Journal of Chromatography, 175 (1979) 141–152

CHROM. 11,843

NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NEOMYCIN SULFATE DERIVATIZED WITH 1-FLUORO-2,4-DINITROBENZENE

KIYOSHI TSUJI, JOHN F. GOETZ, WILLIAM VanMETER and KATHY A. GUSCIORA
Control Analytical Research and Development, The Upjohn Company, Kalamazoo, Mich. 49001 (U.S.A.)
(First received January 24th, 1979; revised manuscript received March 6th, 1979)

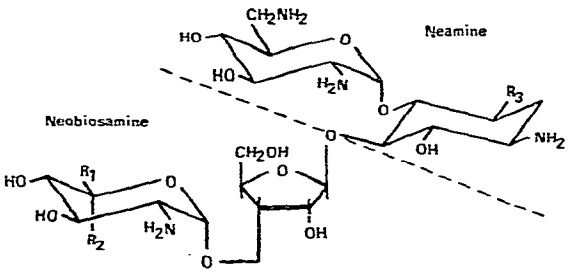
SUMMARY

A high-performance liquid chromatographic (HPLC) assay method has been developed for quantifying neomycin sulfate powders and ointments containing neomycin. A dinitrobenzene derivative of neomycin is formed and then chromatographed isocratically on a normal-phase system. Neomycin B and C can be separated and quantified with a relative standard deviation of approximately 1%. When monitored at 350 nm, the method is sensitive to about 1 ng of neomycin base per column injected. Gradient elution and isocratic HPLC methods for the analysis of derivatized neamine have also been developed. The relative standard deviation is less than 1% for the isocratic method. The HPLC method is applicable to the analysis of other aminoglucoside antibiotics.

INTRODUCTION

Neomycin (Fig. 1) is a medium to broad spectrum aminoglucoside antibiotic used mostly in topical preparations. The term neomycin usually refers to a mixture of two stereoisomers, neomycin B and C, and several chemical methods have been reported for their analysis. These methods include paper chromatography[1], thin-layer chromatography[2], ion-exchange column chromatography[3,4] and ion-exclusion column chromatography[5,6]. A gas–liquid chromatographic assay method[7,8] is being used routinely in our company for the quantification of neamine and neomycin B and C, but it is a complicated method to perform[9]. Recently, however, the method has been applied to monitoring serum levels of aminoglucoside antibiotics[10]. A high-performance liquid chromatographic (HPLC) method has been reported for the analysis of an aminoglucoside antibiotic, gentamicin, with paired-ion chromatography using post-column, on-stream derivatization with *o*-phthalaldehyde for fluorimetric detection[11,12]. The method is reported to be applicable to other aminoglucoside antibiotics[12,13].

One of the major problems is analysing neomycin by HPLC is its detection and its extremely limited solubility in organic solvents. It has no UV or visible absorption characteristics. Both reversed-phase and normal-phase chromatographic systems



Neomycin B	R_1=H	R_2=CH_2NH_2	R_3=NH_2
Neomycin C	R_1=CH_2NH_2	R_2=H	R_3=NH_2
Neomycin LP_B	R_1=H	R_2=CH_2NH_2	R_3=$NHCOCH_3$
Neomycin LP_C	R_1=CH_2NH_2	R_2=H	R_3=$NHCOCH_3$

Fig. 1. Structure of neomycin.

with refractive index and moving-wire flame-ionization detection modes have been examined without success for the separation of neomycin B and C. Thus, an attempt was made to derivatize neomycin to aid in its detection and to modify its solubility characteristics. This paper reports the development of a normal-phase HPLC method for the separation and quantification of neomycin B and C derivatized with 1-fluoro-2,4-dinitrobenzene (DNFB).

Sanger[14] was the first to apply DNFB as a derivatization reagent for identifying the free amino groups of the protein insulin[14]. The method was later used for the determination of the N-terminal sequence of various peptides[15,16]. Aminoglucoside antibiotics possess several primary amines in their molecules. Recently, Bangert and Grossman[17] reported an HPLC assay method for kanamycin by derivatization with DNFB. They presented a chromatogram of a dinitrobenzene derivative of neomycin using reversed-phase HPLC; however, no data on the separation of the isomers or quantification were reported.

