Determination of the relative amounts of the B and C components of neomycin by thin-layer chromatography using fluorescence detection

E. Roets*, E. Adams, I.G. Muriithi, J. Hoogmartens

*Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen. Faculteit Farmaceutische Wetenschappen,
Katholieke Universiteit Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium*

Abstract

The determination of the relative amounts of the B and C components of neomycin sulphate by thin-layer chromatography using silica gel plates from Whatman as the stationary phase is described. The mobile phase consisted of methanol–20% (m/v) sodium chloride solution (15:85). Fluorescence detection was performed after derivatization with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole. The influence of different parameters on the separation was investigated. A number of commercial samples was analysed using this method and the results were compared with results obtained with ion-exchange chromatography and ninhydrin colorimetric detection, which is the official method prescribed by the European Pharmacopoeia. The described method is much easier to perform than the official method.

1. Introduction

Neomycin is a widely used broad spectrum water-soluble aminoglycoside antibiotic produced during fermentation of *Streptomyces fradiae* [1]. It inhibits the growth of Gram-positive and Gram-negative bacteria. It has a narrow therapeutic range, is potentially toxic like other aminoglycosides and may cause ototoxicity and nephrotoxicity.

Neomycin sulphate is mainly composed of a mixture of neomycin B (Fig. 1) and its stereoisomer neomycin C [2]. Neomycin with a content of less than 3% neomycin C is called framycetin.

Another minor component, neomycin A, which was isolated from the mixture [3] and proved to be identical with neamine, can be obtained by partial hydrolysis of neomycins B or C [4,5]. Small amounts of other constituents, less than 1%, are also present in commercial samples. These impurities, formerly called neomycins D, E and F were identified as paromamine, paromomycin I and paromomycin II, respectively [6]. The antimicrobial potency of neomycin C is lower than that of neomycin B and it varies with the microorganism and experimental conditions used in the microbiological assay. The difference in activity necessitates a limit and control of neomycin C in commercial samples [7]. Neamine has no antimicrobial activity. The

* Corresponding author.
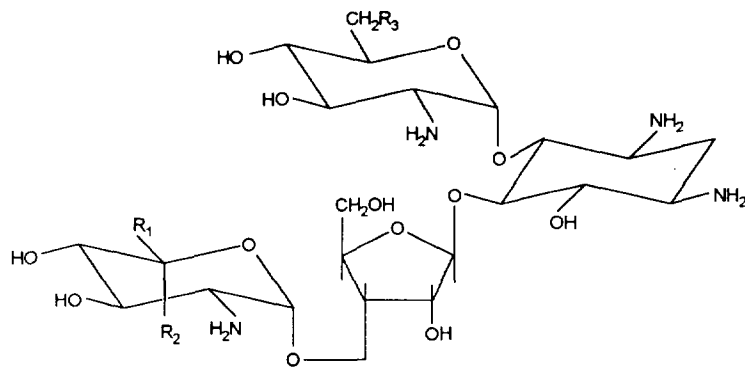
	R_1	R_2	R_3
Neomycin B :	H	CH_2NH_2	NH_2
Neomycin C :	CH_2NH_2	H	NH_2
Paromomycin I :	H	CH_2NH_2	OH
Paromomycin II :	CH_2NH_2	H	OH
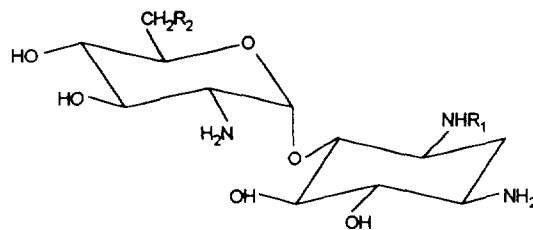
	R_1	R_2
Neamine	H	NH_2
Paromamine	H	OH

Fig. 1. Structures of different neomycin components.

European Pharmacopoeia limits the amount of neomycin C to 3–15% [8]. The United States Pharmacopeia does not distinguish neomycin and framycetin and therefore does not limit neomycin C in a separate test [9].

