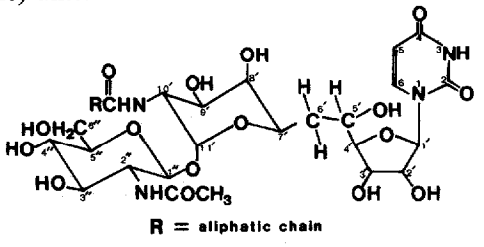
SUMMARY

Resolution of the components of tunicaminyluracil-based antibiotics by reversed-phase high-performance liquid chromatography is described. Two systems are employed using a silica-based ODS bonded-phase support, and gradient elution with either water–methanol or water–methanol–tetrahydrofuran as mobile phase. The ternary mixture reduces analysis time by a factor of 4 whilst retaining resolution, however the increase in background absorption due to introduction of tetrahydrofuran reduces optimum detector sensitivity about sixteen-fold. Both systems are capable of separating the homologues within individual antibiotics and further resolving them into their *anteiso-*, *iso-* or normal-isomers as defined by the termination of the fatty acid portion of their structure. The resultant reproducible pattern of peaks in the chromatograms, and the retention time changes associated with catalytic reduction, have allowed assignment of structures to previously unrecognized or unidentified components in all antibiotics studied, *e.g.*, the 23 components identified in MM 19290, and have permitted correlation of the various nomenclatures published for tunicamycin components.

INTRODUCTION

Related, multicomponent antibiotics have been isolated from several *Streptomyces* species and *Corynebacterium rathayi*[1]. They have a common N-acetylglucosamine-tunicaminyluracil structure and differ from each other only in the fatty acids attached via an amide link to the amino group of the central C_{11} aminosugar (tunicamine) unit:



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Of these antibiotics, high-performance liquid chromatographic (HPLC) separations have been reported for tunicamycin[2–5], streptovirudin[6], MM 19290[7] and mycospocidin[8] from *Streptomyces* species and corynetoxin[9] from *Corynebacterium rathayi.*

We have described, in a preliminary communication[10], an HPLC system which more effectively resolves these antibiotics into their components, and used it to compare the composition of corynetoxin, tunicamycin and streptovirudin in runs lasting about 2 h. In this paper we extend this comparison and present a newly developed system capable of similar resolution in about 30 min.

Both systems offer a means of determining the composition of these multicomponent antibiotics. We have used them to compare and to assign structures to components of MM 19290, streptovirudin, tunicamycin and corynetoxin, and to quantitate their individual components.

EXPERIMENTAL

Apparatus

HPLC was performed using a Model 334 chromatograph (Altex Scientific, Berkeley, CA, U.S.A.). Samples were introduced by a Model 7120 loop injection valve (Rheodyne, Cotati, CA, U.S.A.). Columns used were Ultrasphere ODS, particle size 5 μm (Altex Scientific), and Zorbax SIL, particle size 7 μm (Du Pont, Wilmington, DE, U.S.A.); both were 25 cm × 4.6 mm I.D. Temperature of the column and injection valve was controlled by the heated column compartment of a Model 1010B liquid chromatograph (Hewlett-Packard, Boblingen, G.F.R.). Detection was by UV absorption at 254 nm in a 10-mm path length cell (Altex Scientific). A reporting integrator (Model 3390A; Hewlett-Packard, Avondale, PA, U.S.A.) was used to plot and quantitate reversed-phase chromatograms.

Samples

Corynetoxin was obtained by extraction from annual ryegrass seeds infected by *C. rathayi*[11]. Tunicamycin, streptovirudin and MM 19290 were the generous gifts of Dr. R. Hamill (Eli Lilly, U.S.A.), Dr. K. Eckardt (Academy of Science, D.D.R.) and Dr. M. Kenig (Welcome Pharmaceuticals, U.K.) respectively. Solutions of each were prepared at a concentration of 1 μg/μl in methanol.

Solvents

Methanol, tetrahydrofuran (THF), methylene chloride and glacial acetic acid were chromatographic grade (Ajax Chemicals, Sydney, Australia). Dimethyl sulphoxide (DMSO) was spectroscopy grade (Fluka, Buchs, Switzerland). Water was glass distilled. THF was redistilled prior to use.

Chromatography

Normal-phase HPLC was performed isocratically at ambient temperature, using a mobile phase of methylene chloride–tetrahydrofuran–dimethyl sulphoxide–water (45:45:9.5:0.5) at a flow-rate of 0.5 ml/min[10].

