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The semi-quantitative assay of neamine and neomycin C in neomycin by thin-layer chromatography

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Thin-layer chromatographic methods are described for the separation and assay of neamine and neomycin C in neomycin sulphate and its preparations. The samples are chromatographed on binder-free silica gel plates using, as developing solvents, a 3·85% w/v ammonium acetate solution to separate neamine from the neomycins and a 3·4% w/v ammonium hydroxide solution to separate neomycin C from neomycin B and neamine. The spots are made visible by spraying with a 1% v/v solution of t-butyl hypochlorite in dichloroethane-acetic acid (9:1), removing the excess reagent in a stream of cold air and spraying with a 0·5% w/v solution of potassium iodide in 0·5% w/v starch mucilage. The size and intensity of the spots produced is compared with standards and the neamine or neomycin C content of the sample estimated. The results obtained for neomycin C are comparable with those given by chromatography on an ion-exchange resin. A possible explanation is offered for the lack of agreement in the case of neamine. The chromatographic systems described are also applied to paromomycin and shown to separate paromamine, paromomycin I and paromomycin II, not only from each other, but also from neamine and the neomycins.

NEOMYCIN is a mixture of basic, water-soluble antibiotics, produced by the growth of certain strains of *Streptomyces fradiae* in a suitable culture medium. The major active constituent of the mixture is neomycin B, but large amounts of its less active stereoisomer, neomycin C, may also be present, together with small amounts of neamine, a comparatively inactive degradation product of the neomycins. Although differing in microbiological potency, these substances have similar chemical properties; chemical assays of neomycin are therefore unsatisfactory unless preceded by a separation of the mixture into its components.

Chromatographic separations of the neomycin complex have been described by Leach & Teeters (1951), Pan & Dutcher (1956), Kaiser (1963), Brodasky (1963), Maehr & Schaffner (1964) and Inouye & Ogawa (1964). None of these methods is simple, rapid or sensitive enough for routine use and the following thin-layer chromatographic procedures have therefore been developed.

Experimental

Neomycin sulphate was examined by thin-layer chromatography on cellulose, kieselguhr, alumina and silica gel plates in a variety of developing solvents. No single system was found which would separate all the constituents, including unidentified impurities, but a complete separation was obtained on binder-free silica gel using two developing solvents—an ammonium acetate solution to separate neamine and an ammonium hydroxide solution to separate neomycin C.

The compounds were located by heating the plate to remove ammonium compounds and spraying either with a 0·2% w/v ninhydrin solution in methanol, or with t-butyl hypochlorite followed by starch-iodide solution

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(Schwartz & Pallansch, 1958). The second method was preferred because it was the more sensitive, detecting 0·01 μg of neamine.

The pure neomycin B and C standards used in the assay are usually obtained as the bases, whereas the sample is in the form of the sulphate. This difference does not affect the estimation, if the loadings are adjusted to allow for the fact that neomycin sulphate contains only 70% of base.

APPARATUS AND REAGENTS

Neamine base (*Leach & Teeters*, 1951), *neomycin B base and neomycin C base* (*Ford & others*, 1955) were dried *in vacuo* at 50° before use. No significant amount of any impurity was detected by the recommended thin-layer chromatographic methods or by ion-exchange resin chromatography (Maehr & Schaffer, 1964). Neomycin B was 99·6% pure, neomycin C, 98% pure by a paper chromatographic assay (Kaiser, 1963), and neamine, 100% pure by ion-exchange resin chromatography (Maehr & Schaffner, 1964).

t-Butyl hypochlorite reagent. Mix t-butyl hypochlorite (1 ml) with dichloroethane (90 ml) and glacial acetic acid (10 ml). Store in a refrigerator.

Starch-iodide reagent. A solution of potassium iodide (0·5 g) in freshly-prepared starch mucilage B.P. (100 ml).