Chromatographic separation of the stereoiso-mers neomycins B and C is quite difficult. Ion-exclusion liquid chromatography (IELC) [10], liquid chromatography (LC) on normal phase after dinitrophenylation [11] or gas–liquid chromatography after trimethylsilylation [12] have been described for determination of neomycins

B and C. The IELC method was further studied by Decoster et al. [13] and this method was finally chosen by the European Pharmacopoeia to determine the neomycin C content [8]. More recently LC methods on normal phase combined with pre-column derivatization [14,15] and on reversed phase with post-column derivatization [16] were published. Meanwhile the European Pharmacopoeia [8] method proved to be very laborious and difficult to perform: the column must be packed in the laboratory, the stationary phase is not easily available, the stationary phase is not stable, the efficiency is low, the separation time is about 2 h and the derivatization of the fractions with ninhydrin is carried out manually. Thin-layer chromatographic (TLC) separation was first described on acid-treated carbon black plates, detection was performed by autobiography [17]. Kovacs-Hadady [18] reported on the influence of the interaction of silica gel with metal ions. When the acidic silanol sites were partially saturated with metal ions from the mobile phase, the aminoglycosides migrated while by developing with water no migration occurred. The separation of the components depended on the degree of saturation of the silanol groups by the metal ions [18]. Quantitative densitometric TLC which requires no pre-derivatization has not been reported until very recently [19]. Quantitative results for different neomycins were not reported in this paper. When this method was tried out in our laboratory, the separation was not as good as shown and it was impossible to determine small amounts of neomycin C (3% and less) which is necessary to distinguish neomycin and framycetin.

In this work a TLC method is described for the quantitative determination of the relative amounts of neomycins B and C. The method is based on that described by Kovacs-Hadady [18]. A wide-pore silica gel was used as the stationary phase. The mobile phase consisted of methanol–20% (m/v) sodium chloride solution (15:85). Detection was performed after derivatization with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). The different components were

quantified by densitometry using the fluorescence mode. The results for the different samples are compared with those obtained with the official European Pharmacopoeia method.

2. Experimental

2.1. Chemicals

Methanol and acetone, *n*-hexane and liquid paraffin, sodium chloride of analytical grade and NBD-Cl were all from Janssen Chimica (Beerse, Belgium). Water was distilled twice from glass apparatus. Precoated silica gel layers on glass (20 × 20 cm) 150A K5 and 60A K6 were obtained from Whatman (Maidstone, UK). Other precoated silica gel layers were: DC-fertigplatten Kieselgel 60 (Merck, Darmstadt, Germany), Alugram SilG and DC-fertigplatten SilG 25 (Macherey–Nagel, Düren, Germany), Si 250 (Baker, Phillipsburg, NJ, USA), Silica platten (Woelm, Eschwege, Germany) and Stratocrom SIF$_{254}$ (Carlo Erba, Milan, Italy). Silanized silica gel layers, DC-fertigplatten Kieselgel 60 silanisiert, were obtained from Merck.

2.2. Standards and samples

Neomycin B sulphate, neomycin C sulphate and neamine hydrochloride standards were prepared in the laboratory from commercial samples, as described [20]. Mixtures of the neomycins B and C laboratory standards ranging from 1% (m/m) to 50% neomycin C (m/m) in neomycin B were prepared. Commercial samples were obtained from Sifa (Paris, France), Roussel-Uclaf (Romainville, France), Upjohn (Kalamazoo, MI, USA) and Takeda (Osaka, Japan). Other aminoglycoside antibiotics used in the development of the TLC method were: paromomycin sulphate from Carlo Erba (Italy), gentamicin sulphate from Pierrel (Milan, Italy), kanamycin sulphate from Continental Pharma (Brussels, Belgium), kanamycin B sulphate from the European Pharmacopoeia (Strasbourg, France), tobramycin from Alcon (Puurs, Bel-

gium), apramycin from Eli Lilly (Indianapolis, IN, USA) and amikacin from Myers Squibb (Syracuse, NY, USA). The structures of these aminoglycosides can be found in some reviews [21,22].

2.3. TLC method

The TLC plates were used as received. A narrow band of the layer was removed from both sides of the plate, supposed to be in vertical position during development. Samples were dissolved in water in a concentration of 4.0 mg/ml. Aliquots of 1.0 μl were applied to the TLC plate with a microsyringe (Hamilton, Bonaduz, Switzerland) starting at 10 mm from the edge and at 20 mm from the bottom of the plate. The distance between the lanes was 10 mm. The chromatographic chamber was lined with paper and equilibrated with the mobile phase methanol–20% (m/v) sodium chloride solution (15:85) for at least 1 h prior to use. The plate was developed over a distance of 12 cm with a migration time of about 2 h. After development the plate was dried in an oven at 105–110°C (30 min). Detection of the spots was performed by dipping horizontally for 4 s in a laboratory-made container, filled with a solution of 60 mg NBD-Cl in 300 ml of methanol–acetone (1:1). The plate was allowed to dry on the bench for 5 min and was heated in an oven at 80°C for 30 min to complete the derivatization reaction. The excess reagent was removed by developing the plate twice in methanol–acetone (1:1) over a distance of 12 cm. The plate was dried in an oven at 80°C for 5 min. The sensitivity was enhanced by dipping the plate for 1 s in a 30% (v/v) solution of liquid paraffin in n-hexane. The plate was dried in an oven at 80°C for 5 min. Greenish-yellow fluorescent spots on a colourless background were obtained.

