GLC Assay Method for Neomycin in Petrolatum-Based Ointments

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Abstract □ A GLC assay method is described for neomycin in various petrolatum-based ophthalmic and topical ointments. Other components such as bacitracin, polymyxin, and steroids do not interfere significantly in the assay. The ointment is dissolved in chloroform, and the neomycin is removed by centrifugation. The chloroform layer is discarded, and the neomycin is dissolved in water and freeze dried. The neomycin is then silylated and chromatographed. The relative standard deviation of the method is approximately 2%, with a recovery of neomycin from spiked samples of 98–100%.

Keyphrases □ Neomycin, in petrolatum-based ointments—GLC analysis □ Ointments, neomycin in petrolatum—GLC analysis □ Antimicrobial activity—neomycin in petrolatum-based ointments □ GLC—assay method, neomycin

Neomycin has been incorporated into many topical and ophthalmic ointments because of its wide spectrum of antimicrobial activity. The ointments are generally petrolatum-based, with some containing antibiotics such as bacitracin and polymyxin or anti-inflammatory agents such as methylprednisolone acetate.

The microbiological agar diffusion assay method (1) for determination of neomycin in petrolatum-based ointments has been somewhat troublesome, often giving low results with a relative standard deviation larger than desired. In addition, it has not permitted the quantitation of neomycins B and C which have different biopotencies (2).

The GLC determination of neomycins B and C, developed by Tsuji and Robertson (3), provided the first rapid and precise means of quantitating neomycins B and C and encouraged the development of assays for neomycin in petrolatum-based ointments.

EXPERIMENTAL

Apparatus—An F&M model 402 gas chromatograph with flame-ionization detector was used. The gas flow rates were: hydrogen, 50 ml./min.; air, 600 ml./min.; and helium, 70 ml./min. The chart speed was 0.64 cm. (0.25 in.)/min. Operating conditions were: oven temperature, 290°; detector temperature, 310°; and flash heater, 290°. Since the trimethylsilyl-neomycin is extremely sensitive to moisture, it is important that the carrier gas be dried through an efficient drying agent, *e.g.*, 4A molecular sieve.

Column—Glass, 3-mm. i.d. × 61 cm. (2 ft.) packed with 3% OV-1 on Gas Chrom Q, 100–120 mesh[1], was used. To precondition the column before packing, fill the empty column with a 50% solution of dimethyldichlorosilane[2] in hexane and allow to stand for 5 min. Empty the column and wash with 50 ml. of hexane followed by 50 ml. of chloroform. Dry the column with a stream of dry air. Pack the column to within 8–9 mm. of each end, and fill the remaining portions of the column with a small piece of silylated glass wool.

[1] Applied Science Laboratories, Inc., State College, Pa.
[2] Pierce Chemical Co., Rockford, Ill.

Table I—Recovery of Spiked Neomycin from Topical Ointment Containing Bacitracin and Polymyxin[a]

Milligrams Spiked per Gram of Ointment	Milligrams Found per Gram of Ointment	Percent Recovery
3.053	3.010	98.6
	2.926	95.9
3.452	3.327	96.4
	3.376	97.8
3.852	3.730	96.8
	3.862	100.3
4.234	4.184	98.8
	4.084	96.5
4.625	4.523	97.8
	4.592	99.3
	Average	97.8

[a] Mycitracin, The Upjohn Co.

The trimethylsilyl-neomycin must be injected on the column packing to minimize degradation of the trimethylsilyl-neomycin and to prevent tailing of the solvent.

Heat the column at 350° for 30 min. with the carrier gas off. Cool the column to room temperature and turn on the carrier gas. Heat the column to 300° with a flow of 70 ml./min. When the oven reaches about 250°, inject 40 μl. of Silyl-8[3]. At 300°, inject 50 μl. of trimethylsilyl-neomycin. An injection of trimethylsilyl-neomycin, which is an essential part of the column conditioning, should be made before each day's run to minimize column adsorption. Approximately 265 theoretical plates per foot for trimethylsilyl-neomycin are normally obtained with this column.

Internal Standard-Silylation Reagent—Add 50 μl. *N*-trimethylsilyldiethylamine[2] and 2 mg. trilaurin[3]/ml. TRI-SIL Z[2]. Store the solution in a serum vial tightly capped with a rubber septum and a metal retainer.