EXPERIMENTAL

Instruments

An LDC M19-60066-022 high-pressure mini-pump (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) or a Waters M6000A pump (Waters Assoc., Milford, Mass., U.S.A.) was used to pump the mobile phase at a flow-rate of about 1 ml/min. Analysis was performed using a 5-μm particle size silica column (Cat. No. SI-5A, Brownlee LiChrosorb SI-100 5 μm, 250 × 4.6 mm I.D., Rheodyne, Berkeley, Calif., U.S.A.) at room temperature. A 20-μl sample containing *ca.* 800 ng of neomycin sulfate was injected quantitatively on to a column using a Rheodyne M70-10, 20-μl loop injector. The column effluent was monitored either at 350 nm using an LDC Spectromonitor I variable-wavelength detector with an attenuation setting of 0.32 absorbance unit full scale (a.u.f.s.) or at 254 nm using an LDC UV III fixed-wavelength monitor with an attenuator setting of 0.064 a.u.f.s.

Reagents

Chloroform, methanol, and tetrahydrofuran used were all of UV grade, distilled in glass, obtained from Burdick and Jackson (Muskegon, Mich., U.S.A.). 1-Fluoro-2,4-dinitrobenzene (DNFB) was obtained either from Aldrich (Milwaukee, Wisc., U.S.A.) or Eastman-Kodak (Rochester, N.Y.,U.S.A.). Analytical-reagent grade anhydrous sodium borate (Mallinckrodt, St. Louis, Mo., U.S.A.) was also used to prepare a buffer solution.

The DNFB derivatization reagent was prepared by pipetting 4 ml of DNFB (melted in a 40° water-bath, if solid) into a 250-ml bottle. A 200-ml volume of methanol was added to the bottle to obtain the final DNFB concentration of *ca.* 0.15 *M*. The reagent was prepared fresh daily just prior to the analysis.

Mobile phases

Neomycin. For the analysis of neomycin, the mobile phase was chloroform–tetrahydrofuran (THF)–water (600:392:8).

Neamine. For isocratic elution the mobile phase was chloroform–THF–water (680:314:6). For gradient elution, an LKB Ultragrad M1130 gradient mixer (LKB, Stockholm, Sweden) equipped with a 3-way Teflon valve (Cat. No. 1-28-900, General Valve Corp., E. Hanover, N.J., U.S.A.) was used. The total time for the gradient is 15 min. The gradient program holds at mobile phase A for 3 min, after which a linear gradient is followed for 8 min to reach 100% of mobile phase B. For the last 4 min 100% mobile phase B is used. The compositions of the mobile phases A and B are 680:314:6 and 542:450:8 chloroform–THF–water, respectively.

In the preparation of the mobile phases, THF was first placed in a measuring cylinder, followed by addition of water using a volumetric pipette. Chloroform was then added to volume. The volumes of chloroform, THF and water may have to be slightly modified in order to obtain the optimal chromatographic performance.

Neomycin reference standard solution

USP neomycin sulfate reference standard (Issue J) was dried for 3 h under less than 5 mmHg pressure at 60°. The bottle was capped and placed in a desiccator for 5 min to cool. About 2.0 g of the dried and cooled neomycin sulfate were rapidly and accurately weighed and placed in a 1000-ml volumetric flask. The contents were diluted to volume with 0.02 *M* borate buffer (pH 9.0). The neomycin sulfate reference standard solution thus prepared was divided into 30-ml serum bottles and kept frozen until used. The 0.02 *M* borate buffer solution (pH 9.0) was prepared by weighing approximately 4.02 g of anhydrous sodium borate into a 1000-ml measuring cylinder and adding water to volume.

Sample preparations

Neomycin sulfate powder. Approximately 50 mg of neomycin sulfate powder were accurately weighed "as is" into a 25-ml volumetric flask. A 0.02 *M* borate buffer solution (pH 9.0) was then added to volume.

