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DERIVATIZATION OF PRIMARY AMINES BY 2-NAPHTHALENESULFONYL CHLORIDE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF NEOMYCIN SULFATE

KIYOSHI TSUJI* and KEVIN M. JENKINS
Control Research and Development, The Upjohn Company, Kalamazoo, MI 49001 (U.S.A.)
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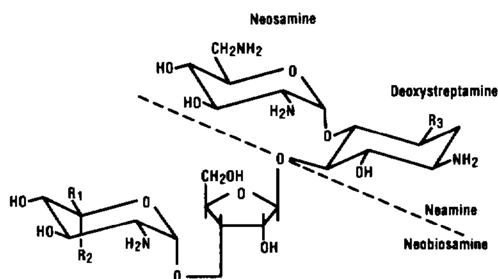
SUMMARY

A normal phase high-performance liquid chromatographic (HPLC) method has been developed for the assay of neomycin sulfate. The method involves precolumn derivatization with 2-naphthalenesulfonyl chloride (NSCl) followed by extraction in chloroform and chromatography using a normal phase silica column with detection at 254 nm. The standard curve for the HPLC assay of neomycin sulfate is linear with a correlation coefficient of 0.9996 over the range of 0.02 to 0.4 mg/ml. Neomycins B, and C, and neamine can be separated and quantified isocratically with relative standard deviations of 0.92% and 1.4% for neomycin (B + 1/2C) and neamine, respectively. Prednisolone is used as an internal standard to aid in quantification. Mass spectrometric data confirms that neomycin is derivatized at all the six primary amines on the neomycin molecule. Eight lots of neomycin sulfate were used to compare the HPLC [NSCl and 1-fluoro-2,4-dinitrobenzene (DNFB)], gas–liquid chromatographic and microbiological assay methods. The average results of the NSCl–HPLC method fell between those of the microbiological and DNFB–HPLC methods. Also, good correlation of the neomycin C contents in neomycin were obtained between the NSCl–HPLC and DNFB–HPLC methods.

INTRODUCTION

Neomycin sulfate is a medium to broad spectrum aminoglucoside antibiotic used mostly in topical preparations and agricultural products. Neomycin is composed of a mixture of two stereoisomers, neomycins B and C (Fig. 1). Cleavage of the ring structure at the ether linkage results in the formation of neobiosamine and neamine. Neobiosamine further degrades to neosamine and ribose. Neomycin, its process intermediates, and degradation compounds all lack UV absorbing chromophore, thereby posing difficulties in the development of a high-performance liquid chromatographic (HPLC) assay method for quantitation of the antibiotic.

Both gas–liquid chromatographic (GLC) and HPLC methods have been reported for the separation and quantification of neomycins B and C. The GLC method



Neomycin B	$R_1 = H$	$R_2 = CH_2NH_2$	$R_3 = NH_2$
Neomycin C	$R_1 = CH_2NH_2$	$R_2 = H$	$R_3 = NH_2$

Fig. 1. Structure of Neomycin.

is difficult to use, as it requires frequent bracketing of samples due to the instability of the silylated derivative[1–3].

HPLC methods for the assay of neomycin may be categorized into the following two classes: (a) reversed-phase ion-pair chromatography with post-column derivatization[4–6] and (b) pre-column derivatization followed with normal phase chromatography[7–10]. Myers and Rindler[4] adapted the paired ion, *o*-phthalaldehyde method for gentamicin[11] to monitor neomycin in fermentation broth. This method requires neomycin sulfate to be converted to the base form by ion-exchange or calcium carbonate treatments prior to chromatography. The pre-column derivatization method with 1-fluoro-2,4-dinitrobenzene (DNFB)[7] requires frequent re-injection of samples due to absence of an adequate internal standard for the method and is beset by the use of a hazardous, DNFB derivatization reagent.

The HPLC method reported in this paper utilizes the 2-naphthalenesulfonyl chloride (NSCl) derivatization method developed for derivatization of secondary amines of spectinomycin[12] and forms a sulfonyl derivative of neomycin for normal phase chromatographic separation and quantification at 254 nm.

