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DETERMINATION OF THE RELATIVE AMOUNTS OF THE B AND C COMPONENTS OF NEOMYCIN BY ION-EXCLUSION CHROMATOGRAPHY USING REFRACTOMETRIC DETECTION

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SUMMARY

Refractometric detection can be used as a convenient alternative to ninhydrin colorimetric or polarimetric detection in ion-exclusion chromatography of neomycin. The determination of the relative amounts of neomycin B and C using different detection methods is examined. The use of a resin of smaller granulometry and a medium-pressure chromatographic apparatus reduces the analysis time to less than 25 min.

INTRODUCTION

Neomycin is a complex mixture of basic water-soluble antibiotics produced during fermentation of *Streptomyces fradiae*[1]. The main active components of this mixture are neomycin B (Fig. 1a) and its stereoisomer neomycin C (b)[2]. Another component, neomycin A, isolated from the mixture[3] and proved to be identical with neamine (g), can be obtained by partial hydrolysis of neomycin B or C[4,5]. Other derivatives isolated from the mixture are neomycin LP-B and LP-C (LP = low potency) which are the mono-N-acetyl derivatives (c, d) of components B and C[6,7]. Related products, isolated from commercial samples of neomycin, are paromamine (h), paromomycin I (e) and paromomycin II (f)[8]. Preparative chromatography of commercial samples in our laboratory confirmed these findings[9]. In addition to the constituents already mentioned, mono-N-acetylneamine (i) was isolated together with minor components designated as G and K. Component G is a O-(diaminodideoxyhexosyl)myoinositol, K is a neomycin B or C molecule lacking the neosamine C part linked to deoxystreptamine.

The antimicrobial potency of component C is lower than that of neomycin B. The potency ratio varies with the microorganism and experimental conditions used in the microbiological assay[10]. An acceptable precision for the latter can only be obtained if the composition of the unknown preparation is fairly similar to that of the standard preparation. Thus a determination of the relative amounts of neomycin B and C has to be included in analysis of commercial neomycin. Chromatographic

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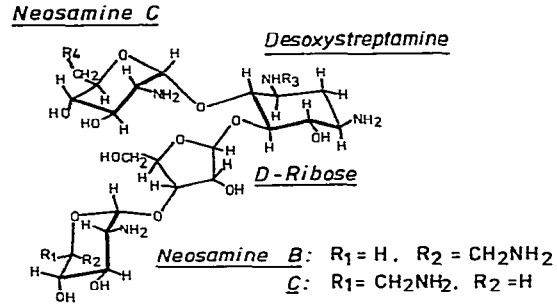
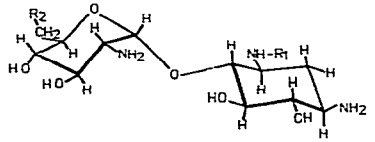


			R_1	R_2	R_3	R_4
a	Neomycin	B	H	CH_2NH_2	H	NH_2
b	Neomycin	C	CH_2NH_2	H	H	NH_2
c	Neomycin	LP-B	H	CH_2NH_2	Ac	NH_2
d	Neomycin	LP-C	CH_2NH_2	H	Ac	NH_2
e	Paromomycin	I	H	CH_2NH_2	H	OH
f	Paromomycin	II	CH_2NH_2	H	H	OH



		R_1	R_2
g	Neamine	H	NH_2
h	Paromamine	H	OH
i	Neomycin LP-A	Ac	NH_2

Fig. 1. Structure of different neomycin components.

separation of the stereoisomers neomycin B and C is quite difficult. Some paper and thin-layer chromatographic systems are successful as mentioned in a recent review[11]. More suitable for routine determinations of neomycin B and C are column chromatography on strongly basic ion-exchange resin (referred to as ion-exclusion chromatography), gas–liquid chromatography (GLC) of trimethylsilyl derivatives[12–14] and high-performance liquid chromatography (HPLC) after dinitrophenylation[15]. In our opinion, chromatography on an ion-exchange column, which requires no pre-column derivatizations, seems to be the simplest procedure. It has been described for the analysis of framycetin[16] (which is neomycin with less than 3% neomycin C) and kanamycin using colorimetric detection after reaction with ninhydrin[17,18], polarimetric detection[19,20] and conductometric detection[21]. It was found that refractometry could be used for the detection of these aminoglycoside antibiotics. So we decided to determine the relative amounts of neomycin B and C in neomycin by the last method and to compare the results with data obtained by previous methods.