Reversed-phase HPLC was performed at 55°C by gradient elution. A system using a mobile phase of water–methanol and a gradient from 50:50 to 10:90 over 160 min was described in a preliminary publication[10]. A new system has been developed,

which uses a two-pump apparatus to generate a gradient in a three-component mobile phase as follows. Two mixtures of solvents were prepared using water, methanol and THF in the ratios 525:450-25 (A) and 450:50:500 (B). After sample introduction the system was held isocratic at 10% B for 0.5 min, then altered linearly to 43% B at 3% per minute, then to 73% B at 1.5% per minute. Total flow-rate was maintained at 1.5 ml/min. The resultant solvent composition profile, water–methanol–tetrahydrofuran (initial ratio of 51.7:41.0:7.3, changed to 49.3:27.8:22.9 over 11 min, and then to 47.0:15.8:37.2 over 20 min), provides a nearly linear spacing of peaks.

Catalytic reduction

The conversion of antibiotics containing unsaturated fatty acids to the corresponding saturated acid form was achieved using a platinum catalyst in methanol at room temperature and pressure[12].

RESULTS AND DISCUSSION

Throughout this discussion we use a nomenclature previously defined for the corynetoxins[9], which incorporates a term indicating the fatty acid present in a particular component. The acid series is indicated first by U ($\alpha\beta$-unsaturated), S (saturated) or H (β-hydroxy). The carbon number of the acid is then given, followed by the terminal methyl branching; a [*anteiso*, $CH_3CH_2CH(CH_3)$–], i [*iso*, $CH_3CH(CH_3)$–] or n (normal, CH_3CH_2–). Thus, for example, the corynetoxin containing an $\alpha\beta$-unsaturated, C_{17} *anteiso* acid is referred to as corynetoxin-U17a. The same system is applicable to other N-acetylglucosamine-tunicaminyluracil antibiotics. Streptovirudin contains components in which the uracil is replaced by dihydrouracil. To take account of this, Eckardt[1] has proposed the addition of U (for uracil) or DU (for

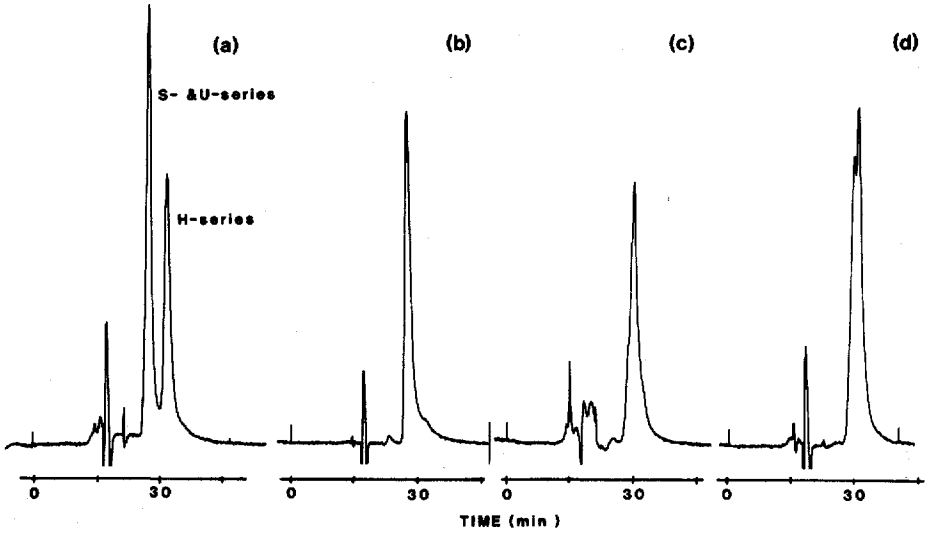

Fig. 1. Normal-phase HPLC chromatograms of (a) corynetoxin (20 μg), (b) tunicamycin (10 μg), (c) streptovirudin (10 μg), (d) MM 19290 (10 μg). Isocratic elution using methylene chloride–tetrahydrofuran–dimethyl sulphoxide–water mobile phase described in text. Detector sensitivity 0.1 a.u.f.s.

