Chromatographic Assay of Neomycin B and C in Neomycin Sulfate Powders

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Abstract □ A chromatographic assay of neomycin sulfate powders on strongly alkaline ion-exchange resin (hydroxide form) is described. D-(+)-α,α-Trehalose was used as an internal standard. The amount of neomycin B and C in commercial samples was determined with the proposed method, and the results are compared with those obtained by microbiological assay. In addition, minor neomycin components were estimated by TLC and GLC methods.

Keyphrases □ Neomycin—ion-exclusion chromatographic assay of neomycin B and C in neomycin sulfate powders □ Chromatography, ion-exclusion—assay of neomycin B and C in neomycin sulfate powders □ Neomycin sulfate powders—ion-exclusion chromatographic assay of neomycin B and C

The microbiological assay of neomycin prescribed by most pharmacopoeias presents more difficulties than usually encountered with other antibiotics. It shows large interlaboratory variation (1) and may be suspected of a lack of accuracy inherent to the mixture assay where the components have markedly different activities. The influence of the composition on the microbiological assay (2) requires a preliminary quantitation of neomycin C as a fraction of neomycin B to ascertain a similar composition of the unknown and standard preparation. Therefore, alternative methods of assaying neomycin have been developed. A GLC method of totally silylated neomycin was reported previously (3). Poor resolution and critical experimental conditions (4) make this method difficult to perform. A high-pressure liquid chromatographic (HLPC) method of *N*-dinitrophenylated neomycins on normal phase was also reported (5).

The present report deals with a chromatographic assay on a strongly basic ion-exchange resin (hydroxide form) using water as the mobile phase and refractometric detection. It was developed from a medium-pressure chromatographic method used in this laboratory for the determination of relative amounts of neomycin B and C (6) and has the advantage of avoiding pre- and postcolumn derivatization. The use of an internal standard allowed the quantitation of absolute amounts of neomycin B and C. With the proposed method, commercial neomycin samples of different origin were assayed and the amount of several minor components were estimated.

EXPERIMENTAL

Apparatus—A stainless steel column[1] (1.0 cm × 30 cm) was provided with the necessary ferules, fittings, and metal low-dead volume tubing. The usual metal fritted disks were replaced by porous polyethylene disks[1]. The outlet was connected to a differential refractometer[2] (attenuator setting, 8×). The strip chart recorder[3] (10-mV range, chart speed, 10 mm/min) was replaced in some experiments by an electronic integrator[4]. The column was held at constant temperature by submersion in a bath of circulating water[5], which also served for thermostatization of the refractometer. The column inlet was provided with an injector[6]. The mobile phase, carbon dioxide-free distilled water, was delivered at a constant rate by a reciprocating piston pump[7]. A Bourdon-type pressure gauge, which also served as a pulse dampener, was placed between the pump and injector, and a stainless steel guard column (1.0 cm × 10 cm) was filled with the same resin as in the analytical column.

The procedure for conversion of the resins into the hydroxide form was discussed in detail in a previous report (6).

Peak areas were determined with a polar planimeter or with an electronic integrator.

Materials—Samples of neomycin sulfates were obtained from different firms. The European Pharmacopoeia CRS of neomycin sulfate was used as received, the USP reference standard (Issue E) was previously dried at 60° for 3 hr *in vacuo* as prescribed. Four resins[8–10] were used without further purification or sizing.

Mobile Phase—Freshly distilled water was used in all experiments. The flasks were closed with a rubber stopper holding a soda-lime trap.

Standard and Sample Solution—Pure neomycin B and C free bases were obtained by preparative chromatography on a carboxylic ion-exchange resin (7). They were converted into sulfates by adding 0.5 *N* sulfuric acid to yield a product containing 30.0% sulfate, expressed on a dry weight basis. The solution was freeze-dried and its sulfuric acid content and weight loss on drying were determined. From these values the free base content of the neomycin B and C sulfates was calculated.

The $[\alpha]_D$ of D-(+)-α,α-trehalose dihydrate[11] (+181°) was in close agreement with the value given by the manufacturer (+179.9°). Weight loss on drying was 9.73% (theoretical water content 9.67%). The internal standard stock solution was prepared by dissolving 4.00 g of trehalose in 50.0 ml of distilled water.

Neomycin sulfate (100 mg) was weighed accurately in a test tube, and 1.000 ml of internal standard stock solution was added with a micrometer syringe[12]. A calibration curve was obtained by chromatographing mixtures of pure neomycin B and C sulfate in 1 ml of internal standard stock solution. Then, 100 μl of all solutions was injected onto the column.