Standard neamine solutions. Dissolve neomycin B base (70 mg) in water (5 ml). To 0·5 ml aliquots of this solution, add 0, 0·1, 0·2, 0·3, 0·4 and 0·5 ml of a solution of neamine base (2·5 mg) in water (5 ml) and dilute to 1 ml in each case. These standards are equivalent to neomycin sulphate containing 0, 0·5, 1·0, 1·5, 2·0 and 2·5% respectively of neamine.

Standard neomycin C solution. To 0, 0·05, 0·10, 0·15, 0·2, 0·25 and 0·3 ml of a solution of neomycin C base (2 mg) in water (4 ml), add 0·50, 0·475, 0·45, 0·425, 0·40, 0·375 and 0·35 ml respectively of a solution of neomycin B base (4 mg) in water (4 ml) and dilute to 1 ml in each case. These standards are equivalent to neomycin sulphate containing 0, 5, 10, 15, 20, 25 and 30% respectively of neomycin C sulphate.

Developing solvent 1. *Neamine assay.* A freshly-prepared solution of Analar grade ammonium acetate (3·85 g) in water (100 ml). 2. *Neomycin C assay.* Strong solution of ammonia B.P. is assayed (British Pharmacopoeia, 1963) and diluted immediately before use to give a solution containing 3·4% w/v of ammonia.

Thin-layer chromatographic plates. Spread a 0·25 mm layer of Kieselgel H (Merck) on 20 × 20 cm glass plates and activate by heating for 1 hr at 110°.

PROCEDURE FOR NEOMYCIN SULPHATE

Sample solution 1. *Neamine assay.* Dissolve 10 mg of sample in water (1 ml). 2. *Neomycin C assay.* Dissolve 5 mg of sample in water (7 ml).

Method. Line a chromatographic tank with filter paper, add the appropriate developing solvent, saturating the paper lining with the solvent, and allow the tank to equilibrate (1 hr). Spot 1 μl of the sample

solution and of each of the appropriate standard solutions on a thin-layer plate and score the surface of the plate 15 cm from the line of application to limit the extent of the solvent run. Stand the plate in the tank with the silica gel layer facing the paper lining and as close to it as practicable, and allow the solvent to run to the scored line. Remove the plate from the tank and dry in a stream of hot air. Allow the plate to cool and spray uniformly with t-butyl hypochlorite reagent (about 10 ml). Remove the excess reagent by standing the plate in a stream of cold air, until a sprayed portion below the original application line gives little or no blue colour with a spot of the starch-iodide reagent (prolonged exposure to the air stream reduces the intensity of the spots). Finally, spray the plate with starch-iodide reagent and compare the size and intensity of the appropriate spot obtained from the sample with that from the standards.

PROCEDURE FOR NEOMYCIN SULPHATE TABLETS

Shake a quantity of powdered tablets equivalent to 500 mg neomycin sulphate with water (50 ml) and filter (Solution A). Dilute 5 ml of Solution A to 70 ml (Solution B). Use 1 μl of Solution A and 1 μl of Solution B for the assay of neamine and neomycin C respectively.

PROCEDURE FOR CREAMS AND OINTMENTS OF NEOMYCIN SULPHATE

Transfer a quantity of sample equivalent to 10 mg neomycin sulphate to a 10 ml graduated centrifuge tube, add chloroform (5 ml), shake vigorously and centrifuge. Note the volume of any separated aqueous phase and dilute to 2 ml with water. Shake vigorously and recentrifuge to separate the aqueous phase (Solution A). Dilute 1 ml of Solution A to 7 ml (Solution B). Use 2 μl of Solution A for the assay of neamine, comparing the spots obtained with those from 2 μl of a 1 + 1 dilution of each of the standard neamine solutions. Use 1 μl of Solution B for the assay of neomycin C.

Results and discussion

The results obtained when neamine, neomycin B and neomycin C were examined by thin-layer chromatography in the ammonium acetate and ammonium hydroxide systems are given in Table 1. Paromamine, paromomycin I and paromomycin II—the constituents of paromomycin, an antibiotic closely related to neomycin—also separate in the recommended systems and results for these compounds are also quoted.