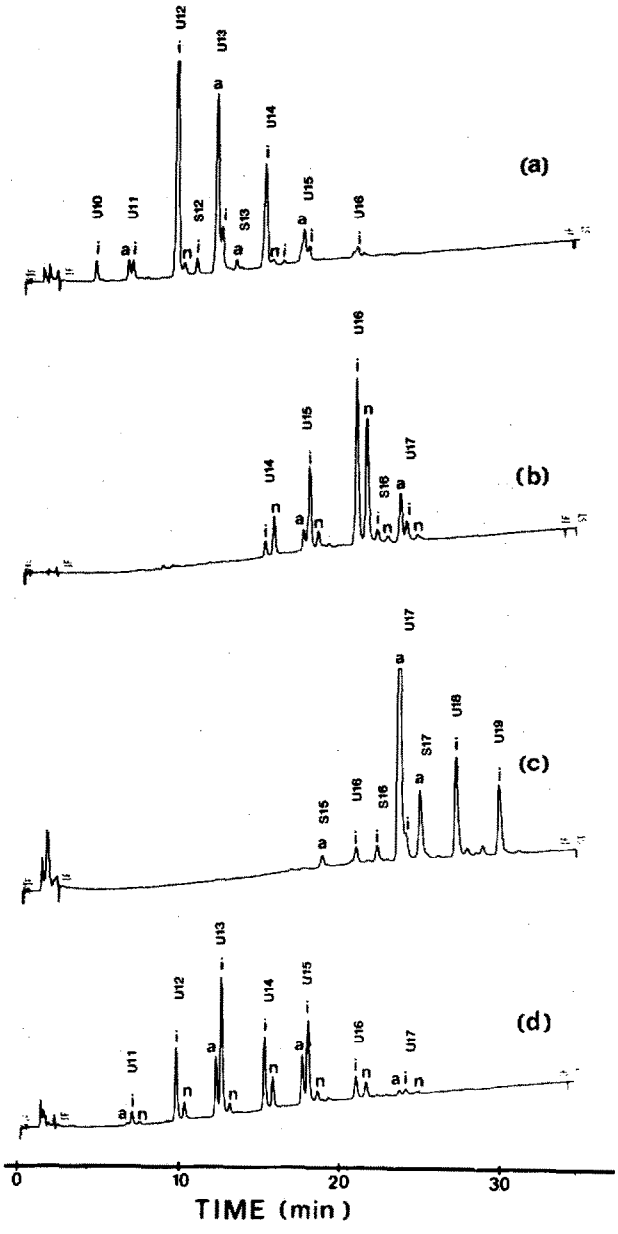

Fig. 2. Comparative reversed-phase HPLC chromatograms of (a) streptovirudin, (b) tunicamycin (c) corynetoxin (S and U series), (d) MM 19290. The series and C-number designations, *e.g.*, U17, refer to the cluster of isomer peaks (a, i, n) immediately under them. Gradient elution using water–methanol–tetrahydrofuran mobile phase as described in text. Detector sensitivity 0.32 a.u.f.s., 50-μg sample applied in each case.

dihydrouracil) to the coding we have proposed, *e.g.*, U13aU and U13aDU. However simple addition of a terminal D to indicate the presence of dihydrouracil would seem adequate. Since this work described HPLC using UV detection the presence of dihydrouracil-containing components cannot be assessed and the original form of coding is retained. Use of a single stem name, *e.g.*, tunicamycin (which has historical precedence), for these antibiotics, regardless of origin, cannot be justified at present because a rigorous comparison involving definition of the stereochemistry of the glycouracil portion of individual components from different antibiotic mixtures has not yet been reported. In order to indicate the origins of the material under discussion we have therefore continued to use the names corynetoxin (CT), tunicamycin (TM), streptovirudin (SV) and MM 19290 (MM), followed by designation of the acid, when indicating individual components.

H-series components, which tend to co-chromatograph with S or U series components with one carbon less, have only been reported as constituents of CT[9,12] and were not demonstrated in normal-phase HPLC of the samples of TM, SV and MM examined here (Fig. 1). We have therefore used isolated H-series and S- and U-series CT fractions, obtained by preparative normal-phase HPLC, when comparing antibiotics. The reversed-phase profiles produced by the water–methanol–tetrahydrofuran system are shown in Fig. 2, and Table I summarises our interpretation of this data.