Weight Loss on Drying—Weight loss was determined after heating a 100-mg sample in a drying pistol[13] at 60° in a vacuum of 0.1 mm Hg for 3 hr over phosphorus pentoxide. The mean absolute standard deviation of the method was 0.13, calculated for five sets of four assays[14].

Sulfuric Acid Content—A previously reported titrimetric method (8) was modified slightly. A 100-mg sample of neomycin sulfate was applied to a 1 × 5 cm column of strongly acidic ion-exchange resin[15]. The column was washed with water and the eluate collected in a 50-ml volumetric flask. A 20-ml aliquot was diluted with 20 ml of methanol and buffered with 2 drops of 0.5 *M* magnesium acetate. After addition of 3 drops of 0.25% thorin[16] solution and 3 drops of 0.0125% aqueous methylene blue solution, the titration was carried out under vigorous stirring with 0.01 *M* barium chloride until the color changed from green to pink. The mean absolute standard deviation of the method was 0.15, calculated for five sets of four assays[14].

Thin-Layer Chromatography of Minor Components—Some eluate fractions from the resin column were evaporated to dryness and dissolved

[1] Alltech Europe, Belgium.
[2] Waters Differential Refractometer R 403.
[3] Kipp BD 40.
[4] Hewlett-Packard 3390 A.
[5] Varian type 4100.
[6] Valco loop injector.
[7] Milton Roy MiniPump.
[8] Bio-Rex 9 resin (200–400 mesh), Bio-Rad Laboratories, Richmond, Calif.
[9] Bio-Rad AG 1-X2 and AG 1-X4 resins, Bio-Rad Laboratories, Richmond, Calif.
[10] Durrum DA-X2 resin, Pierce Chemical Co., Rockford, IL 61105.
[11] Fluka, Büchi, Switzerland.
[12] AGLA-Burroughs, Wellcome & Co., London, U.K.
[13] Desaga, Heidelberg, Germany.
[14] H. Vanderhaege, unpublished results.
[15] Dowex 50-X8 (100–200 mesh).
[16] Disodium salt of *O*-(2-hydroxy-3,6-disulfo-1-naftylazo)benzene arsonic acid.

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Table I—Chromatographic Parameters on Different Strongly Alkaline Resins [a]

Parameter \ Resin	Resin[b]	Resin[c]	Resin[d]	Resin[e]	Two-Column System
t_R neomycin C, min	9.7	8.0	5.1	6.6	9.2
t_R neomycin B, min	20.4	17.0	9.7	14.2	18.4
t_R Internal Standard, min	30.0	43.0	17.0	25.1	29.3
N (calculated on neomycin B peak)	277 (923/m)	125 (417/m)	192 (1920/m)	200 (666/m)	220 (733/m)
Rs neomycin B – neomycin C	2.8	2.0	2.4	2.5	2.4
Rs neomycin B – paromomycin I	1.3	3.3	2.0	2.3	1.7
Rs paromomycin I–Internal Standard	Poor	Good	Poor	Good	Good
Symmetry factor neomycin B	0.77	0.91	0.95	0.92	0.90

[a] Hydroxide form resins in a 1 × 30-cm column at a flow rate of 270 ml/hr. [b] Bio-Rad AG I-X2 resin, minus 400 mesh. [c] Bio-Rad AG I-X4 resin, minus 400 mesh. [d] Durrum DA-X2, 1 × 10-cm column, flow rate 170 ml/hr. [e] Bio-Rex 9, 200–400 mesh.

in 0.5 ml of water. This solution (1, 2, 3, and 4 μl) was spotted on precoated silica gel plates[17], activated for 2 hr at 130°, and developed over 12 cm with a 15% (w/v) aqueous solution of potassium dihydrogen phosphate. It has been shown that these TLC plates[17] contain a polycarboxylic acid binder which is responsible for the separation of the aminoglycosides (9). After development, the dried plates were sprayed with a ninhydrin reagent (0.3 g in 100 ml of *n*-butanol containing 3% acetic acid) and heated for 5–7 min at 130°. Reference solutions of paromamine, mono-*N*-acetylneamine, mono-*N*-acetylneomycin B, and neamine contained 400 μg/ml. Comparison of the color intensities of the reference spots and the spots from the commercial samples allowed a semiquantitative determination of minor neomycin components.