Petrolatum-based ointments. The sample preparation procedure used was similar to that reported by VanGiessen and Tsuji[8]. Approximately 5 g of ointment were accurately weighed into a 30-ml conical centrifuge tube. After the addition of 15 ml of chloroform, the tube was loosely capped and placed in a 60° water-bath

to melt and dissolve the ointment. The contents were then shaken vigorously for 15 min on a reciprocating platform shaker (Eberbach, Ann Arbor, Mich., U.S.A.). After centrifugation at 4000 *g* for 15 min, the chloroform was carefully aspirated off. The neomycin pellet on the bottom of the tube should not be disturbed. The contents of the tube were re-suspended by the addition of 25 ml of chloroform with vortexing. The tube was centrifuged and chloroform was aspirated off by using the procedure described above.

A 10-ml volume of 0.02 *M* borate buffer (pH 9.0) was added quantitatively to the tube, followed by 15 ml of *n*-heptane. The tube was shaken vigorously for 10 min on a reciprocating platform shaker (Eberbach). The top *n*-heptane layer was aspirated off and the aqueous layer was taken for analysis.

Fermentation purification samples. The aqueous samples were diluted by the addition of 0.02 *M* borate buffer (pH 9.0) so as to contain *ca.* 1.2 mg/ml of neomycin base.

Derivatization

Five milliliters each of the neomycin reference standard solution and sample solutions were quantitatively transfered into a 250-ml volumetric flask. A 15-ml volume of 0.15 *M* DNFB reagent was added to the flask and the opening of the flask was covered with aluminum foil. The flask was then placed into a 100° silicone oil-bath for 45 min to form dinitrobenzene (DNB)-neomycin. The amount of DNFB is more than 30 times in excess of the amount required for the derivatization of neomycin. The flask was then cooled and the mobile phase was added until the lower, yellow organic phase reached the 250-ml mark. The DNB-neomycin is insoluble in water. The top, aqueous layer was aspirated off and the yellow organic phase chromatographed using the chromatographic conditions described above. The derivatized neomycin is stable for over a week at room temperature when stored in the dark.

Calculations

The concentrations of neomycin B and C and the bioequivalent potency[18] can be calculated by using the following equations:

$$\text{Neomycin B } (\mu\text{g/mg}) = \frac{B_{smp}}{B_{std}} \cdot \frac{W_{std}}{W_{smp}} \cdot F_1 \cdot F_2$$

$$\text{Neomycin C } (\mu\text{g/mg}) = \frac{C_{smp}}{B_{std}} \cdot \frac{W_{std}}{W_{smp}} \cdot F_1 \cdot F_2$$

$$\text{Bioequivalent potency } (\mu\text{g/mg}) = [\text{neomycin B}] + \tfrac{1}{2}\,[\text{neomycin C}]$$

where: B_{std} and B_{smp} are the peak area of neomycin B in the reference standard and samples, respectively, C_{smp} is the peak area of neomycin C in samples, W_{std} and W_{smp} (mg/ml) are the weight of the reference standard and samples, respectively, F_1 is the dilution factor and F_2 is the assigned potency of the USP neomycin sulfate reference standard (767 μg/mg for Issue J).

RESULTS AND DISCUSSION

Derivatization of neomycin

The major difficulty encountered in establishing the reaction conditions was the differing solubility characteristics of neomycin and the derivatization reagent, DNFB. Many solvents were examined for mutual solubility and proper control of pH during the reaction without success. The optimal reaction conditions established uses an aqueous buffer solution to dissolve neomycin and to react with a DNFB-methanol solution.

The 1% tris(hydroxymethyl)aminomethane (THAM) buffer used by Bangert and Grossman[17] to control the pH of the reaction mixture at the optimal pH of 9.0 gave a peak that interfered in the neomycin determination. The use of a 0.02 *M* borate buffer eliminated the interference peak.

Reaction rate studies were carried out at 50°, 80° and 100° to select the proper temperature and time for completion of the derivatization reaction. Neomycin C formed doublet peaks when reacted at 80° or below. However, when reacted at 100° for 30 min, a single neomycin C peak was obtained. Neomycin B can be completely derivatized in 25 min at 100°. Thus, 100° for 45 min was selected as the routine derivatization condition.

The reaction condition described above uses about a 30-fold excess of DNFB reagent to derivatize neomycin. Therefore, a minor modification in the ratio of the reagent to neomycin can be made without undue effect on the quantification of neomycin.