Reference Standard—Prepare a water solution containing 5.0 mg./ml. of neomycin sulfate, USP Lot I Reference Standard. Before using, dry the neomycin sulfate standard at 60° in a vacuum oven (< 5 mm. Hg) for 3 hr. Pipet 1-ml. aliquots into 2-ml. serum vials[4] and freeze dry. Cap the vials using red rubber closures[5] and metal retainers[6].

Chloroform—Analytical reagent grade was used. Wash with two portions of water to remove any ethyl alcohol present.

Sample Extraction Procedure—Accurately weigh 5 g. of ointment or the equivalent of 25 mg. neomycin sulfate into a 35-ml. centrifuge tube. Add 25 ml. of chloroform and stopper. Place in a 60° water bath for 3 min. and then shake vigorously until the ointment is well dispersed. Centrifuge at 2000×*g* for 10 min. Remove the chloroform by aspiration; the neomycin remains in the bottom of the tube. Wash the neomycin with 15 ml. of chloroform, centrifuge, and remove the chloroform. Add 5.0 ml. of water and 15 ml. of *n*-heptane to the tube and shake until the neomycin is dissolved in the water. Centrifuge at 220×*g* for 3 min. and discard the heptane. Pipet 1 ml. of the water solution into a 2-ml. serum vial and freeze dry. Cap the vial with a red rubber closure and metal retainer.

[3] Supelco, Inc., Bellefonte, Pa.
[4] Kimble Glass No. 62113-3174.
[5] No. V-35, West Co., Phoenixville, Pa.
[6] No. 12-FA-003, West Co., Phoenixville, Pa.

Table II—Recovery of Spiked Neomycin from Ophthalmic Ointment Containing Cortisone Acetate[a]

Milligrams Spiked per Gram of Ointment	Milligrams Found per Gram of Ointment	Percent Recovery
3.088	3.089	100.0
	3.122	100.1
3.516	3.557	101.2
	3.581	101.9
3.833	3.872	101.0
	3.886	101.5
4.203	4.189	99.7
	4.230	100.6
4.617	4.606	99.8
	4.506	97.6
	Average	100.4

[a] Neosone, The Upjohn Co.

Silylation Procedure—Add 1 ml. of internal standard–silylation mixture to each vial, using a 1-ml. tuberculin syringe. Heat the vials in a 75° oil bath for 35 min., swirling occasionally.

Calculation of Biopotency—Measure each peak area. Add one-third of the area of neomycin C[7] to the area of neomycin B, and determine the peak area ratio of neomycin to trilaurin. The biopotency is determined by comparing area ratios between sample and standard solutions.

RESULTS

The topical ointments used in this work contained white petrolatum, microcrystalline wax, mineral oil, methylparaben, butyl-*p*-hydroxybenzoate, cholesterol, and neomycin sulfate. The ophthalmic ointments were prepared from white petrolatum, mineral oil, anhydrous lanolin, and neomycin sulfate. In addition, some ointments contained bacitracin and polymyxin or an anti-inflammatory steroid such as prednisolone acetate and methylprednisolone acetate.

The method described in the "Code of Federal Regulations" for the extraction of neomycin from ointments (1) uses ethyl ether to dissolve the ointment, followed by extraction of the neomycin with water. Unfortunately, the ethyl ether does not completely dissolve the ointment, which results in an incomplete extraction of the neomycin and a recovery of about 90% by GLC. Other solvents were tested, such as hexane, heptane, toluene, and chloroform, with chloroform proving the most efficient in dissolving all of the ingredients except the neomycin. If water is used to extract the neomycin from the chloroform, a precipitate forms in the ophthalmic ointment extracts. The precipitate comes from the lanolin and hinders the extraction of neomycin, giving recoveries of about 90% by GLC.

Ointments containing bacitracin and polymyxin in addition to neomycin[8] pose another problem. With this product the bacitracin and polymyxin are extracted along with the neomycin and interfere with the silylation of neomycin. In the presence of these two antibiotics, only 80–90% of the neomycin is recovered. Increasing the amount of *N*-trimethylsilyldiethylamine or TRI-SIL Z did not improve the recovery of neomycin.

Since ointments are usually anhydrous, and the neomycin is insoluble in chloroform, the neomycin can be removed physically by suspending the ointments in chloroform and centrifuging. Centrifugation separates the neomycin from bacitracin and polymyxin; the bacitracin floats on the top of the chloroform, the polymyxin remains suspended, and the neomycin settles on the bottom.