GLC Determination of Acetyl Groups—In addition to the TLC method, the presence of acetylated products in commercial neomycin was also determined by a GLC method, preceded by acid hydrolysis and extraction of the liberated acetic acid with *tert*-butylmethyl ether. The stationary phase was 10% SP 1200[18] and 1% H_3PO_4 on 800/100 Chromosorb W AW (152.5 cm; o.d., 6 mm; i.d., 4 mm) at 95°. The flow rate of the carrier gas (nitrogen) was adjusted to 60 ml/min. The flame ion detector oven was set at 220°. Acid hydrolysis was performed on 100 mg of neomycin sulfate to which were added 0.4 ml of butyric acid internal standard solution (containing 1 mg/ml), 1 ml of concentrated sulfuric acid, and 0.8 ml of water. The test tube was sealed and heated at 100° for 4 hr in an oil bath. The reaction mixture was then extracted three times with 2.5 ml of *tert*-butyl methyl ether. The extracts were dried on anhydrous sodium sulfate, filtered, and reduced to ~0.5 ml with a stream of dry nitrogen; 5 μl of this concentrated extract then was injected on the column.

A calibration curve in the range of 50–500 μg/ml acetic acid was obtained by running known dilutions of acetic acid through the procedure described for the unknown samples. The peak height of acetic acid (retention time ~1 min) over the peak height of butyric acid (retention time ~2 min) was plotted against the amount of acetic acid present. The linear regression was $y = 0.471x - 0.008$ where the correlation coefficient is 0.9993.

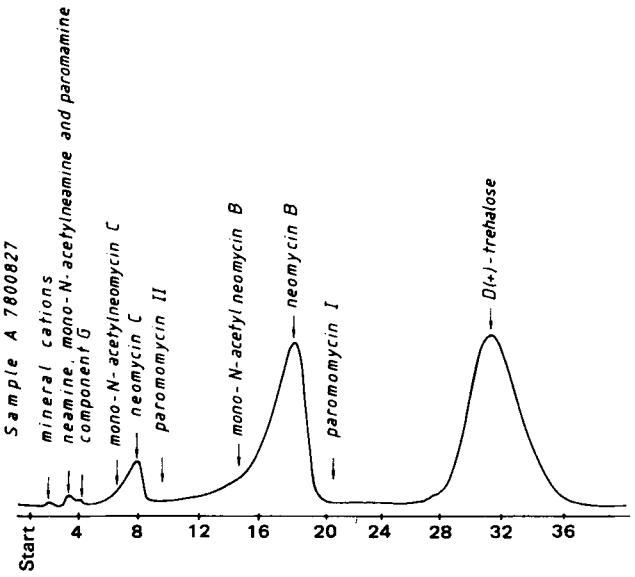

Figure 1—*Chromatogram of 10 mg of neomycin sulfate and 8 mg of internal standard, obtained on a 1 × 10 cm column of resin[21] connected to a 1 × 20 cm column of another resin[20]; flow rate, 270 ml/hr.*

[17] Merck Si60 precoated plates.

[18] Supelco, Bellefonte, Pa.

RESULTS AND DISCUSSION

Internal Standard—When examining a chromatogram of neomycin on strongly alkaline resin in the hydroxide form (Fig. 1), an internal standard should elute after neomycin B. The only aminoglycoside that is commercially available and has a larger retention time than neomycin B is paromomycin I (10). This product cannot be used, as it may be present in some neomycin samples. Since refractometric detection does not necessitate a compound with amino groups, as would be the case with ninhydrin–colorimetric or fluorimetric detection, nonreducing oligosaccharides were examined. Reducing sugars cannot be used because they react with the strongly basic resin (11). The retention times of saccharose and raffinose were too long, but D-(+)-α,α-trehalose was suitable (Fig. 1).

Selection of the Resin—Three resins with benzyltrimethylammonium groups and one resin with methylpyridinium groups were examined (Table I). This last type of resin[8] gave good separations of neomycin B and C and the internal standard, trehalose. However, it was observed that the resin in the hydroxide form deteriorated rapidly. When the column was run for 1 week, injecting only two samples a day, the area of the neomycin B peak, relative to that of the trehalose peak, decreased regularly (Fig. 2). The resin degradation also could be seen from the drastically changing column parameters. The t_R of neomycin C, B, and the internal standard decreased after 1 week to retention times of 5.3, 10.2, and 15.2 min. The resolution between neomycin B and C changed from 2.5 to 1.7, and the resolution between neomycin B and the internal standard changed from 2.3 to 1.1. It is probable that the resin decomposes at the