The order of elution of individual components reflects their relative polarity and molecular shape and size. This has been established by work with the corynetoxins[12]. It is thus the nature of the fatty acid that determines the elution position. An increase in the carbon number of the acid proportionately increases retention time, U-series components emerging before those of the less polar S-series with the same carbon number. In both series a component with an *anteiso*-acid appears before the *iso*-acid isomer which, in turn, preceeds the normal-acid isomer. Components containing even-carbon-number fatty acids are found only as *iso*- and normal-acid isomers, whilst components with odd-carbon-number fatty acids also occur as the *anteiso*-acid isomer. This is to be expected on biosynthetic grounds[13] and gives rise to an alternating pattern of two and three peak multiplets in the chromatograms, as is clearly shown for MM in Fig. 2d. Where S- and U-series components occur together, the U-series multiplet is immediately followed by a duplicate multiplet for the S-series. This is demonstrated in Fig. 3, which shows the chromatogram resulting from partial (*ca.* 50%) reduction of the tunicamycin sample shown in Fig. 2b. Thus the assignment of structures indicated in Fig. 2 is based on a predictable pattern of peaks and retention times, the use of catalytic reduction to distinguish U- and S-series components and co-chromatography of the different antibiotics[10].

Our results correlate well with reports of the main TM[2] and uracil-containing SV[14] components. They also demonstrate previously unrecognised small amounts of U10i, U11a, U11i and U16i in SV (Fig. 2a) and we are able to assign U15a and U17a structures to two TM components, TM IV and TM IX[2], with previously undefined methyl branching of their fatty acids. The composition of MM had not been ascertained prior to this investigation, although co-chromatography with a sample of TM had indicated some common components[7]. Fig. 2d shows resolution of eighteen components, all of which we have shown, by catalytic reduction and retention time, to be in the U-series. The more intense chromatogram in Fig. 4 run using the water–methanol system reveals five S-series components as well.

TABLE I

COMPOSITION OF TUNICAMINYLURACIL-BASED ANTIBIOTICS

Data obtained from chromatograms using water–methanol–tetrahydrofuran gradient elution system. Components are listed in order of increasing retention time.

*Structure**	t_R *(min)*	*Percentage composition*				
		Streptovirudin	*Tunicamycin*	*Corynetoxin*		*MM 19290*
				S + U series	*H series*	
U10i	4.7	2.3				
U11a	6.8	2.3				0.3
U11i	7.0	2.3				2.1
U11n	7.5					0.3
U12i	9.8	31.4				11.4
U12n	10.3	0.7				2.5
S12i	11.1	1.7				
U13a	12.4	23.6				8.1
U13i	12.7	6.1				21.7
U13n	13.2					1.3
S13a	13.6	1.1				
S13i	13.9	0.3				
U14i	15.4	15.7	2.1			11.0
U14n	15.9	1.1	5.5			4.8
S14i	16.6	0.5				
U15a	17.9	5.6	3.2			8.5
U15i	18.2	1.9	14.2			15.4
U15n	18.8		3.2			1.6
S15a	19.0			0.8		
H16i	19.2				1.7	
U16i	21.2	2.1	29.4	1.6		4.1
U16n	21.8	0.7	22.4			3.1
H17a	21.8				26.2	
H17i	22.0				1.2	
S16i	22.5		1.9	2.0		
S16n	23.2		0.9			
U17a	24.0		8.8	33.5		1.0
U17i	24.4		3.8	2.4		1.2
U17n	25.0		1.0			0.4
S17a	25.2			6.1		
H18i	25.5				3.3	
U18i	27.5			10.0		
U18n	28.0			1.1		
H19a	28.0				1.3	
S18i	29.1			1.1		
U19a	30.2			6.7		
S19a	31.5			0.3		

* See text for explanation of nomenclature.

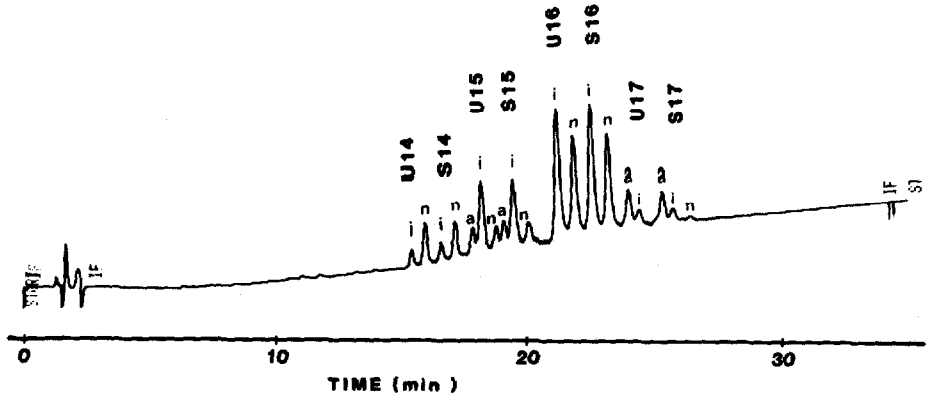

Fig. 3. Reversed-phase HPLC chromatogram of 50% reduced tunicamycin. Gradient elution using water–methanol–tetrahydrofuran mobile phase as described in text. Detector sensitivity 0.32 a.u.f.s., 50-μg sample applied.