When monitored at 350 nm, the absorption maximum of a DNB derivative of neomycin, the sensitivity of the detection was approximately 1 ng of neomycin base per column injected. However, the DNB-neomycin can also be monitored at 254 nm without significantly affecting the quantification of neomycins.

Characterization of dinitrobenzene derivatives of neomycin

Pure samples of neomycin B and C and neamine were derivatized with DNFB. After derivatization, the sample floating on the aqueous phase as droplets was purified by washing with toluene. The purified samples thus prepared were dried under a stream of dry nitrogen.

The DNB derivatives of neamine and neomycin B and C were examined by field desorption mass spectrometry. Examination of the mass spectrum of neamine indicated the presence of a relatively strong molecular ion peak at *m/e* 986 (M^+), indicating that all four available amino groups in the molecule are derivatized. A much stronger peak was observed at *m/e* 494 ($M^+ - 2DNBC_6H_{11}O_3N_2$), representing neosamine C and/or deoxystreptamine with one less oxygen atom. A peak at *m/e* 956 ($M^+ - NO$) was weak.

The mass spectrometric analysis of derivatized neomycin B and C was extremely difficult owing to the high mass involved. However, examinations of the mass spectra of both neomycin B and C showed a peak at *m/e* 1610 (M^+), which is the theoretical, totally derivatized molecular weight.

Chromatographic parameters

DNB-neomycin was first chromatographed on a reversed-phase column, and

no separation of neomycin B and C was obtained. Chromatography of neomycin on a normal-phase silica gel column gave an excellent separation of neomycin B and C and other impurities (Fig. 2). Use of a 5-μm particle size silica gel column resulted in more than 3000 theoretical plates per 25-cm column. The relative retentions of some compounds are presented in their order of elution in Table I.

Fig. 2. HPLC chromatogram of DNB derivatives of neomycin using a 5-μm particle size silica column. Mobile phase: chloroform–THF–water (600:392:8). Peaks: 1 = neomycin C; 2 = neomycin B; 3 = mono-N-acetylneomycin B (LP_B).

TABLE I

RELATIVE RETENTIONS OF NEOMYCIN B AND C AND IMPURITIES

Compound	*Relative retention*
Neamine	0.15
Neomycin C	0.71
Deoxystreptamine	0.91
Neomycin B	1.00
Neobiosamine	1.69
N-Acetylneomycin B (LP_B)	1.90

The mobile phase used has three components, chloroform, THF and water. The effects of each component on the chromatographic parameters, such as HETP, resolution between neomycin B and C peaks, neomycin B peak asymmetry and capacity factor (k') for the neomycin B peak were studied (Table II). In general, chloroform had a considerable effect on k': however, its effects on the other chromatographic parameters was minimal. THF had significant effects on HETP

and k'. Water, however, was the most important factor affecting the chromatographic parameters examined. An increase in the water content of the mobile phase was essential for increasing resolution, minimizing peak tailing, decreasing the chromatographic time and, above all, increasing the theoretical plate number. A water content of 0.8% is near the saturation point in the mobile phase studied. Thus, the optimal composition of the mobile phase established was 600:392:8 chloroform–THF–water.

TABLE II

EFFECTS OF MODIFIERS ON CHROMATOGRAPHIC PARAMETERS

Modifier examined	*$CHCl_3$:THF:water ratio in mixture*	*HETP (Neo B) (mm)*	*Peak resolution (Neo B/Neo C)*	*Asymmetry (Neo B)*	*k' (Neo B)*
Chloroform	520:392:8	0.081	3.9	1.50	3.8
	560:392:8	0.087	4.2	1.43	4.9
	600:392:8	0.091	4.4	1.38	6.0
	640:392:8	0.110	4.6	1.45	7.8
	680:392:8	0.118	4.7	1.54	9.4
Tetrahydrofuran	600:312:8	0.142	4.1	1.22	12
	600:352:8	0.122	4.4	1.00	7.5
	600:392:8	0.106	4.5	1.38	5.6
	600:432:8	0.096	4.1	1.40	4.4
	600:472:8	0.091	3.6	2.08	3.9
Water	600:392:4	0.449	2.5	3.40	11.1
	600:392:6	0.176	3.6	2.33	7.0
	600:392:7	0.123	3.8	2.00	6.6
	600:392:8	0.095	4.3	1.38	5.6