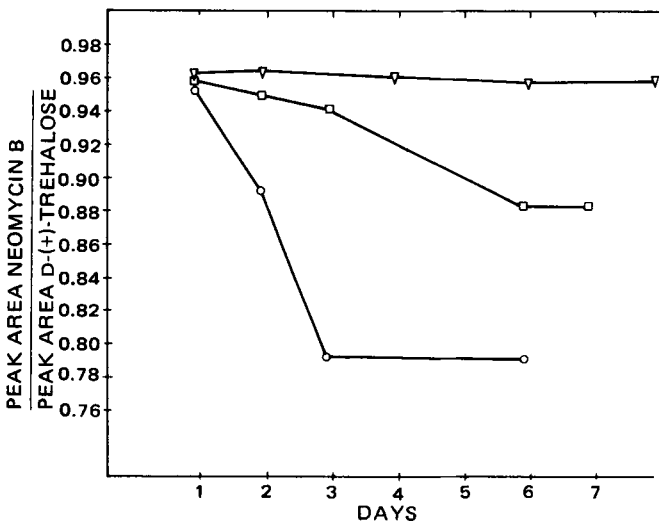

Figure 2—*Resin stability as a function of time. Key: (▽) resin[20] with guard column (>400 mesh); (□) resin[20] (>400 mesh); (○) resin[8] (200–400 mesh).*

Table II—Composition of Neomycin Samples of Different Origin

Sample	Number of Assays	Neomycin B Base[a], %	Neomycin C Base[a], %	Sulfuric Acid[b], %	Weight Loss on Drying[b], %	Paromomycin I, %	Mono-*N*-acetylneamine or Paromamine, %	Neamine, %	Component G	SUM[a], %	Mono-*N*-acetyl Neomycin B, % (TLC)	(GLC)	$[\alpha]_D^{20}$[c]
A	3	54.6 ± 1.0	7.0 ± 0.2	29.4	6.3	1–1.5	<0.5	–	–	98.6 ± 1.5	±0.75		+55.8°
B	9	54.4 ± 0.5	6.9 ± 0.2	30.1	5.9	1.2–1.7	–	–	–	98.7 ± 0.9			+56.8°
C	6	53.6 ± 0.7	8.9 ± 0.2	27.6	7.5	0.5–1	–	–	–	98.4 ± 1.2			+57.6°
D	8	59.4 ± 0.7	5.4 ± 0.3	26.5	9.4	–	<0.5	<0.5	+	100.6 ± 0.7	±3	4.2	+56.3°
E	8	56.3 ± 1.1	5.6 ± 0.2	26.0	11.9	–	<0.5	<0.5	+	99.8 ± 1.2	4 to 5	4.8	+55.9°
F	3	58.2 ± 1.8	4.9 ± 0.7	29.9	4.1	–	1.5–2	<0.5	+	97.7 ± 1.6	±0.75		+53.4°
G	8	56.1 ± 0.9	5.5 ± 0.4	30.8	6.5	–	2–3	<0.5	+	101.4 ± 1.7	±1		+54.4°
H	3	52.4 ± 0.7	6.0 ± 0.7	23.7	13.6	<0.5	±1	<0.5	+	96.5 ± 1.5	±1	0.9	+55.7°
I	8	55.2 ± 0.6	6.8 ± 0.7	25.6	13.0	<0.5	±1	<0.5	+	100.5 ± 0.8	±0.25		+55.6°
USP Reference Standard (Issue E)	4	70.8 ± 0.2	Trace	29.7	0	–	–	<0.5	–	100.5 ± 0.2	–	–	–
Standard European Pharmacopoeia	6	64.6 ± 0.2	6.3 ± 0.25	29.3	0	–	–	–	–	100.1 ± 0.5	–	–	+56.3°
First International Reference Standard Preparation	4	51.4 ± 1.6	16.4 ± 0.6	29.0[d]	0	–	–	3%[d]	–	99.8	–	–	–

[a] Confidence limits (95%) (*t* test). [b] Values represent the mean of two separate determinations. [c] Calculated on dry basis (*c* 1, H_2O). [d] Ref. (1).

site of the methylpyridinium groups yielding acidic functions. This would account for the regular decrease of the neomycin peaks.