A series of recovery studies was conducted on a variety of topical and ophthalmic ointments. Table I shows recovery data for a topical ointment that contained bacitracin, polymyxin, and neomycin. The ointment was spiked with five different levels of neomycin, and assays were performed in duplicate. Table II shows recovery data for an ophthalmic ointment that contained cortisone acetate and neomycin. The ointment was spiked with five levels of neomycin. Recoveries better than 98% were obtained from topical and ophthalmic ointments containing either hydrocortisone, prednisolone, methylprednisolone, or fluorometholone (topical only). The grand average recovery was 99.6%. A production lot of each type of ointment was assayed six times to give a grand average relative standard deviation of 1.5%.

[7] The antimicrobial activity of neomycin C is 33% of neomycin B (2).
[8] Mycitracin is the registered trademark of The Upjohn Co. for the mixture of these three antibiotics.

Table III—Assay of Production Lots of Topical Ointment Containing Prednisolone Acetate[a]

	GLC			Microbiological		
Lot	Number of Assays	$\bar{X}$, mg./g.[b]	*SD*	Number of Assays	$\bar{X}$, mg./g.[b]	*SD*
1	2	4.02	0.007	2	3.88	0.042
2	2	3.60	0.035	2	3.71	0.057
3	2	3.48	0.035	2	3.74	0.127
4	2	3.56	0.156	4	3.45	0.119
5	2	3.79	0.099	4	4.21	0.210
6	2	3.86	0.120	2	3.85	0.028
7	2	4.00	0.113	2	4.08	0.035
8	2	3.98	0.050	2	3.86	0.014
9	2	3.84	0.113	2	3.76	0.050
All	18	3.79[c]	0.094	22	3.84[c]	0.114
		RSD = 2.47%[d]			*RSD* = 2.97%[d]	

[a] Neo-Delta-Cortef, The Upjohn Co. [b] Average of replicate assays within a lot. [c] Label is 3.5 mg. neomycin base/g. ointment. [d] A pooled estimate of the within-sample variance.

Production lots of each type of ointment were assayed by GLC and by microbiological assay. The microbiological data were obtained using the extraction method as described in the "Code of Federal Regulations" (1), with *Staphylococcus aureus* ATCC 6538P as the test organism. The data for a topical and an ophthalmic ointment are shown in Tables III and IV. No significant differences were found in the potencies obtained by the two assay methods when they were compared using an approximate paired *t*-test ($p < 0.05$). A typical chromatogram of neomycin extracted from a topical ointment is shown in Fig. 1.

Several 5-year-old ointments were assayed to see if aging caused any interferences in the performance of this GLC method. None was observed. The degradation of neomycin can be followed by the appearance of neamine and neobiosamine, which are silylated and chromatographed along with the neomycin (3).

DISCUSSION

The ointments used in this study contained 3.5–4.2 mg. neomycin base/g. ointment. A straight-line standard curve was obtained over

Table IV—Assay of Production Lots of Ophthalmic Ointment Containing Hydrocortisone Acetate[a]

	GLC			Microbiological		
Lot	Number of Assays	$\bar{X}$, mg./g.[b]	*SD*	Number of Assays	$\bar{X}$, mg./g.[b]	*SD*
1	2	3.90	0.042	4	4.14	0.209
2	2	3.76	0.049	2	3.84	0.021
3	2	3.97	0.014	2	3.80	0.050
4	2	3.93	0.057	3	3.76	0.156
5	2	4.00	0.042	2	3.66	0.177
6	2	4.12	0.078	5	3.70	0.512
7	2	3.78	0.042	4	3.83	0.298
8	2	3.65	0.092	2	3.92	0.092
9	2	4.03	0.042	2	3.92	0.092
10	2	3.72	0.049	2	3.71	0.042
All	20	3.89[c]	0.055	28	3.84[c]	0.268
		RSD = 1.41%[d]			*RSD* = 7.16%[d]	

[a] Neo Cortef, The Upjohn Co. [b] Average of replicate assays within a lot. [c] Label is 3.5 mg. neomycin base/g. ointment. [d] A pooled estimate of the within-sample variance.