A problem faced during the initial developmental work was solvent-induced anomalies in the chromatography of samples dissolved in THF. Formations of doublets and even triplets of neamine and neomycin C were observed. THF can strip away water from the silica surface, resulting in an irregular water content on the silica particle. Similar injection solvent-induced anomalies for the assay of vitamin D_2 have been reported previously[19]. The use of the mobile phase to dissolve the derivatized neomycin culminated in an increase in the theoretical plate number (from 1700 to 3200), baseline stability and peak resolution. A typical chromatogram of a neomycin is shown in Fig. 2.

Quantification

Linearity of response to DNB-neomycin was examined by weighing a neomycin sulfate powder in the range 0.45–4.0 mg/ml. The response was linear up to 4.0 mg/ml (1.6 μg per 20-μl injection) with a correlation coefficient (r) of 0.9997 and 0.9990 when monitored at 254 and 350 nm, respectively. The sensitivity of detection at 254 nm was about half that at 350 nm; however, the attenuation needed to monitor DNB-neomycin at 254 nm was still a reasonable 0.064 a.u.f.s.

The precision of the HPLC assay method was determined by use of seven individually weighed (at an approximate concentration of 2 mg/ml) and derivatized neomycin sulfate powder samples (about 800 ng per injection). The relative standard deviation of the assay for the determination of neomycin B was about 1%; 0.9% when monitored at 254 nm (Table III) and 1.1% when monitored at 350 nm.

TABLE III

PRECISION OF THE NEOMYCIN HPLC ASSAY METHOD

Weight of neomycin sulfate (mg/ml)*	*Peak area/weight ratio*	
	Neomycin B	*Neomycin C*
2.038	35,810	4780
2.014	35,160	4757
2.032	36,010	4687
2.030	35,910	4709
2.034	35,940	4757
2.027	36,010	4767
2.024	35,480	4723
	RSD** = 0.90%	RSD** = 0.72%

* 2 mg/ml neomycin level is equivalent to 800 ng per 20-μl injection.
** RSD = relative standard deviation.

Various batches of neomycin sulfate powder were then analysed (Table IV). The bioequivalent potencies calculated from the HPLC data were compared with those obtained by gas–liquid chromatography (GLC)[7] and microbiological assay[20]. The results indicated that the average of the bioequivalent potencies obtained by GLC was the highest (691 μg/ml) and that of the microbiological assay was the lowest (670 μg/mg), with that of the HPLC method (683 μg/mg) between them. The 3%

