Quantitative Analysis of Neomycin Sulfate by Thin-Layer Chromatography

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A thin-layer chromatographic method for the assay of neomycin sulfate is presented that simultaneously provides an identification test and a potency assessment with an average deviation of about 2 per cent.

NEOMYCIN SULFATE assay procedures currently practiced require time-consuming microbiological techniques and facilities often not available to industrial control laboratories. The present broad-scale use of thin-layer chromatographic methods prompted the development of this approach as a rapid and reproducible alternative. The studies demonstrated that the variability of the thin-layer chromatographic method is no greater than that of the microbiological procedure and that substantial laboratory time may be saved by the adoption of this technique as a guide to the microbiologist. While thin-layer chromatography has only been applied to the neomycin sulfate powders, the possibility of applying this method to in-process control for the manufacture of dosage forms would have some obvious merit.

The elution system found to be best suited to determine total potency of neomycin sulfate was 3% ammonia–acetone (160:40) using silica plates. Allowed to develop for 1 hour, the neomycins B and C move together and show an R_f of 0.33. Visualization with ninhydrin at this point provides a rapid identification procedure. For quantitative determination, the neomycin zone was removed from the plate, and a colorimetric assay of its ribose moiety was employed using an orcinol–ferric chloride–hydrochloric acid–acetic acid reagent (1, 2). The neomycin-silica zone was mixed with this reagent and held in boiling water for 1 hour, cooled, filtered, and the absorbance of the filtrate read at 665 mμ. The coloration produced was linearly proportional to the concentration of neomycin present.

A linear standard curve was made by applying concentrations of from 40–250 mcg. of the U.S.P. neomycin sulfate B reference standard to silica coated plates (0.35–0.375 mm. thick) in the 3% ammonia–acetone (160:40) system, developing for 1 hour, drying for 0.5 hour at 110°, removing the respective zones, hydrolyzing in the presence of the orcinol reagent for 1 hour, and reading the absorbance against a blank at 665 mμ. The curve was constructed in terms of neomycin B base.

Commercial samples of neomycin sulfate vary in their neomycin C content from about 5 to 20%; these varying ratios of B:C contribute, in part, to the variation of assay results between laboratories using different test methods. The Food and Drug Administration cylinder-plate assay method, utilizing *S. aureus* (3, 6) and used in this laboratory, indicates values of about 50% when neomycin C, prepared by the method of Ford *et al.* (4), is assayed against a pure neomycin B sulfate standard (5). Similarly, this same neomycin C gives a value of the same order of potency when run through the thin-layer chromatographic procedure. Consequently, in comparing the results of these two assay methods, both should account for the neomycin C content on an equipotency basis.

Direct assay of neomycin sulfate by application of the above orcinol–ferric chloride reagent procedure without recourse to the silica plate was simultaneously studied. Each sample invariably gave higher results ranging from 0.5 to 13% above the comparable thin-layer plate values. These differences are being investigated further, though it would appear that the specificity of the R_f value and the greater assurance that interfering substances are removed by the chromatographic system justifies the additional effort of the full procedure.

EXPERIMENTAL

Preparation of Plates

Glass plates, 20 × 20 cm., were used after degreasing with cleaning solution. A slurry of 40 Gm. of silica gel in 80 ml. of distilled water was applied to the plates with a Desaga variable applicator (Brinkmann) set to deliver a layer 0.35 mm. thick. The plates were air-dried for a few minutes, then dried at 110° for 1 hour and stored in a desiccator until used.