One of the resins[19], which has a narrow particle size range (20 ± 5 μm for the resin in the chloride form) gave sharp peaks and a good separation of neomycin B and C. The separation of paromomycin I and the internal standard was not optimal (Table I). The main drawback was the low resistance to pressure of the resin in the hydroxide form. After 3 days at a pressure of 5 kg/cm², the resin bed was retracted to two-thirds of its original volume.

A second resin[20] gave good separation of neomycin B and C, but a poor resolution of paromomycin I and the internal standard, whereas for another resin[21] the opposite was true. With these resins a decrease with time of the neomycin peaks occurred also, although to a lesser extent than with a different resin[8] (Fig. 2). The pronounced change in column parameters was not observed.

Nonelution of a portion of the neomycin load is probably due to some carbon dioxide present in the water used as the mobile phase. Low concentrations could have an influence as large volumes of water are pumped through the columns during 1 week. When an aqueous solution of neomycin B base was saturated with carbon dioxide and applied to the column, no peak could be detected. When a freshly prepared column of resin[20] was loaded with 3 ml of carbon dioxide-saturated water or a 0.025% $NaHCO_3$ solution, the peak area of neomycin B relative to the peak area of trehalose decreased by 15 to 20%, although neomycin sulfate was applied. Formation of a carbonate link of the type $-\phi-CH_2-N^+(CH_3)_3$ CO_3^{-2} neomycin H^+ could explain the influence of carbon dioxide and carbonates on the elution of aminoglycosides. Similar problems have been encountered previously (12) when analyzing kanamycin free base on resin[22] in the hydroxide form. It was found that kanamycin base in aqueous solution converted partially into kanamycin carbonates by reaction with atmospheric carbon dioxide. The carbonates could only be eluted from the resin with water at pH 13 or 14 (12). The problem of carbonate accumulation on the analytical column was solved by connecting a guard column filled with one of the two resins[20,21] in the hydroxide form between the pump and the injector. This ensured a constant neomycin–internal standard ratio for at least 1 week (Fig. 2).

None of the two resins[20,21] gave an entirely satisfactory separation. Good separation of all components was obtained by replacing the 1 × 30 cm of resin[20] by a 1 × 10 cm column of resin[21] connected to a 1 × 20 cm column of resin[20] (Table I).

Calibration—Pure free bases of neomycin B and C were converted into their sulfates for reasons described. Since neomycin B and C had the same detector response in the experimental conditions (6), a mixture of known composition provided two points of the calibration curve per chromatogram. The peak area of the neomycin components over the internal standard peak area was plotted *versus* the calculated amount of free base present in the sample solution. In the 7–70 mg range of neomycin free base, the calibration curve was linear. The regression equation is $y = 0.0036 + 0.0130x$ and the correlation coefficient is 0.9995.

Interfering Compounds—Commercial neomycin contains mono-*N*-acetyl derivatives of the B and C compounds and of neamine (13) (the acetyl group is located at the amino function in the 3-position of the deoxystreptamine moiety) (14). Less active mono-*N*-acetylneomycins cause an overestimation of the B and C components, since they are eluted in the ascent of the main peaks (Fig. 1). Nevertheless, a TLC system allows the separation of neomycin B (or C) and mono-*N*-acetylneomycin B (or C) (Fig. 3). The eluate fractions corresponding to the first half of the neomycin B peak were collected and evaporated to dryness. The res-

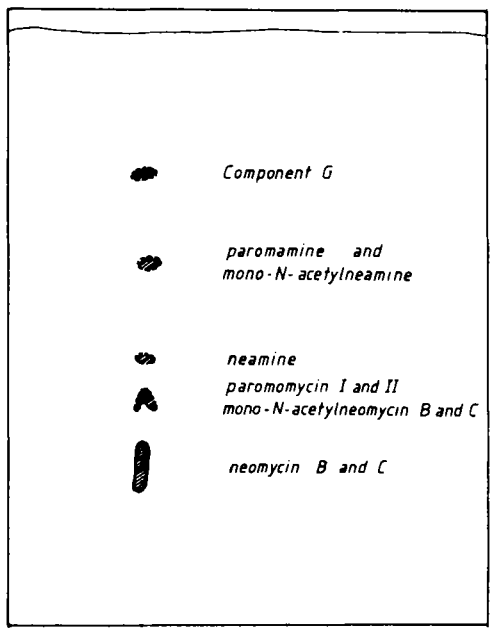

Figure 3—*TLC of a 10-μg sample of neomycin sulfate (silicagel plate[17], 15% potassium dihydrogen phosphate).*

[19] Durrum DA-X2 resin, Pierce Chemical Co., Rockford, IL 61105.
[20] Bio-Rad AG I-X2 resin, Bio-Rad Laboratories, Richmond, Calif.
[21] Bio-Rad AG I-X4 resin, Bio-Rad Laboratories, Richmond, Calif.
[22] Dowex 1-X2.