TABLE IV

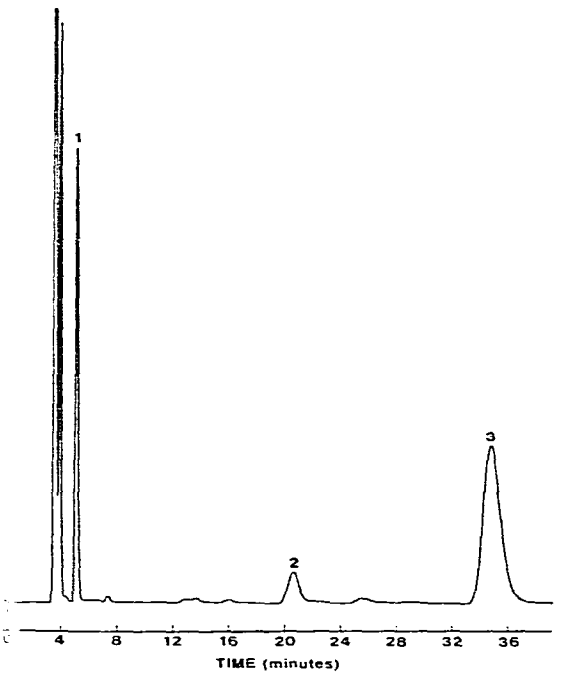
ANALYSIS OF NEOMYCIN SULFATE POWDER

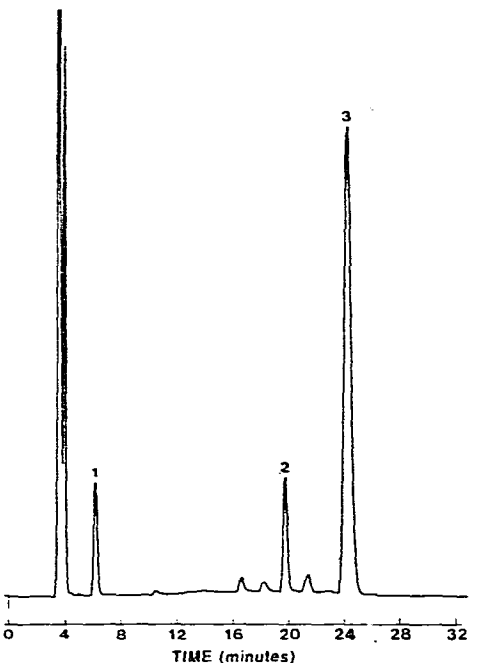
Neomycin batch no.	*HPLC*			*GLC*			*Microbioassay (μg/mg)*
	Neomycin (%)		*Potency (μg/mg)*	*Neomycin (%)*		*Potency (μg/mg)*	
	B	*C*		*B*	*C*		
1	88.2	11.8	660	91.3	8.7	684	666
2	88.3	11.7	687	91.7	8.3	675	704
3	88.6	11.4	672	91.9	8.1	649	691
4	87.1	12.9	694	89.1	10.9	685	658
5	88.7	11.3	700	90.8	9.2	682	633
6	88.4	11.6	705	90.3	9.7	692	670
7	89.1	10.9	682	92.6	7.4	687	658
8	86.6	13.4	681	90.6	9.4	704	637
9	86.1	13.0	697	91.2	8.8	724	711
10	87.9	12.1	696	91.2	8.8	674	620
11	90.0	10.0	706	93.1	6.9	726	689
12	86.5	13.5	696	89.9	10.1	720	708
13	86.6	13.4	701	92.2	7.8	700	695
14	87.1	12.9	692	91.7	8.3	706	658
15	83.1	16.9	683	81.8	18.2	675	625
16	80.1	19.9	632	82.8	17.2	646	674
17	86.1	13.9	652	91.1	8.9	696	691
18	90.6	9.4	641	95.0	5.0	668	695
19	85.7	14.3	696	88.2	11.8	732	641
Average	87.2	12.9	682.8	90.3	9.7	690.8	669.7

TABLE V

RECOVERY OF NEOMYCIN FROM PETROLATUM-BASED OINTMENT

Neomycin (% of production target)	*Neomycin (% of production target)*		*Recovery (%)*
	Added	*Recovered*	
85	84.67	84.67	100.0
	85.17	84.67	99.4
	85.33	86.50	101.4
	85.83	85.83	100.0
100	100.0	99.67	99.7
	100.8	100.2	99.3
	100.8	101.7	100.8
	102.8	104.2	101.3
110	111.0	110.3	99.4
	111.2	112.7	101.3
	111.3	111.5	100.1
	111.7	114.8	102.8
120	120.7	124.2	102.9
	121.8	117.8	96.7
	121.8	123.0	100.9
	122.2	126.2	103.3





ig. 3. Isocratic HPLC chromatogram of DNB derivatives of neamine and neomycin B and C using 5-μm particle size silica column. Mobile phase: chloroform–THF–water (680:314:6). Peaks: 1 = amine, 2 = neomycin C; 3 = neomycin B.

g. 4. Gradient elution HPLC chromatogram of neamine and neomycin B and C. A 15 min gradient ution from mobile phase A (680:314:6) to B (542:450:8) chloroform–THF–water. Peaks: = neamine; 2 = neomycin C; 3 = neomycin B.

TABLE VI

PRECISION OF THE NEAMINE HPLC ASSAY METHOD

Sample	*Concentration (mg/ml)*	*Peak area*
1	0.112	67,710
2	0.112	67,040
3	0.112	67,910
4	0.112	67,700
5	0.112	68,360
6	0.112	67,970
7	0.112	68,100
		RSD* = 0.61%

* RSD = relative standard deviation.

difference in potency between the GLC and the microbiological assay values was statistically significant; however, no significant difference existed between the HPLC and the GLC or between the HPLC and the microbiological assay values.