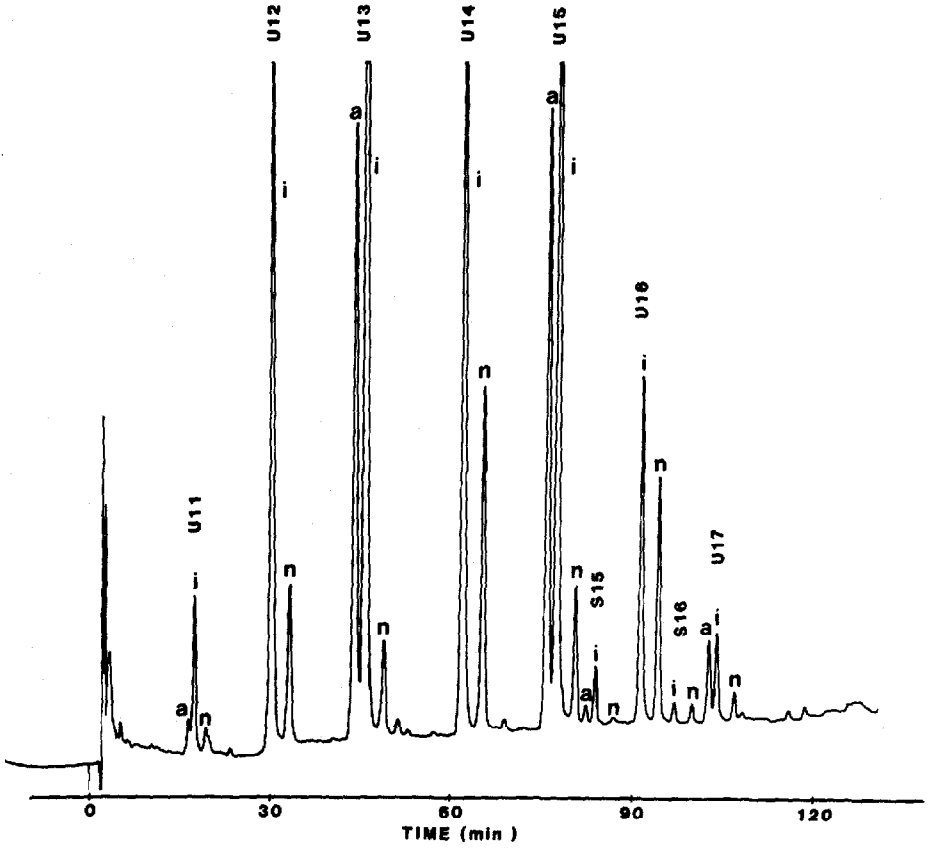

Fig. 4. Reversed-phase HPLC chromatogram of antibiotic MM 19290. Gradient elution using water–methanol mobile phase as described in text. Detector sensitivity 0.02 a.u.f.s., 10-μg sample applied.

TABLE II

IDENTIFICATION OF TUNICAMYCIN COMPONENTS

Correlation of nomenclature used by several authors to identify individual components with a systematic nomenclature based on structure*.

Cockrum and Edgar (this work and ref. 10)	*Ito et al.*[2]	*Mahoney and Duksin*[3,4,15]	*Keenan et al.*[5]
U13a**			
U13i**	I	A_0	
U13n**			
U14i	II	A_1 (I)***	TM 6 (A_1)
U14n	III	A_2 (II)	TM 7 (A_2)
U15a	IV§	B_1§§	
U15i	V	B_2 (IV)	TM 9 (B_1)
U15n		B_3§§§	
S15a†		B_4§§	
S15i†	VI	B_5§§ (V)	
S15n†		B_6§§	
U16i	VII	C_1 (VI)	TM 12 (C_1)
U16n	VIII	C_2 (VII)	TM 13 (C_2)
S16i		C_3§§ (VIII)	
S16n			
U17a	IX§	D_1§§ (IX)	TM 16 (D_1)††
U17i	X	D_2 (X)	
U17n			

* Fig. 3 shows the range of likely components in TM, their order of elution under reversed-phase HPLC conditions and the resolution of the systems described here.