Table III—Potencies of Analyzed Samples Expressed on Dry Basis

Sample	Average Neomycin B Content, %	Average Neomycin C Content, %	Potency, U/mg	Microbiological Assay	
A	59.6[a]	7.5	725	724 IU/mg[c]	749 IU/mg[e]
B	59.3[a]	7.3	721	732 IU/mg[c]	721 IU/mg[e]
C	57.9[a]	9.6	718	696 IU/mg[c]	746 IU/mg[e]
D	62.3[b]	6.0	747	775 IU/mg[c]	
E	59.3[b]	6.4	715	744 IU/mg[c]	770 IU/mg[e]
F	60.7	5.1	724		
G	60.0	5.9	720		
H	60.6	6.9	733	700 μg/mg[c]	788 IU/mg[e]
I	63.4	7.9	770	710 μg/mg[c]	782 IU/mg[e]
USP Reference Standard (Issue E)	70.8		810	767 μg/mg[d]	
Standard European Pharmacopoeia	64.55	6.25	775	775 IU/mg[d]	
First International Reference Standard Preparation	51.4	16.4	682	680 IU/mg[d]	

[a] Including paromomycin I. [b] Corrected for mono-*N*-acetylneomycin B. [c] Declared by manufacturer. [d] Assigned potency. [e] Determined by Dr. Dubost, Rhône-Poulenc, Paris, France.

idue was then dissolved in 0.50 ml of water, and 1, 2, 3, and 4 μl were spotted on the activated TLC plates, together with 1, 2, 3, and 4 μl of the mono-*N*-acetylneomycin B reference solution. After development and resolution with ninhydrin, a semiquantitative determination of the amount of mono-*N*-acetylneomycin B was made by estimating which spot of the unknown series corresponds best to a spot of the reference dilution. The amount of acetylated products can also be estimated by GLC assay of acetic acid liberated by acid hydrolysis. The correlation between TLC and GLC figures is good, as can be seen from Table II. Mono-*N*-acetylneomycin C, if present, would only represent a fraction of neomycin C, so it was not necessary to estimate such a small amount. If no mono-*N*-acetylneomycin B is available as the reference, it can be replaced by paromomycin, taking into account the sulfuric acid and moisture content of this commercial product. No detectable difference of the ninhydrin response between mono-*N*-acetylneomycin B or C and paromomycin I or II was found.

Although not interfering directly in the assay of the main products, other minor components were analyzed also by TLC. The eluate fractions containing neamine, paromamine, *N*-acetylneamine, and component G were also evaporated to dryness and dissolved in 0.50 ml of water. TLC and semiquantitative determination of these components were performed in a similar way as for mono-*N*-acetylneomycin B. Component G, an *O*-(diaminodideoxyhexosyl)-*myo*-inositol (7) was available, but it was not sufficiently pure to allow such an estimation. Its presence in neomycin samples will be indicated only qualitatively. As can be seen from Fig. 3, it cannot be distinguished whether mono-*N*-acetylamine or paromamine is present. However, from results obtained in the laboratory with ion-pair chromatography on a reversed-phase column[23] it can be seen that mono-*N*-acetylneamine is generally present in a larger quantity than paromamine.

Assuming an equal detector response of paromomycin I and neomycin B (or C) to the differential refractometer, the paromomycin I content of neomycin samples was calculated as for the main products.

Composition of Commercial Samples—A survey of the composition of recent neomycin sulfate samples of French (A–C), Norwegian (D, E), Japanese (F, G), and American (H, I) origin is given in Table II. The neomycin B free base content ranges from 52–59%. These values include mono-*N*-acetylneomycin B which is eventually present. The amount of neomycin C free base is between 4 and 9%. In addition to neomycin B and C, sulfuric acid content, weight loss on drying, and an estimation of the minor components are tabulated. Disregarding components that represent <0.5%, a summation of all other components closely approaches 100%.

Although an assay or estimation of neamine is prescribed by many pharmacopoeias, the present results prove this substance to be virtually absent from all samples analyzed. However, mono-*N*-acetylated neomycins and paromomycin I may be present in appreciable amounts. The nature of the minor neomycin components is typical for a particular manufacturer, possibly as a result of different fermentation and isolation processes.