The percentages of neomycin C in neomycin sulfate powder detected by HPLC were 3.2% higher than those assayed by GLC (Table IV). As HPLC gives a better resolution of the neomycin B and C peaks (Fig. 2) than GLC[7–9], the neomycin C content detected by HPLC method can be considered both dependable and accurate.

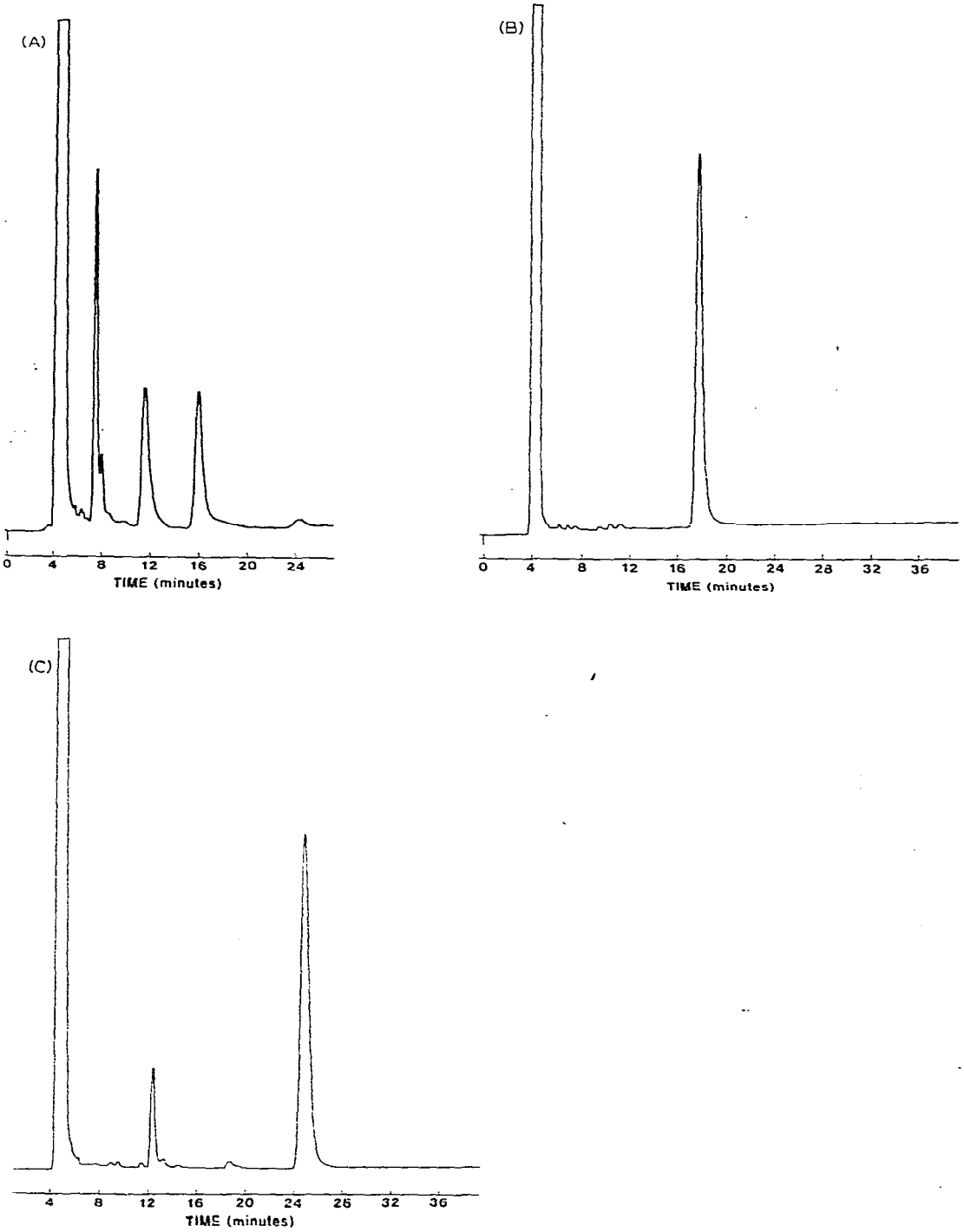
The recovery of neomycin in petrolatum-based ointments was examined by the addition of neomycin to a placebo, which contained bacitracin and polymyxin in the petrolatum base. As shown in Table V, the recoveries of neomycin were 100.2, 100.3, 100.9 and 101.0% when spiked at 85, 100, 110 and 120% of the production target, respectively. The ointment base showed no effect on quantification, regardless of whether monitoring was carried out at 254 or 350 nm.