Assay of Neomycin Base in Neomycin Sulfate

Standard Curve.—U.S.P. neomycin sulfate (potency 730 mcg./mg.), dried at 60° in an Abderhalden for 2 to 3 hours, was applied from a standard solution of 50 mg./10 ml. water, containing 50 mcg. in 10 μl. Application of more than 50 mcg. in one spot on the thickness of the plate described above resulted in excessive elongation of the neomycin spot. Consequently, to construct the standard curve over the

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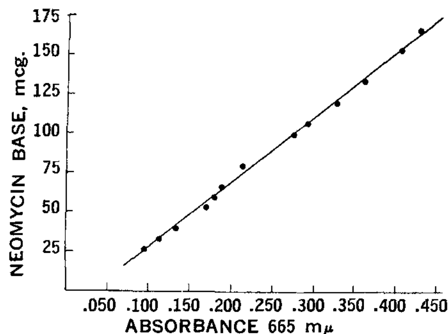

Fig. 1.—U.S.P. neomycin sulfate reference standard. Orcinol color test absorbance in thin-layer chromatographic system; 3% ammonia–acetone (160:40); 60-minute color development.

range of 40–250 mcg., applications were made in groups of one to five spots to achieve the neomycin levels desired. Thirteen levels were used to construct the curve shown in Fig. 1. Each of the 13 groups was spaced on the plates to allow for easy removal and assay. Individual spots within a group were applied 10 mm. apart; groups were separated at least 20 mm. apart from each other. Usually three groups were accommodated on one plate. One end of the plate was reserved for two or three applications of neomycin for visualization by spraying with ninhydrin. This technique along with observation under ultraviolet light was used to determine the zone areas to be scraped off for assay. (See below.) The standard curve thus obtained was used to determine the neomycin content of the samples to be assayed.

Neomycin Sulfate Test Samples.—The neomycin sample to be assayed was dried similarly at 60° for 3 hours, and a solution of 125-mg., accurately weighed, in 25 ml. of water at 20° was prepared. The solution was applied as above in groups of three to five spots in the following microliter levels: (3 × 8), (3 × 10), (4 × 8), (4 × 10), and (5 × 10) on two plates.

Development.—The eluting system was obtained by diluting 16 ml. of concentrated ammonia solution to 160 ml. with distilled water; 40 ml. of acetone was added and the contents mixed and transferred to a chamber to equilibrate. The plates were allowed to develop for 1 hour, after which they were removed and dried at 110° for 0.5 hour.

Removal of Zones.—A glass plate was used to cover the groups to be assayed, while the remaining portion of exposed plate was sprayed while hot with ninhydrin spray described below. The neomycin spots are visualized almost immediately. Using the indicated red-purple neomycin spots as a guide, the unsprayed spots to be assayed were centered within an inch high band above the origin points of the particular group. This position was usually within $1^1/_4$–$2^1/_4$ in. above the origin to $1^1/_2$–$2^1/_2$ in. above the origin, but the exact position is determined by the indicated neomycin. This band is the section scraped out for assay. The unwanted silica below the zones was scraped away and discarded, and a straight edge spatula was used to scrape off the test zones individually onto glassine paper; each was transferred into a glass-stoppered test tube. A further aid in assuring complete removal of the neomycin is to examine the plate under a long wave ultraviolet lamp; the neomycin is generally visible, and any traces can be added to the test zone scrapings. Care must be taken to exclude any silica which may have come in contact with the ninhydrin spray reagent.

Color Development.—To each of the glass-stoppered test tubes containing the silica-neomycin zones and one used for a blank, 1 ml. of distilled water and 4 ml. of the orcinol reagent described below were added and gently mixed. The tubes were heated in boiling water for 1 hour, then cooled and filtered gently by vacuum through a little filter aid. The heating time chosen as the optimum condition for this color assay was 60 minutes at 100°. The color may change on continued heating, so precise attention to heating time is required to correlate with the standard curve constructed at these same conditions. The absorbance of the filtrate was obtained at 665 mμ using a Beckman DU ultraviolet spectrophotometer. (If crystallization in the filtrate occurs during or before the reading is made, the crystals should be filtered off for a correct reading.)

Calculation.—After the absorbance is read, the quantity of neomycin base is obtained from the standard curve. The number of micrograms of neomycin base obtained divided by the microliters used for the assay gives the number of micrograms of neomycin in 5 mcg. of neomycin sulfate. This result divided by 5 and multiplied by 1000 gives the potency in micrograms per milligram.