EXPERIMENTAL

Instruments

A Perkin-Elmer Series 4 solvent delivery system (Perkin-Elmer, Norwalk, CT, U.S.A.), a Waters Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.) or an LDC minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used to pump the mobile phase at flow-rates of 1–2 ml/min. Analysis was performed using a 5-μm particle size high-speed silica column (Part No. 0258-1000, Perkin-Elmer P-E HS-5 silica, 125 × 4.6 mm I.D.) or conventional 5-μm irregular particle silica columns (Spheri-5, Brownlee Labs., Santa Clara, CA, U.S.A. or LiChrosorb Si 60, E. Merck, Darmstadt, F.R.G., both 250 × 4.6 mm I.D.) at ambient temperature. A 50-μl sample of derivatized neomycin was injected quantitatively onto a column utilizing a Waters WISP 710B injector. The column effluent was monitored at 254 nm using either a LDC Spectromonitor D variable-wavelength detector with an attenuation setting of 0.1 absorbance units full scale (a.u.f.s.) or with a LDC UV III

Monitor fixed-wavelength detector (Model 1203, LDC) at an attenuation setting of 0.064 a.u.f.s. Quantitation of peak area responses was performed by a VAX computer system (Digital Equipment, Maynard, MA, U.S.A.), and/or a Hewlett-Packard 3390A integrator (Hewlett-Packard, San Diego, CA, U.S.A.). A Cahn electronic balance (Model 21, Cahn, Division of Ventron Corporation, Cerritos, CA, U.S.A.) was used to weigh neomycin powders. A Labline oil bath (Catalogue No. 3005-7, Labline, Chicago, IL, U.S.A.) or a Thermolyne Dri Bath (Model No. D816525, Thermolyne, Division of Sybron, Dubuque, IA, U.S.A.) equipped with a heating block was used for derivatization of samples.

Reagents

Chloroform and methanol (both acetone and ethanol free), UV-grade, distilled in glass, were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Acetic acid was analytical reagent grade from Mallinckrodt and 2-naphthalenesulfonyl chloride (NSCl) was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Analytical reagent grade anhydrous dibasic sodium phosphate and monobasic sodium phosphate (Mallinckrodt) were used to prepare a phosphate buffer solution. The phosphate buffer solution was prepared by adding 475 ml of 0.2 *M* dibasic sodium phosphate and 25 ml of 0.2 *M* monobasic sodium phosphate into a 1-l flask and diluting to volume with double distilled water to obtain a 0.1 *M* solution with a pH of approximately 8.0.

The derivatization reagent was prepared by dissolving the NSCl in acetonitrile at a concentration of 40 mg/ml. The reagent was prepared fresh daily prior to derivatization.

The extraction solution was prepared by dissolving the internal standard, prednisolone, in a small amount of tetrahydrofuran (THF) to obtain a final concentration of 2.0 mg per 100 ml of chloroform. It is critical that the internal standard be first dissolved in THF to assure complete dissolution.

The mobile phase was prepared by combining 950 ml of chloroform, 23 ml of methanol, and 25 ml of acetic acid. The amount of methanol was adjusted to obtain optimum chromatographic performance.

Reference standard and sample preparation

Neomycin reference standard solution. Approximately 20 mg of neomycin sulfate reference standard (USP issue K, 765 μg base per mg sulfate) was placed in a Cahn balance aluminum weighing cup. This cup containing the neomycin reference standard was dried for 3 h under less than 5 mmHg pressure at 60°C. After drying, the standard in the aluminum cup was immediately weighed and the contents placed in a 100-ml volumetric flask and diluted to volume with 0.1 *M* sodium phosphate buffer (pH 8.0).

Neamine reference standard solution. Approximately 10 mg of neamine in-house reference standard was dried at 60°C in a vacuum oven for 3 h under less than 5 mmHg pressure and immediately weighed into a 100-ml volumetric flask and filled to the volume with 0.1 *M* phosphate buffer. The solution was further diluted by transferring 3.0 ml to another 100-ml volumetric flask and filled to volume with 0.1 *M* phosphate buffer to obtain a 0.3 mg/100 ml neamine reference standard solution. The neamine concentration, after derivatization, was equivalent to *ca.* 1.5% by weight of neomycin preparation.

Neomycin sulfate sample preparation. Approximately 20 mg of neomycin sulfate bulk powder were accurately weighed "as is" into a 100-ml volumetric flask. A 0.1 *M* sodium phosphate buffer solution (pH 8.0) was then added to volume.