Assay of neamine

The HPLC procedure developed for quantification of neomycin B and C was slightly modified for the assay of neamine. For isocratic analysis, the mobile phase is modified by increasing the chloroform concentration to retard the chromatography. A typical chromatogram indicating separation of neamine spiked into a neomycin sulfate sample is shown in Fig. 3. There is a pronounced broadening of the neomycin B and C peaks with this isocratic technique. The precision of the neamine assay was determined by independently derivatizing seven samples of a solution containing 0.1 mg/ml of neamine with 15 ml of DNFB solution at 100° for 45 min. This neamine concentration would correspond to 5% by weight in a neomycin sample. The relative standard deviation of the method was less than 1% (Table VI). In gradient elution HPLC, however, the assay is rapid with no loss of resolution of neomycin B and C (Fig. 4).

Application to the analysis of other aminoglucoside antibiotics

The HPLC methods developed can be used to analyse varieties of other aminoglucoside antibiotics. Chromatograms of gentamicin, kanamycin and paromomycin illustrating such an application are shown in Fig. 5.



ig. 5. HPLC chromatograms of DNB derivatives of the aminoglucoside antibiotics (A) gentamycin, 3) kanamycin and (C) paromomycin using a silica column. Mobile phase, chloroform–THF–water: , 650:175:2; B, 210:282:8; C, 250:282:8.

ACKNOWLEDGEMENTS

K. L. Rinehart of the University of Illinois and L. Baczynskyj are acknowledged for the mass spectrometric anlyses. A. R. Lewis is thanked for the statistical analysis.

REFERENCES

1 D. G. Kaiser, *Anal. Chem.*, 35 (1963) 552.
2 T. F. Brodasky, *Anal. Chem.*, 35 (1963) 343.
3 A. E. Kaptionak, E. Biernacka and H. J. Pazdera, *Technicon Symposia*. Mediad Press, New York, 1965, p. 27.
4 G. Nomine and L. Penasse, *U.S. Pat.*, 3,062,807 (1962).
5 H. Maeher and C. P. Schaffner, *Anal. Chem.*, 36 (1964) 104.
6 S. Inouye and H. Ogawa, *J. Chromatogr.*, 13 (1964) 536.
7 K. Tsuji and J. H. Robertson, *Anal. Chem.*, 41 (1969) 1332.
8 B. VanGiessen and K. Tsuji, *J. Pharm. Sci.*, 60 (1971) 1068.
9 M. Margosis and K. Tsuji, *J. Pharm. Sci.*, 62 (1973) 1836.
10 J. W. Mayhew and S. L. Gorbach, *J. Chromatogr.*, 151 (1978) 133.
11 J. P. Anhalt, *Antimicrob. Ag. Chemother.*, 11 (1977) 651.
12 J. P. Anhalt, F. D. Sancilio and T. McCorkle, *J. Chromatogr.*, 153 (1978) 489.
13 H. N. Myers and J. V. Rindler, Upjohn Co., Kalamazoo, Mich., personal communication, 1977.
14 F. Sanger, *Biochem. J.*, 39 (1945) 507.
15 R. R. Porter and F. Sanger, *Biochem. J.*, 42 (1948) 287.
16 F. Sanger, *Biochem. J.*, 40 (1946) 261.
17 F. K. Bangert and D. M. Grossman, *4th Annual FACSS Meeting, Detroit, Mich., 1977*, Abstr. No. 164.
18 J. H. Robertson, R. Bass, R. L. Yeager and K. Tsuji, *Appl. Microbiol.*, 22 (1971) 1164.
19 C. Y. Wu and J. J. Wittick, *Anal. Chim. Acta*, 79 (1975) 308.
20 *Code of Federal Regulations*, U.S. Government Printing Office, Washington, D.C., 1977, Vol. 21, Parts 141–599.