EXPERIMENTAL

Column and pump

(A) Pyrex glass columns (40 × 0.6 cm I.D. or 20 × 0.6 cm I.D.), with jackets allowing temperature control by circulation of water, were provided with a glass tube outlet (2 mm I.D.) sealed with PTFE and a stainless-steel capillary. The outlet was plugged with acid-washed glass wool. The top of the column was also plugged with glass wool and closed with a rubber septum. Carbon dioxide-free distilled water was delivered at a constant rate by a Pharmacia P 3 peristaltic pump through a side-inlet at the top of the column (Fig. 2).

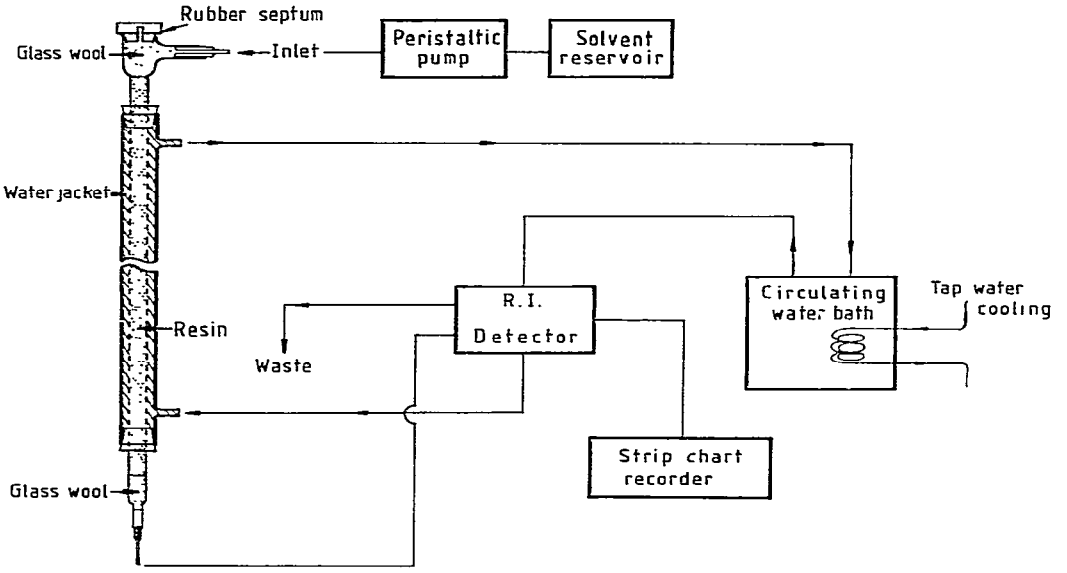


Fig. 2. Low-pressure chromatographic apparatus using a glass column (40 × 0.6 cm I.D.).

(B) A stainless-steel column (Li-Chroma 30 × 1.0 cm I.D.; Alltech Europe, Eke, Belgium) was provided with the necessary end fittings and low dead volume metal tubing. The usual fritted metal discs were replaced by porous polyethylene discs of the same diameter, which had a considerably lower back-pressure. Sheets of this material were purchased from Alltech Europe. The column inlet was connected to a Model CV-6-UHPa-N60 injector (Valco, Houston, TX, U.S.A.). Carbon dioxide-free water was delivered at a constant rate (46–460 ml/h) by a Milton-Roy reciprocating piston Mini pump, provided with a Bourdon-type pressure gauge, which also served as a pulse-damper. A stainless-steel column (10 × 1.0 cm), filled with AG 1-X2 resin (OH^-), was placed between the pressure gauge and injector to remove all traces of CO_2 from the eluent. The column temperature was held constant by immersing the column vertically in a bath of circulated water.