Derivatization

Volumes of 10 ml each of the neomycin reference standard solution, neamine reference standard solution or sample solutions were quantitatively transferred into 40-ml disposable screw cap vials. A 10-ml aliquot of the derivatization solution was added to the vial using a repipet dispenser. The vial was capped tightly to avoid sample loss during derivatization, shaken briefly and placed in a 100°C silicone oil bath or 105°C heating block for 10 min to form the derivative. The amount of NSCl is more than 90 times in excess of the amount required for derivatization. The flask was then cooled to room temperature and approximately 15 ml of the extraction solution was added to each vial for quantitative extraction. The vial was shaken vigorously for 10 min, and centrifuged, if necessary, at low speed (< 300 *g*) for 3–5 min to obtain a clear lower organic layer. Portions of the lower organic layer were then chromatographed.

RESULTS AND DISCUSSION

Derivatization of neomycin

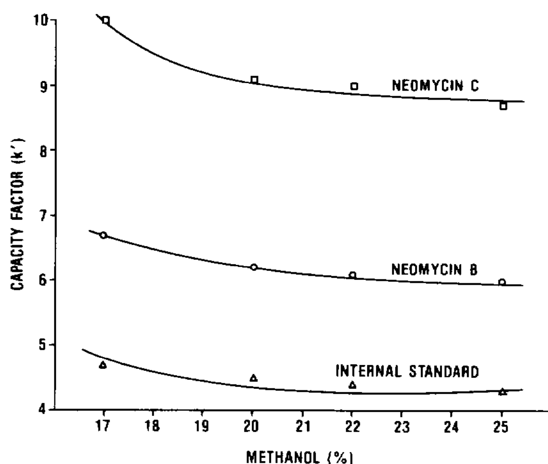
The lack of UV absorbing chromophore of neomycin presented a problem for easy detection by UV. Since there are six primary amines and seven hydroxyl groups on the neomycin molecules, derivatization of neomycin with NSCl[12], which was used to successfully form derivatives of the secondary amines of spectinomycin, was attempted. Neomycin sulfate was reacted with the NSCl reagent by heating at 100°C for 10 min. After cooling, samples were extracted in chloroform and then spotted on thin-layer chromatographic (TLC) silica plates. Plates were developed using a chloroform–tetrahydrofuran (60:40) mixture. Upon drying, the plates were examined by use of a short-wave UV lamp. Formation of a derivative was observed as a spot on TLC plates.

Development of chromatographic condition

The following columns were evaluated: 5-µm LiChrosorb Si 60, 5-µm Spheri-5, Zorbax SIL 5-µm (Du Pont, Wilmington, DE, U.S.A.) and P-E HS-5 silica columns.

Initial attempts to chromatograph NSCl derivatized neomycin utilized the LiChrosorb Si 60 silica column with a mobile phase composition of 70% chloroform and 30% acetonitrile–acetic acid (7:1). A large frontal peak eluted at approximately 5 min followed by a larger peak at about 11.6 min; however, no other peak was eluted. Further work revealed that inclusion of methanol (*ca.* 3%) in the mobile phase was essential for elution of the neomycin C peak.

The P-E HS-5 silica column showed the best separation of the major peaks with short chromatographic run times and high theoretical plates among the columns examined. The capacity factor (k') for the internal standard prednisolone, neomycin B and neomycin C on the column indicated adequate separation of the three components over a wide range of mobile phase compositions, thus suggesting ruggedness of the chromatographic system (Fig. 2). Although the optimum mobile phase com-



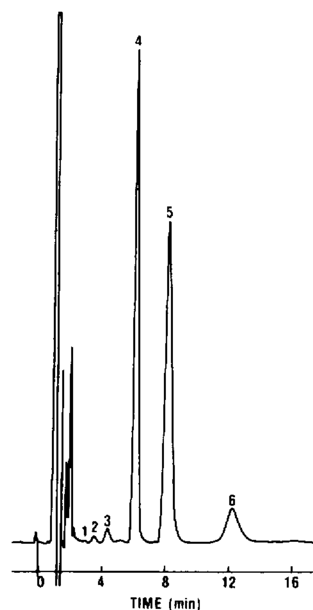


Fig. 2. Effect of methanol (%) in the mobile phase on capacity factor (k').