Correlation Between Chromatographic and Microbiological Assay—To make a correlation of the amounts of neomycin B and C found for different samples with the results of the microbiological assay, it is necessary to know the relative response of neomycin C to neomycin B. However, this response depends on the microorganisms and media used in a diffusion or a turbidimetric assay. Relative responses of 30–40% have been obtained (15). Past experience indicates that the variation is even larger, and it has been assumed that the potency of neomycin C is half that of neomycin B (5). Using the amount of neomycin B and C in the European Standard (which is from the same batch as the International Standard) and its titer (775 IU/mg), it can be calculated that 1 μg of free base of the European (or International) Standard corresponds to:

$$\frac{775}{645.5 + \frac{62.5}{2}} = 1.145 \text{ potency units.}$$

Although potency units correspond to international units, an alternative symbol is preferred since international units are, strictly speaking, only valid for microbiological assays.

This conversion was applied to the different neomycin samples (Table III). If they contained paromomycin I, the amount of this component was added to the amount of neomycin B, because their antimicrobial activity is almost identical. It is interesting to note that the potency units of the First International Standard are nearly identical to the assigned IU value. For samples A–E, H and I, the correlation is also acceptable. The results of the microbiological assays of two samples (C and E) differ somewhat from one laboratory to another, even if they were obtained by analysts having much experience with this type of assay.

An attempt can be made to correlate the International Standard with the USP Reference Standard, although the potency in micrograms per milligram is defined in a different way and despite the fact that the latter preparation contains almost exclusively neomycin B. From the calculated 810 potency U/mg and the 767 μg/mg figures, it can be deduced that 1 μg/mg = 1.056 IU/mg

These calculations are not essential for the proposed chromatographic assay, which gives information concerning relative and absolute amounts of neomycin B and C and related compounds. Nevertheless, the calculations indicate that the proposed method could replace a microbiological assay.

The proposed method allows a chromatographic quantitation of neomycin B and C in neomycin sulfate powders without cumbersome sample derivatizations. The absolute standard deviation (mean value) is 0.67% for the determination of neomycin B and 0.33% for neomycin C. It can be performed with relatively simple apparatus which can be automated easily. Taking the precaution of not analyzing free bases, reliable values can be obtained.

[23] Method described in a private communication of Dr. A. Sezerat, Roussel-UCLAF Co., Romainville, France.

REFERENCES

(1) J. W. Lightbown, A. H. Thomas, B. Grab, and H. Dixon, *J. Biol. Stand.*, **7**, 227 (1979).

(2) W. T. Sokolski, C. G. Chichester, and D. G. Kaiser, *J. Pharm Sci.*, **53**, 726 (1964).

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

(4) M. Margosis and K. Tsuji, *J. Pharm. Sci.*, **62**, 1836 (1973).

(5) K. Tsuji, W. F. Goetz, W. Van Meter, and K. A. Gusciora, *J. Chromatogr.*, **175**, 141 (1979).

(6) W. Decoster, P. Claes, and H. Vanderhaeghe, *ibid.*, **211**, 223 (1981).

(7) P. Claes, F. Compernolle, and H. Vanderhaeghe, *J. Antibiot.*, **27**, 931 (1974).
(8) E. Roets and H. Vanderhaeghe, *J. Pharm. Pharmacol.*, **24**, 795 (1972).
(9) M. Dubost, C. Pascal, B. Terlain, and J. P. Thomas, *J. Chromatogr.*, **86**, 274 (1974).
(10) S. Inouye and H. Ogawa, *J. Chromatogr.*, **13**, 536 (1964).
(11) A. Neuberger and A. P. Fletcher, *Carbohydr. Res.*, **17**, 89 (1971).
(12) K. S. Andrews and H. W. Coleman, *Annual General Meeting of Electrophoresis and Chromatography*. London, Nov. 12, 1975.
(13) W. S. Chilton, *Ph.D. Thesis*, University of Illinois, 1963.
(14) K. L. Rinehart, "The Neomycins and Related Antibiotics," Wiley, New York, N.Y., 1964.
(15) K. Tsuji, J. H. Robertson, R. Baas, and D. J. McInnis, *Appl. Microbiol.*, **18**, 396 (1969).