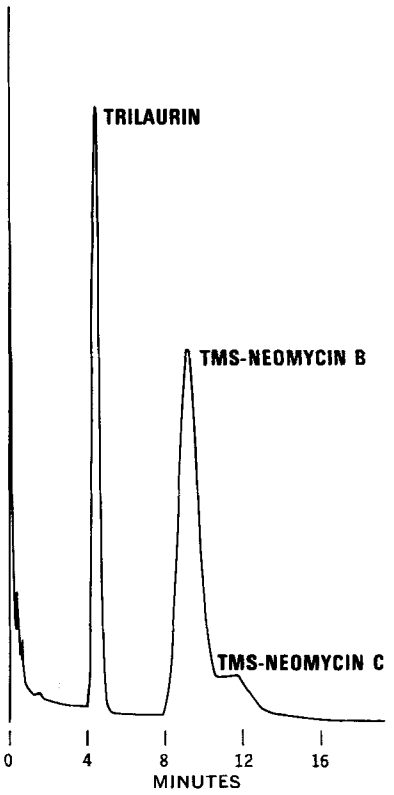

Figure 1—*Typical chromatogram of neomycin extracted from a topical ointment.*

the range of 3.0–4.6 mg. when a silylation mixture of 50 μl. of *N*-trimethylsilyldiethylamine in 1 ml. of TRI-SIL Z was used. The amount of *N*-trimethylsilyldiethylamine was found to be quite critical in obtaining a sharp symmetrical peak of trimethylsilyl-neomycin. If the amount of *N*-trimethylsilyldiethylamine was increased to 60 μl., a trailing shoulder on the neomycin peak was produced. Mass spectrometric data on the major trimethylsilyl-neomycin peak indicated all 13 sites on the neomycin molecule were silylated (3). It is possible that the trailing shoulder was due to silylation of an additional hydrogen of one or more nitrogen groups. Increasing the concentration of *N*-trimethylsilyldiethylamine or silylating reagent did not produce a single peak with the retention time of the shoulder. The amount of *N*-trimethylsilyldiethylamine must be determined experimentally if samples outside the range of 3.0–4.6 mg. neomycin base are to be analyzed.

A 3-day stability study at room temperature was conducted with trimethylsilyl-neomycin contained in sealed vials. The silylated samples were stable for 1 day at room temperature, with an estimated degradation of 3% after 8 hr. Degradation was characterized by the broadening of the trimethylsilyl-neomycin peak. Samples may be kept for longer periods with a minimum of degradation if they are refrigerated at 5° or placed at a freezer temperature of −18°. After 7 days at 5°, the trimethylsilyl-neomycin degraded 10%. After 7 days at −18°, 5% degradation was detected. The silylated samples must always be protected from moisture. Differences in the rate of degradation of neomycins B and C were not detectable.

The most reliable results were obtained with a 61-cm. (2-ft.) column packed with 3% OV-1 on Gas Chrom Q, 100-120 mesh. A 122-cm. (4-ft.) column of 3% OV-1 gave better separation of neomycins B and C, but the increased column temperature needed to chromatograph neomycin reduced column life, with a higher frequency of repair of the gas chromatograph. Increasing column adsorption was noted with 2% OV-1 or less. Column life using the 61-cm. (2-ft.), 3% OV-1 column is around 3 months.

REFERENCES

(1) "Code of Federal Regulations, Title 21, F.D.A.," U. S. Government Printing Office, Washington, D. C., 1967, pp. 67, 71.

(2) K. Tsuji, J. H. Robertson, R. Baas, and D. J. McInnis, *Appl. Microbiol.*, **18**, 396(1969).

(3) K. Tsuji and J. H. Robertson, *Anal. Chem.*, **41**, 1332(1969).

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Cathode Ray Polarography of Riboflavin, Thiamine Hydrochloride, and Niacinamide Content of Pharmaceutical Preparations

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Abstract □ A polarographic method for the determination of riboflavin, thiamine hydrochloride, and niacinamide in multivitamin preparations was evaluated. Distinct polarographic waves of the three vitamins from single extracts were obtained after adjusting the pH to 5.7–6.0. Polarographic results were compared with official chemical analyses. Results indicate that the polarographic method has potential for rapidly screening the vitamin content of multivitamin preparations.

Keyphrases □ Riboflavin, thiamine HCl, niacinamide in dosage forms—determination □ Multivitamin products—riboflavin, thiamine HCl, niacinamide determination □ Polarography, cathode ray fast sweep—analysis

Several investigators described the basic design, application, and advantages of single-sweep cathode ray polarography (1, 2). Several polarographic methods for vitamins require solutions between pH 7 and 10 because of the interference from hydrogen and its catalytic wave in acid solution, especially with thiamine and niacinamide (3–7). In preliminary studies, using conventional polarography, waves of several vitamins in