Fig. 3. Typical HPLC analysis of neomycin. Peak identification: 1 = neamine; 2 and 3 = unidentified; 4 = internal standard; 5 = neomycin B; 6 = neomycin C. Mobile phase, chloroform–methanol–acetic acid (950:23:25). Column: P-E HS-5 Silica, 5-μm, 125 × 4.6 mm I.D.

position was determined to be chloroform–methanol–acetic acid (950:23:25), the amount of methanol may be slightly modified to compensate for changes in column conditions. This chromatographic condition resulted in isocratic elution of neamine, the internal standard, neomycin B and neomycin C at 2.5, 6.2, 8.3 and 12.3 min, respectively (Fig. 3). The use of the high-speed column required a flow-rate of 1.7 ml/min for optimum performance.

For the LiChrosorb Si 60 column the optimum mobile phase composition was determined to be 85% chloroform, 12% acetonitrile and 3% methanol–acetic acid (10:1). Neomycin B, the internal standard, prednisolone, and neomycin C eluted at 8.7, 11.0, and 20.6 min, respectively.

For the Zorbax SIL column, the optimum mobile phase composition was chloroform–methanol–acetic acid (96:2:2). The internal standard, neomycin B, and neomycin C eluted at 21.1, 26.0 and 31.2 min, respectively.

For the Spheri-5 column an optimum mobile phase composition was determined to be chloroform–methanol–acetic acid (95:2.5:2.5). The internal standard, neomycin B and neomycin C eluted at 13.7, 16.2 and 24.2 min, respectively. However, both the Zorbax and Spheri-5 columns necessitated long chromatographic run times (25 min or more) to achieve adequate resolution of neomycin peaks.

Derivatization

Selection of buffer. The optimum pH for the NSCl derivatization of amines was shown to be between pH 8.0 and 9.0 in either 0.1 *M* phosphate or 0.05 *M*

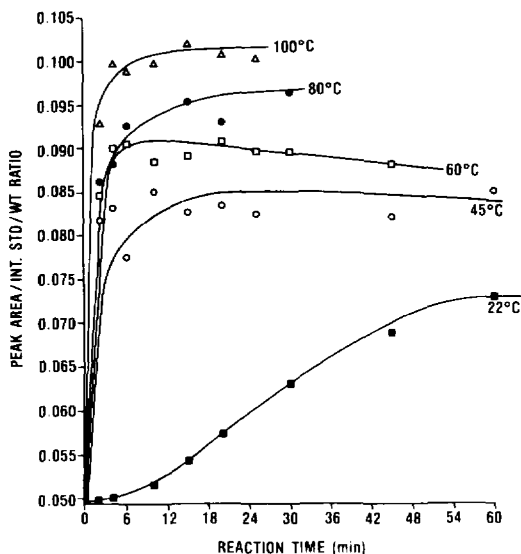


Fig. 4. Derivatization time–temperature for the formation of naphthalenesulfonyl neomycin.

bicarbonate buffer[12]. These two phosphate and bicarbonate buffers were examined for the derivatization of neomycin. The peak area of neomycin derivatized in phosphate buffer was larger (*ca.* 8%) than that of the bicarbonate buffer. Thus, the phosphate buffer (0.1 *M*) was utilized for further work.

Amount of derivatization reagent. The NSCl concentrations of 5, 25, 50, 75, 100, 200 and 400 mg/10 ml were examined for derivatization of neomycin when reacted at 100°C for 10 min. At each concentration of NSCl the peak area ratio of neomycin B and C to internal standard were examined. The data indicated that the peak area ratio increased rapidly to NSCl concentration of 200 mg/10 ml and reached the maximum thereafter while the peak area of neomycin C reached maximum at NSCl concentration of 100 mg/10 ml. To assure complete derivatization, the concentration of 300 mg/10 ml was chosen for time–temperature study. The NSCl concentration of 300 mg/10 ml is approximately a 90-fold excess for derivatization of neomycin; therefore, a minor deviation in the concentration of NSCl would have no undue effect on the quantification of neomycin.