** We observed these components in one early sample of TM, but not in more recently acquired samples.

*** Roman numeral nomenclature used by Mahoney and Duksin[3] and subsequently correlated by these authors with their later nomenclature in ref. 4.

§ Methyl branching of fatty acid, unspecified by Ito *et al.* Assignment of TM IX as U17a based on co-chromatography with CT-U17a. Assignment of TM IV as U15a based on co-chromatography with SV-U15a.

§§ Unassigned by Mahoney and Duksin[4,15]. Our assignments are based on elution pattern and retention time (see text).

§§§ Assigned the structure S15i by Mahoney and Duksin[4,15]. Elution position as shown in Fig. 2 of ref. 4 indicates it to be U15n. Compare with Fig. 2c this paper and Fig. 3 of ref. 10.

† These components are barely visible in Fig. 2c but are readily observed in the more intense chromatogram in Fig. 3 of ref. 10.

†† Assigned as U17n by Keenan *et al.*[5], however the NMR evidence adduced to identify it as the n-isomer is possibly inadequate to distinguish it from the a-isomer. The elution position shown in Fig. 2 of ref. 5 indicates it to be U17a. Compare Fig. 2c of this work.

During the course of this work it became clear that the naming of TM components has become somewhat confused, with various workers each using their own nomenclature[2–5]. In Table II we have correlated the several reported designations with the systematic nomenclature based on structure described above, in an effort to clarify the situation.

The two HPLC systems described here for the separation of tunicaminyluracil-based antibiotics are complementary. The water–methanol–tetrahydrofuran system

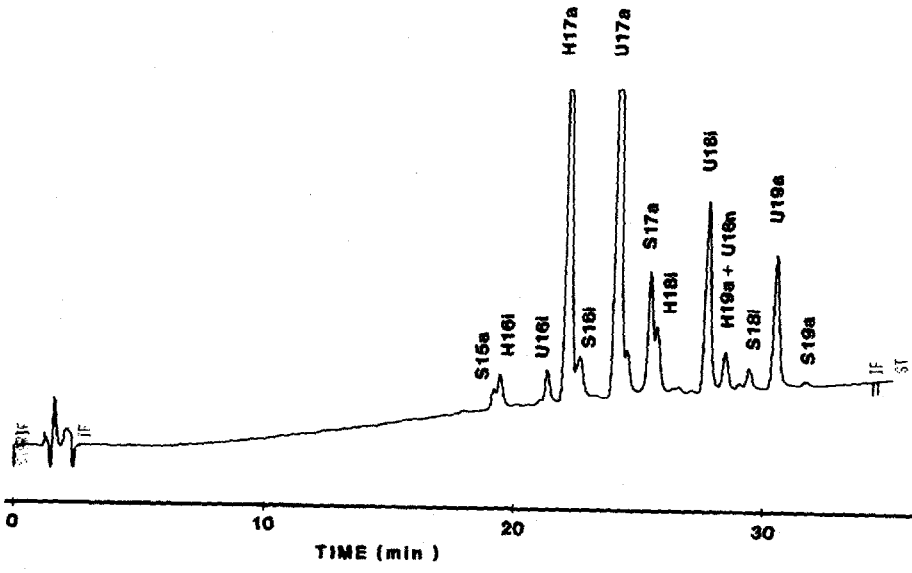

Fig. 5. Reversed-phase HPLC chromatogram of total corynetoxin. Gradient elution using water–methanol–tetrahydrofuran mobile phase as described in text. Detector sensitivity 0.32 a.u.f.s., 75-μg sample applied.

gives partial resolution of H16i from S15a and H18i from S17a when applied to the total CT complex (Fig. 5), a result not accomplished by the water–methanol system[12]. However, the introduction of THF with its appreciable UV absorption at 254 nm reduces the sensitivity at which the detector can be operated whilst restricting the baseline rise to an acceptable level. For example, for the chromatogram of MM shown in Fig. 4, generated using the water–methanol system, the detector setting was sixteen-fold more sensitive than that used for the same sample (Fig. 2d) using water–methanol–tetrahydrofuran. In addition to the greater sensitivity, general resolution is also somewhat better with the water–methanol system, but at the expense of a four-fold increase in run time. Clearly, the application will determine the system of choice.

The main biological effect of these antibiotics is inhibition of protein N-glycosylation. Inhibition of protein synthesis[1,15] has been reported for some individual members, an effect that appears to be influenced by the structure of the fatty acid[15]. The HPLC systems described here provide improved definition of components and improved potential for their isolation, both of which should aid structure–activity studies.

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