Detector

Glass and metal columns were connected by PTFE tubing (0.3 mm I.D.) to a Waters R-403 Differential Refractometer. The refractometer was thermostatted at 10 or 20°C using a Varian 4100 water-bath, which operated continuously to avoid base-line drifting. For work at 10°C, cooling of the water-bath by a Haake-Cryostat instead of tap-water was necessary. The trapped reference cell of the refractometer was filled with water. With a usual sample load of 10 mg neomycin sulphate, the attenuation setting was $\times 8$. The refractometer is a semi-preparative type which has the advantage of a lower back-pressure than the analytical R-401 model (*ca.* 4 kg/cm^2 at a flow-rate of 270 ml/h). The analytical type can be used with steel columns when the connection between the column outlet and refractometer is made of metal tubing instead of PTFE. Both types gave the same results.

The detector signal was recorded on a Kipp BD40 recorder with a chart speed of 10 mm/min. This high speed was chosen because the precision is higher when measuring larger peak areas. The peak areas were approximated by triangulation or determined with a HAFF 317 Polar Planimeter.

A Thorn PL Type 243 photo-electric polarimeter was provided with a flow-through cell (2 × 0.4 cm I.D.) which was thermostatted at 10°C (to avoid bubble formation in the cell). The output of the polarimeter was shunted to give a 1-cm deviation on the recorder for a $\Delta\alpha$ of 0.001°.

Reagents and materials

Neomycin sulphate was obtained from Roussel-Uclaf (Romainville, France), SIFA (Paris, France) and Upjohn (Kalamazoo, MI, U.S.A.). The free bases of neomycin B and C were isolated as described previously[9]. The weight loss on drying these products was 10.0 and 5.5%, respectively.

The concentration of sample solutions was 100 mg/ml. Bio-Rad AG 1-X2 resin (Cl^-), 200–400 mesh and −400 mesh, was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Carbon dioxide-free water was obtained by boiling and cooling double glass-distilled water.

Procedure

The resin (Cl^-) was suspended in carbon dioxide-free water and poured into a glass column (3 cm I.D.), provided with a fritted glass disc (porosity No. 2) and a 500-ml solvent reservoir. The settled resin bed was washed with 1 *N* sodium hydroxide (*ca.* 500 ml per 15 g resin) until the eluate was free from Cl^-. In order to exclude CO_2 from the atmosphere, a soda-lime trap was placed on top of the column. The flow-rate was adjusted to 20–25 ml/min by moderate vacuum or nitrogen pressure. After removing the chloride ion, the resin was washed with carbon dioxide-free water until the eluate was neutral to universal indicator. A slurry of the resin in carbon dioxide-free water was used immediately for filling the analytical columns.

(a) The top of the glass analytical column was connected to a glass tube (70 × 0.6 cm), in which the slurry was poured. When the resin-bed had settled, the excess of resin above the inlet was discarded and replaced by acid-washed glass wool. The opening was closed with a tightly fitting serum cap. Sample injections (100 µl solution) were made through this septum with a 100-µl Hamilton syringe.

(b) A 50-cm metal tube was connected to the metal analytical column. Five

millilitres of carbon dioxide-free water, followed by the slurry of resin (OH^-), were poured into the column. Carbon dioxide-free water was pumped for about 20 min at a maximum flow-rate of 460 ml/h.