Derivatization time and temperature. A reaction time–temperature study was performed to determine the optimum derivatization conditions for complete derivatization of neomycin. Samples of neomycin sulfate bulk powder were dissolved in the buffer solution and derivatized for 2, 4, 6, 10, 15, 20, 25, 30, 45 and 60 min at each of 22, 45, 60, 80 and 100°C. The results, displayed in Fig. 4, indicated that the peak area/internal standard/weight ratio for derivatization of neomycin was highest when reacted at 100°C and that the reaction reached maximum at 7 min and remained at a plateau from 7 to 30 min. Thus, the derivatization condition of 100°C for 10 min were utilized for further study.

Catalysts, such as 1-methylpyrrole and 2-acetylpyrrole, found essential for the sulfonylation of secondary amines[12] were then examined for the NSCl derivatization of neomycin. The catalysts showed no effects on the rate nor quantity of the deri-

vatization of primary amines nor the relative standard deviation (R.S.D.) of the neomycin assay method.

Extraction of derivatives. After derivatization, the sulfonyl neomycin is extracted in an organic phase to minimize the excess derivatization reagent and excipients for ease of chromatography. Extraction with chloroform yielded the best recovery and ease of operation. The derivatized neomycin is extracted in the lower, organic layer and the commonality of extraction solution and mobile phase yields an uncluttered chromatographic result. The refractive index negative peak found just after the major solvent front peak is due to the acetonitrile used to dissolve the derivatization reagent (Fig. 3). The acetonitrile is not common to the mobile phase.

Internal standard

The lack of an internal standard in the DNFB-HPLC assay method for neomycin was a major disadvantage of the method[7]. The problem encountered for selection of an internal standard was finding a compound that elutes fast enough not to increase the chromatographic time and yet not to interfere with the elution of neomycins B and C, process intermediates, and degradation compounds. Steroids such as prednisolone, triamcinolone and 25 other various steroids (*e.g.*, 6α-fluoroprednisolone-21-hydrogen succinate; 11β,17α,21,21-tetrahydroxy-1,4-pregnadiene-3,20-dione; prednisolone-21-hydrogen succinate) with polar characteristics were evaluated for elution time and peak resolution.

Triamcinolone (6.8 min) eluted later than prednisolone (5.5 min) but was characterized by a broad peak. The other steroids examined were unsatisfactory; they either eluted too early or were characterized by broad peaks. Prednisolone was therefore chosen due to its availability and sharp resolution, although it eluted approximately 5 min after the solvent front (Fig. 3). In order to ensure optimum resolution of the internal standard peak from the neomycin B peak, a slight adjustment of the mobile phase with methanol may be required.

Incorporation of the internal standard in the derivatization reagent was also attempted to improve assay efficiency. However, presence of the internal standard in the derivatization solution resulted in high R.S.D. (2–3%), while the internal standard added in the extraction solution resulted in R.S.D. of less than 1%.

Chromatography

Fig. 3 is a typical chromatogram of neomycin sulfate with the internal standard, prednisolone. Retention times for the internal standard, neomycin B, and neomycin C were 6.2, 8.3 and 12.3 min, respectively.

One of the goals of this assay development was to achieve isocratic assay of neamine, a degradation compound, along with neomycins. This objective was accomplished using the mobile phase composed mainly of chloroform (Fig. 3). Neamine eluted at 2.5 min.

The reagent blank eluted no compound which interfered with chromatography of the neomycins.

Linearity and precision

Neomycin. The linearity of the HPLC method for the assay of neomycin B was determined by analyzing seven different quantities of the neomycin reference stan-

TABLE I

PRECISION OF NSCl–HPLC NEOMYCIN ASSAY METHOD

Sample	*Weight (mg)*	*Peak area*		
		Neomycin (B+1/2C)	*Int. Std.*	*Neomycin/Int. Std/wt.*
1	20.56	4861000	3136000	0.07541
2	20.29	4939000	3137000	0.07758
3	20.72	4904000	3099000	0.07638
4	20.21	4774000	3078000	0.07675
5	20.50	5022000	3184000	0.07693
6	20.27	5020000	3203000	0.07732
7	20.36	4842000	3111000	0.07645
			Average	0.07669
			R.S.D.	0.92%

dard ranging from 12.5% to 200% of the mid-point of the standard curve (20 mg/100 ml). The plot of amount recovered *versus* amount assayed was linear with a correlation coefficient of 0.9996 and an intercept not significantly different from zero.