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Potential Alkylating Agents Derived from Benzimidazole and Benzothiazole

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Abstract □ Several benzimidazole and benzothiazole alkylating agents, bearing structural modification of certain drugs, were synthesized and evaluated for anticancer activity. Among the products, the dihydrochloride salt of 2-[*p*-[2-(bis(2-chloroethyl)amino)ethoxy]phenyl]benzimidazole (VI) exhibited a high antileukemic activity in P-388 lymphocytic leukemia.

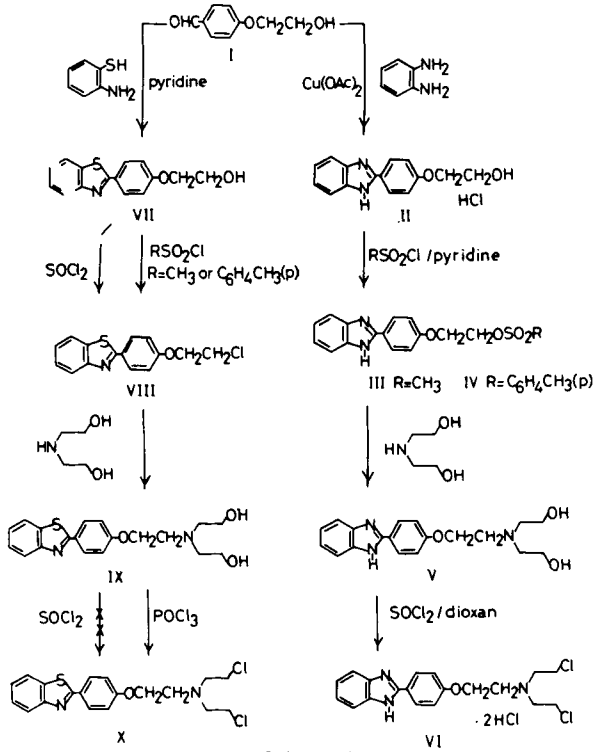
Keyphrases □ Alkylating agents—derived from benzimidazole and benzothiazole, potential □ Benzimidazole—potential alkylating agents, benzothiazole □ Benzothiazole—potential alkylating agents, benzimidazole

The studies of benzimidazole alkylating agents reported the production of a variety of compounds (1–5) of which 4 - [2 - [5(6) - bis - (2-chloroethyl)amino]benzimidazolyl]-butyric acid[1] (6) and 2-[bis(2-chloroethyl)aminoethyl]-benzimidazole[2] (7, 8) are the most effective and clinically useful anticancer agents. In a previous investigation of the effect of structural modification on the anticancer activity of these compounds, several benzimidazole-2-thioethylsulfonic esters and nitrogen mustard derivatives were synthesized and evaluated for antileukemic properties (9). In continuation of these studies, this study describes the preparation of several new benzimidazole and benzothiazole alkylating agents (III, IV, VI, and X) and reports on the results of their evaluation against P-388 lymphocytic leukemia (Scheme I).

RESULTS AND DISCUSSION

Chemistry—*p*-(2-Hydroxyethoxy)benzaldehyde (I), prepared by etherification of *p*-hydroxybenzaldehyde with ethylene chlorohydrin (10) in the presence of sodium methoxide, was reacted with *o*-phenylenediamine and copper acetate, in accordance with the modified Weidenhagen reaction (11), to give 2-[*p*-(2-hydroxyethoxy)-phenyl]-benzimidazole hydrochloride (II). This was reacted with methanesulfonyl chloride or *p*-toluenesulfonyl chloride in pyridine to produce the corresponding sulfonic esters (III and IV). The *p*-toluenesulfonic ester (IV) was fused with excess diethanolamine and the produced 2-[*p*-[2-(bis-(2-hydroxyethyl)amino)ethoxy]phenyl]benzimidazole (V) converted into the dihydrochloride salt of the nitrogen mustard 2-[*p*-[2-(bis-(2-chloroethyl)amino)ethoxy]phenyl]benzimidazole (VI) by boiling with thionyl chloride in dioxane.

The reaction of *p*-(2-hydroxyethoxy)benzaldehyde (I) with o-aminothiophenol in pyridine gave 2-[*p*-(2-hydroxyethoxy)phenyl]benzothiazole (VII). This, on treatment with *p-toluenesulfonyl* chloride or methanesulfonyl chloride, yielded 2-[*p*-(2-chloroethoxy)phenyl]benzothiazole (VIII) rather than the corresponding sulfonic esters. Compound VIII was also obtained when the hydroxybenzothiazole derivative



Scheme I

[1] Cytostasan, Imet 3393, A.
[2] Benzimidazole mustard NSC 23891, B.

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