RESULTS

All chromatograms were run on anion-exchange resins (OH^-), Dowex 1-X2 or the analytical grade (AG) produced by Bio-Rad. These resins consist of a polystyrene lattice cross-linked with 2% divinylbenzene, carrying quaternary ammonium groups, $-CH_2-N^+(CH_3)_3$. The chromatogram obtained on a column (40 × 0.6 cm) of Bio-Rad AG 1-X2 (200–400 mesh) at 20°C with a flow-rate of 60 ml/h, requiring 1 h 45 min, is shown in Fig. 3a. Lowering the column temperature to 10°C increases the

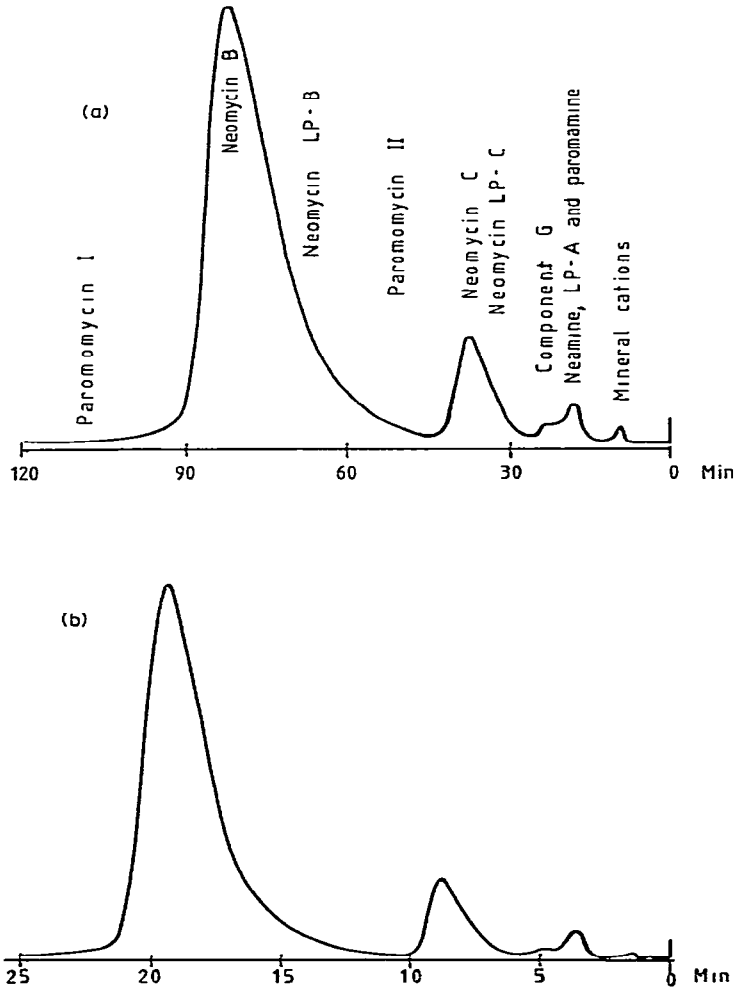


Fig. 3. a, Chromatography of neomycin sample U XZ-336 obtained on Bio-Rad AG 1-X2 (OH^-, 200–400 mesh) in a 40 × 0.6 cm column at a flow-rate of 60 ml/h. The position of the different neomycin components is indicated. b, Chromatography of neomycin obtained on Bio-Rad AG 1-X2 (OH^-, −400 mesh) in a 30 × 1 cm column at a flow-rate of 270 ml/h.

capacity factors, resolution between neomycin B and C, the number of theoretical plates, but also the analysis time. The use of a shorter column (20 cm) at 10°C reduces the elution time (Table I), but in our opinion the increased column efficiency at lower temperature does not justify a more complicated thermostating procedure. The analysis times in Table I include the elution of paromomycin I, which may be present in some samples. Since only the neomycin B and C peaks are of interest in our experiments, two columns can be used alternately, one being connected to the detector while the other is rinsed.