When samples of neomycin sulphate were examined in the ammonium acetate system, an unidentified impurity was detected on the chromatogram between neamine and the neomycins. This was shown to be active against *Bacillus pumilus* on seeded agar plates (Brodasky, 1963). In the ammonia system, the material gives two partially-separated spots running in front of neamine and it may be a mixture of "low potency neomycins," which are thought to be mono-*N*-acetyl derivatives of the neomycins (Rinehart, 1964).

An ion-exchange column chromatographic assay, in which the eluting fractions are determined with ninhydrin, was used as a reference method (Maehr & Schaffner, 1964, as modified by De Rossi, personal communication). The column and thin-layer assays gave similar results for neomycin C, but for neamine higher results were obtained by ion-exchange chromatography than by the thin-layer method (Table 2). Accordingly,

TABLE 1. RUNNING DISTANCES OF NEOMYCIN AND PAROMOMYCIN COMPOUNDS IN AMMONIUM HYDROXIDE AND AMMONIUM ACETATE SOLUTIONS

Compound	Running distances in mm*			
	Compounds run individually	Compounds run as a mixture (1)	(2)	(3)
	Ammonium hydroxide solution			
Neamine	65–75	63–72	—	60–69†
Neomycin C	61–70	49–58	—	47–55
Neomycin B	51–61	37–46	—	36–45
Paromamine	93–102	—	91–102	92–101
Paromomycin II	85–93	—	80–89	79–88
Paromomycin I	73–82	—	67–76	69–74†
	Ammonium acetate solution			
Neamine	49–59	49–60	—	49–59
Neomycin C	13–23	14–25 (Neomycin C and B together)	—	13–23 (Neomycin C and B together)
Neomycin B	13–24		—	
Paromamine	78–88	—	78–89	78–88
Paromomycin II	37–46	—	36–46 (Paromomycin II and I together)	35–45 (Paromomycin II and I together)
Paromomycin I	36–45	—		

* Distances from the starting line to the rear and front of the spot are given to enable the effectiveness of the separation to be judged. Figures quoted are for loadings of 0·1 μg of each compound in 1 μl water; running distance of solvent front, 15 cm.
† Separation of neamine and paromomycin I incomplete.

the fractions of eluate which contained neamine were examined by thin-layer chromatography in the ammonium acetate system. In addition to neamine, three other compounds were detected, all of which reacted with a ninhydrin reagent spray. They are probably responsible for the high neamine result given by the column assay.

TABLE 2. COMPARISON OF THIN-LAYER AND COLUMN CHROMATOGRAPHIC ASSAYS FOR NEAMINE AND NEOMYCIN C

Sample	Thin-layer assay: Neamine % (mean and range)	Thin-layer assay: Neomycin C sulphate % (mean and range)	Column assay: Neamine %	Column assay: Neomycin C sulphate %
1	Nil	8 (7·5–10·0) [9]*	Nil	8
2	2·5 (2·3 –2·5) [4]	15 (12·5–17·5) [8]	2·9	14
3	0·3 (0·25–0·5) [4]	17 (15·0–17·5) [8]	1·9	16
4	0·4 (0·25–0·5) [7]	15 (15·0–17·5) [11]	1·5	13
5	2·0 [4]	19 (15·0–20·0) [10]	3·4	16

* Figure in [ ] is number of assays.

The thin-layer assay procedures were applied to neomycin sulphate and to tablets, creams and ointments prepared from it. Recovery experiments were made by adding known amounts of neamine, neomycin C and neomycin B to blank formulations and submitting them to the assay; complete recoveries of the added neamine and neomycin C were obtained.

The neamine contents of neomycin sulphate (26 samples), neomycin sulphate tablets (13 samples) and neomycin creams and ointments (15 samples) from several manufacturers were determined. None of the samples contained more than 2·5% neamine relative to the neomycin sulphate present and only three samples contained more than 1·5%. The neomycin C contents of the samples were also determined. Between 5% and 30% of the neomycin sulphate content was present as neomycin C sulphate, with most results falling in the range 15% to 25%. Each sample was assayed between four and ten times: replicate results fell within ±25% of the mean.

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