Example.—A group of three applications of 10 μl. neomycin sulfate solution put through the assay gave a reading of 0.295 at 665 mμ. The neomycin base content (obtained from the standard curve) for the reading was 108 mcg.

$$\frac{108}{30} = 3.6 \text{ mcg.}$$

$$\frac{3.6}{5} \times 1000 = 720 \text{ mcg./mg.}$$

Time.—A time schedule for the assay is shown in Table I.

TABLE I.—NEOMYCIN TLC ASSAY TIME SCHEDULE[a]

Steps	Expired Time	Man-Hr. Time, Min.
Drying sample[b]	3 hr.	5
Weighing sample	10 min.	10
Prepn. of plates[b]	1 hr. 20 min.	20
Prepn. of tank	10 min.	10
Application	30 min.	30
Development	1 hr.	.. } 5
Dry at 110°C.	30 min.	.. }
Zone removal	30 min.	30
Reagent (prepare and deliver)	30 min.	30
Hydrolysis	1 hr.	10
Filtration	15 min.	15
Absorbance reading	15 min.	15
Total	9 hr. 10 min.	3 hr. (30 min. per assay)

[a] For six assays on two plates in one tank. [b] Previous preparation of plates and drying of sample cuts expired time to 4 hours and 50 minutes.

TABLE II.—ANALYSIS[a] OF ASSAY DATA FOR U. S. P. NEOMYCIN SULFATE—THIN-LAYER CHROMATOGRAPHY *versus* BIOASSAY

Lot No.	TLC Assay		Microbiological Assay S. B. Penick		Microbiological Assay FDA	
U.S.P. reference standard	720	715				730
	745	724				
	735	710				
	732					
	Av.	726 ± 14				
264-NAF	671	659	603	661		
	668	687	694	714		
	664	688	655	599	663	627
	692	668			676	676
	Av.	675 ± 13	Av.	654 ± 43	Av.	660 ± 20
303-NAF	688	691				
	701	700	704	676		
	714	685	646	673		
	692	700	645	704	662	722
	700				695	675
	Av.	697 ± 6	Av.	675 ± 23	Av.	688 ± 23
347-NAF-1	708	710	700	687		
	699	690	742	653		
	700	692	701		725	689
	686				682	650
	Av.	698 ± 9	Av.	697 ± 28	Av.	687 ± 27
398-NAF-1	689	706	669	647		
	699	692	664	677	686	696
	711	688	650		689	748
	Av.	698 ± 9	Av.	661 ± 11	Av.	705 ± 25
182-NBF-1	659	656	630	679		
	641	648	700	700	672	672
	687		700	623	706	632
	Av.	658 ± 22	Av.	672 ± 33	Av.	670 ± 26
113-NBF-1	652	690	697	670		
	654	637	724	663	674	649
	652		737	634	667	690
	Av.	657 ± 18	Av.	687 ± 36	Av.	670 ± 15

[a] ± = Standard deviation.

Ninhydrin Spray, Reagent

To 50 ml. of 1 *M* citrate buffer (21.0 Gm. citric acid and 200 ml. in *N* sodium hydroxide diluted to 1 L.) was added 80 mg. of stannous chloride ($SnCl_2 \cdot 2H_2O$). This solution was added to 50 ml. of methyl cellosolve containing 2 Gm. of ninhydrin.

Orcinol Reagent

A 0.1% solution of orcinol in concentrated HCl–acetic acid (1:3 v/v) was prepared by dissolving 40 mg. of orcinol in a mixture of 30 ml. of glacial acetic acid and 10 ml. of concentrated hydrochloric acid and adding 0.4 ml. of molar ferric chloride solution (2.7 Gm. $FeCl_3 \cdot 6H_2O$ dissolved in a 10-ml. volumetric flask with distilled water). This reagent should be prepared just before use.

Statistical Analysis

Replicate assays of six production lots of neomycin sulfate U.S.P. (Table II) show that the variability of the thin-layer chromatographic method is no greater than that of the standard microbiological procedure (3). The average per cent deviation for the TLC procedure is 1.9% compared to 4.3% for the bioassay technique.

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