TABLE I

CHROMATOGRAPHIC PARAMETERS ON BIO-RAD AG 1-X2 (OH^-) UNDER DIFFERENT EXPERIMENTAL CONDITIONS

	40 × 0.6 cm column, 200–400 mesh resin, 60 ml/h, 10°C	*40 × 0.6 cm column, 200–400 mesh resin, 60 ml/h, 20°C*	*20 × 0.6 cm column, 200–400 mesh resin, 60 ml/h, 10°C*	*30 × 1 cm column, −400 mesh resin, 270 ml/h, 20°C*
Capacity ratio, neo B	13.6	8.0	13.1	7.9
Capacity ratio, neo C	4.9	3.0	5.0	3.2
Resolution, (neo B–neo C)	2.9	1.9	2.1	2.9
Peak symmetry factor (neo B)	0.74	0.80	1.0	0.77
Number of theoretical plates (neo B)	205 (513/m)	102 (255/m)	105 (525/m)	277 (923/m)
Analysis time	2 h 15 min	1 h 45 min	1 h 45 min	25 min

Separation of the main components can also be improved by using the Bio-Rad resin (−400 mesh). With this resin a metal column and a medium-pressure pump must be used. The improved separation of neomycin B and C allows higher flow-rates and a reduction of the analysis time to 25 min (Table I). A chromatogram obtained at a flow-rate of 270 ml/h on a column (30 × 1 cm) at 20°C is shown in Fig. 3b.

The mobile phase for all chromatographic systems is carbon dioxide-free water at a pH of *ca.* 6.5. We observed that adjustment to pH 7.0, 10.0 and 11.5 by adding sodium hydroxide has no influence on column parameters such as resolution, capacity factors and peak symmetry. It should be noted that capacity factors and resolution decrease with time. This is due partially to retention of sulphuric acid from the neomycin sulphate, but also to degradation of the functional groups of the resin in the OH^- form. A continuous flow of water, even when no samples are applied, has a favourable effect on column lifetime. The resin should be replaced when the calculated resolution is lower than 1.5, since in this case the skewed peaks of neomycin B and C are no longer baseline-separated. This replacement should take place after 1–2 weeks of use or application of 25–30 samples of 10 mg neomycin sulphate. Regeneration of the inexpensive resins is not recommended because of increased peak asymmetry.

Analysis of samples of known composition confirmed the identical response of neomycin B and C to the refractometric detection. These samples were prepared by dissolving known amounts of the pure neomycin B and C free bases in water, taking into account their weight loss on drying.

TABLE II

EXPERIMENTAL CONDITIONS FOR ION-EXCLUSION CHROMATOGRAPHIC ANALYSIS OF NEOMYCIN

Column dimensions and resin	*Flow-rate (ml/h)*	*Temperature (°C)*	*Sample load (mg)*	*Detection*	*Analysis time*	*Ref.*
A 27 × 2.5 cm Dowex 1-X2 (200–400 mesh)	300	Ambient	15	Conductimetric*	*ca.* 2 h	21
B 15 × 1 cm Bio-Rad AG1-X2 (200–400 mesh)	1.5–1.7	20	10	Ninhydrin (manual)	18–21 h	16
C 15 × 1 cm Bio-Rad AG1-X2 (200–400 mesh)	4.5	20	75	Polarimetric	7 h	20
D 40 × 0.6 cm Bio-Rad AG1-X2 (200–400 mesh)	60	20	2	Ninhydrin	1 h 45 min	23
E 20 × 0.6 cm Bio-Rad AG1-X2 (200–400 mesh)	60	10	10	Polarimetric	1 h 45 min	This paper
F 40 × 0.6 cm Bio-Rad AG1-X2 (200–400 mesh)	60	20	10	Refractive index	1 h 45 min	This paper
G 30 × 1 cm (metal) Bio-Rad AG1-X2 (−400 mesh)	270	20	10	Refractive index	25 min	This paper

* Determination of peak height instead of area.

Relative amounts of neomycin B and C in neomycin sulphate were also determined with polarimetric detection. The sample load (10 mg) is lower than in a previous study[20] where 75 mg were used. This is possible because of the use of a larger flow-cell (inner volume of 250 μl instead of 32 μl), with which a more stable baseline is obtained. The difference in $[\alpha]_D$ values of neomycin B and C requires a correction for the peak areas. Instead of the $[\alpha]_D$ values measured in 0.02 *N* H_2SO_4 (+83° for neomycin B and +121° for neomycin C) employed by de Rossi[19], we used the values determined in 0.02 *N* NaOH (neomycin B, +71°; neomycin C, +110°) as reported by Ford *et al.*[22]. We confirmed these values for neomycin B and C prepared in our laboratory.