The precision of the assay was determined by the use of seven individually weighed and derivatized neomycin sulfate powders. The relative standard deviation of the assay for the determination of neomycin sulfate was 0.92% (Table I).

Neamine. The linearity for the assay of neamine was determined by analyzing six different quantities of neamine reference standard ranging from 16.6% to 333% of the mid-point of the neamine standard curve (0.3 mg/100 ml or equivalent to 1.5% of the neomycin). The plot of amount recovered *versus* amount analyzed was linear with a correlation coefficient of 0.9977 and an intercept not significantly different from zero.

TABLE II

BIO-EQUIVALENT POTENCIES (NEOMYCINS B + 1/2C) OF NEOMYCIN SULFATE BULK DRUG (μg/mg); COMPARISON BETWEEN HPLC (NSCl AND DNFB), GLC, AND MICROBIOLOGICAL ASSAY METHOD

Lot No.	*HPLC*		*GLC*	*Microbiological*
	NSCl	*DNFB*		
A	622	636	625	602
B	625	638	632	621
C	629	645	655	604
D	654	666	670	592
E	632	638	647	590
F	649	657	681	593
G	644	655	654	596
H	659	673	688	615
Average	639	651	657	602

The precision of the neamine assay was determined by individually derivatizing seven samples of a solution containing 0.3099 mg/100 ml of neamine reference standard. This neamine concentration corresponds to 1.5% by weight in a neomycin sample. The relative standard deviation of the assay was 1.4%.

Neomycin sulfate powder assay

Neomycin potency. Eight current lots of neomycin sulfate bulk drug were assayed by the NSCl–HPLC, DNFB–HPLC, GLC, and microbiological assay methods and the results, expressed as biological equivalencies (neomycins B + 1/2 C)[13], are summarized in Table II. The average results of the NSCl–HPLC method was 639 μg/mg, while those of the DNFB–HPLC, GLC, and microbiological methods were 651, 657, and 602 μg/mg, respectively.

The paired *t*-test indicated that there is a statistically significant difference at the 95% confidence level between the methods compared. The difference between the NSCl–HPLC and microbiological methods represent a 5.8% difference. This significant difference may be rationalized given the high variability associated with the microbiological method. It is interesting to note that the results of the NSCl–HPLC method falls between those of the microbiological and DNFB–HPLC methods. The average potency determined by the NSCl–HPLC and DNFB–HPLC methods (639 and 651 μg/mg) represented a 1.8% difference. The cause of this minor, 1.8% difference between the two HPLC methods has been traced to the difference in the preparation (weighing after drying) of the reference standard as practiced in two laboratories performing the two different assays; it is extremely difficult to precisely weigh neomycin reference standard since the neomycin standard absorbs 8% moisture in 5 min.

The average percentage neomycin C in neomycin was determined by the NSCl–HPLC method to be 13.8%, while those by the DNFB–HPLC and GLC methods was 14.0% and 20.1%, respectively (Table III). Inadequate separation of neomycin C peak from the neomycin B peak by the GLC method could be the cause

TABLE III

ASSAY OF NEOMYCIN C (%) IN NEOMYCIN SULFATE BULK DRUG; COMPARISON BETWEEN HPLC (NSCl AND DNFB) AND GLC ASSAY METHOD

Lot No.	*HPLC*		*GLC*
	NSCl	*DNFB*	
1	16.2	15.8	22.0
2	16.1	16.2	22.0
3	13.0	13.5	19.7
4	12.3	12.4	18.3
5	13.4	13.1	19.1
6	14.5	15.4	21.1
7	14.5	15.2	21.6
8	10.2	10.1	16.9
Average	13.8	14.0	20.1

TABLE IV

ASSAY OF NEAMINE (%) IN NEOMYCIN SULFATE BULK DRUG

Lot No.	*Day 1*	*Day 2*	*Average*
Current lot			
A	0.107	0.101	0.104
B	0.133	0.117	0.125
C	0.084	0.096	0.090
Old lot			
D	0.353	0.378	0.370
E	0.324	0.328	0.326
F	0.387	0.389	0.388

of the difference obtained by the NSCl–HPLC and GLC methods. The percentage of neomycin C content determined by the two HPLC methods showed no significant difference indicating that the two methods are compatible for the detection and qualification of both neomycin B and neomycin C.