A comparison of the methods described in this report with other detection methods is possible, since some samples have been examined in other laboratories also. The experimental conditions for ion-exclusion chromatographic determination of neomycin C are given in Table II. Good correlation was found between the results obtained with ninhydrin detection (Dr. A. Sezerat, Roussel-Uclaf), polarimetric detection (Dr. J. Lightbown, National Laboratory for Biological Standards and Control, London, Great Britain) and our method. This is illustrated in Table III which gives the relative amounts of neomycin C in eight commercial samples, determined with different detection methods. The confidence limits of the mean were calculated with a *t*-test. Conductometric detection[21] used previously in our laboratory gives a systematic overestimation of neomycin C, possibly due to the non-linear response of the detector. Four rather old samples were analysed by preparative chromatography on a carboxylic ion-exchange resin[9]. The percentages of neomycin C found in that

TABLE III

RELATIVE AMOUNTS OF NEOMYCIN C IN COMMERCIAL SAMPLES

Values expressed as:

$$\frac{\text{neo C}}{\text{neo C + neo B}} \times 100$$

Sample	*Method*						
	A	*C**	*D***	*E*	*F*	*G*	*Preparative chromatography on Amberlite CG-50*[9]
U. XZ-336	11.6	10.8	9.8	9.1–10.3***	9.5–10.6***	9.7–10.5***	9.5
S. 52001	39.0	32.2	37.8	34.5–37.0***	–	–	34.5
U. TRO-32	12.2	9.8	9.6	8.2–9.2***	–	–	8.4
R. 7S-1251	12.5	8.9	10.0	9.2–10.6***	11.0	9.7–10.4***	9.1
R. 9S0560	–	–	12.4	–	11.8	11.0–11.8***	–
R. 9S0572	–	–	–	–	11.8	–	–
R. 9S0581	–	–	15.3	–	15.1	14.0–14.7***	–
R. 9S0594	–	–	–	–	16.5	–	–

* Figures furnished by Dr. J. Lightbown.

** Figures furnished by Dr. A. Sezerat.

*** 95% confidence limits.

study are included in Table III. A comparison between GLC of trimethylsilylated neomycins, HPLC of N-dinitrophenylated neomycin and ion-exclusion chromatography with refractometric detection (Table IV) was made possible by analysis of samples kindly provided by Mr. Tsuji (Upjohn, Kalamazoo, MI, U.S.A.). Our results are intermediate between the values obtained by GLC and HPLC reported by Tsuji *et al.*[15].

TABLE IV

COMPARISON BETWEEN HPLC, GLC AND ION-EXCLUSION CHROMATOGRAPHY

The relative amount of neomycin C is expressed as in Table III.

Sample	*Method G*	*HPLC*[15]	*GLC*[15]
6	8.9–10.4*	11.6	9.7
9	12.4	13.1	8.8
13	11.6–12.4*	13.4	7.8
16	17.9–20.1*	19.9	17.2
17	11.9–12.9*	13.9	8.9

* 95% confidence limits.

It should be mentioned that, in all chromatograms using an anion-exchange resin, the acetyl derivatives of neomycin B and C (LP-B and LP-C) are located in the ascending parts of the neomycin B and C peaks (Fig. 3a). These components, which may be present in some samples at concentrations up to 5%, are determined together with the non-acetylated main products.

CONCLUSION

The results reported show that refractometric detection can be used as an alternative to ninhydrin colorimetry or polarimetric detection in ion-exclusion chromatography of neomycin. It is simple to perform and gives an identical response for the B and C components. Medium-pressure chromatography using a −400 mesh resin permits a reduction of the analysis time to *ca.* 25 min. This makes the ion-exclusion chromatographic determination of neomycin C competitive with the HPLC method described recently by Tsuji *et al.*[15].

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