Neamine. Three current and three old neomycin sulfate lots were assayed for neamine content by the NSCl–HPLC method (Table IV). The percentage of neamine in three current lots examined were *ca.* 0.1% while the old lots were *ca.* 0.3%.

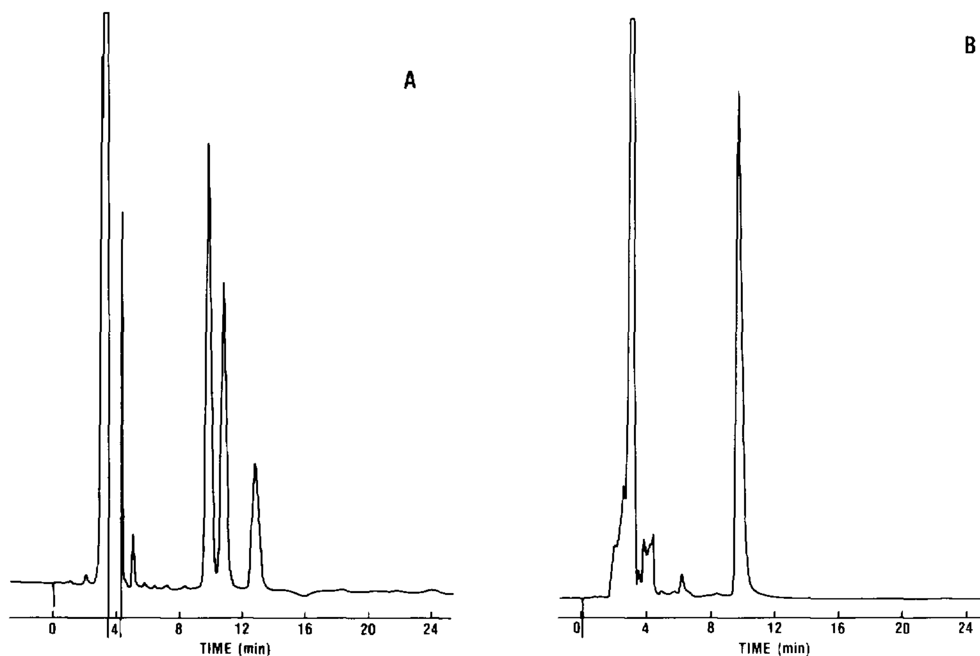


Fig. 5. HPLC analysis of naphthalenesulfonyl derivatives of aminoglucoside antibiotics (A) gentamicin and (B) kanamycin using a silica column. Mobile phase, chloroform–methanol–acetic acid: (A) 700:10:7; (B) 560:400:40.

Mass spectrometric identification of sulfonyl-neomycin

NSCl derivatized neomycin B, neomycin C and neamine were concentrated by extraction and evaporation under a stream of nitrogen for analysis by fast atom bombardment mass spectrometry (FAB-MS). Column fractions corresponding to derivatized neomycins were also collected and analyzed by MS.

Examination of the mass spectrum of neamine indicated the presence of a relatively strong molecular ion plus $2H^+$ at *m/e* 1083, indicating that all four primary amines in the molecule were derivatized. A weak ion observed at *m/e* 893 is the three substituted neamine plus $2H^+$ and the ion at *m/e* 543 represents neosamine and/or deoxystreptamine plus $2H^+$.

The mass spectrum of neomycin C showed a relatively strong molecular ion plus Na^+ at *m/e* 1777 indicating a 6 substituted molecule. The *m/e* peaks at 1566, 1376 and 1185 indicated 5, 4 and 3 substitutions. Also the *m/e* peaks of 1105, 893 and 543, contributed from the neamine portion of the neomycin molecule, were observed. MS of neomycin B and the HPLC column fractions were essentially identical with those of the solvent extracted samples described above.

Potential application to analyze other aminoglucoside antibiotics

The HPLC method developed may be used to analyze varieties of other aminoglucoside antibiotics. Chromatogram of gentamicin and kanamycin illustrating such an application are shown in Fig. 5. Sensitivity for the detection of these antibiotics improves when the column effluent is monitored at 227 nm ($\log \varepsilon = 4.98$).

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