The chromatograms were analysed with a CS-990 TLC scanner (Shimadzu, Kyoto, Japan) using the following parameters: linear scan, scan step in the y-direction 0.05 mm, beam size 6 mm × 0.4 mm, fluorescence mode with emission at $\lambda = 475$ nm and filter 4; linearizer off, background correction on; drift-line integration 0.05.

The ratio of the peak areas of the two isomers was compared to the ratios obtained for the mixtures of the house standards.

3. Results and discussion

3.1. Influence of the brand of silica gel

The different brands of silica gel plates were examined for their selectivity towards the separation of neomycins B and C. Precoated plates from Macherey–Nagel and Carlo Erba gave insufficient retention and the spots moved close to the front. The layers from Merck and Baker gave separation with considerable streaking. Both the silica gels from Whatman gave good separation of the two isomers. Since the separation and spot shape on Whatman K5 plates with 150 Å pores were better, these plates were preferred. Experiments with silica gel from Merck with pore sizes of 60, 200 and 1000 Å indicated that the use of silica gel with wider pores did not improve the separation.

The silanized silica gel layers from Merck gave results comparable to those obtained with bare silica gel. The difference in result between the different brands of plates may be due to the quality of the silica gel and the nature of the binder.

3.2. Composition of the mobile phase

Different salts such as sodium chloride, sodium bromide, sodium fluoride, lithium chloride, sodium bromide and triethylammonium acetate were used to prepare the mobile phase. Sodium chloride was found to be the best in terms of symmetry of the spots and retention characteristics. Decreasing the sodium chloride concentration deteriorated the symmetry, but with higher concentrations the baseline noise increased. A final concentration of 20% was adopted. An explanation for the mechanism was given by Kovacs-Hadady. The retention is connected with the adsorption of the metal ions on the surface of the stationary phase. As long as the surface of the silica gel layer is not saturated by adsorbed

metal ions, the free silanol groups interact strongly with the basic aminoglycosides [18]. The R_F values decreased with increase of the methanol content but the resolution decreased at the same time. A final concentration of 15% was selected. Substitution of methanol by ethanol did not improve the separation. Good separations were obtained after a saturation time of the chromatographic chamber for at least 1 h prior to use. Activation of the plate at 105°C for 30 min did not improve the separation. Predevelopment of the plate with methanol, followed by drying at 105°C for 30 min did affect neither the separation nor the noise during scanning. The plates were always developed at room temperature over a distance of 12 cm. Table 1 shows the R_F values for different neomycins and some related aminoglycoside antibiotics. With the described method the relative amount of paromomycin II in commercial samples of paromomycin sulphate can also be determined.

3.3. Resolution and peak symmetry

The resolution between the peaks of neomycins B and C and their symmetry factors were measured using a mixture of equal amounts of both components. The resolution was 1.7 and

Table 1
R_F values of the different neomycins and some related aminoglycoside antibiotics

Aminoglycoside antibiotic	R_F
Neomycin A	0.33
Neomycin B	0.24
Neomycin C	0.16
Paromamine	0.59
Paromomycin I	0.51
Paromomycin II	0.40
Kanamycin A	0.47
Kanamycin B	0.21
Amikacin	0.68
Tobramycin	0.30
Apramycin	0.47
Gentamicin	0.53, 0.57, 0.62

the symmetry factor of the peak of neomycin B was 1.2 and that of neomycin C 1.1.

3.4. Detection method

Since fluorescence is more sensitive and gives higher slopes than colorimetric procedures, a fluorogenic technique was investigated. Fluorescamine was not investigated as the fluorescence decays rapidly. NBD-Cl, which reacts only with primary and secondary amines, while yielding a non-fluorescent hydrolysis product, was found to be the fluorogenic reagent of choice [23]. The NBD-Cl solution used may be stored in the dark at room temperature for at least two months. Horizontal dipping using a 0.02% solution of NBD-Cl in methanol–acetone (1:1) and a dipping time of 4 s, was determined to be optimal. The heating time to complete the derivatization was varied from 20 to 60 min at 80°C. A reaction time of 30 min was proved satisfactory. Higher temperature did not increase the response. Spraying the plate instead of dipping has the drawback that unstable baselines are obtained and integration is difficult to standardize. The excess NBD-Cl was removed by developing the plate twice in a mixture methanol–acetone (1:1). Treatment of the plate with sodium acetate or sodium carbonate as buffer before reaction with NBD-Cl was described to enhance the sensitivity [24]. However, the loading of more salt on the plate increased the baseline noise during the scanning procedure and therefore this technique was not retained. Measurements at 475 nm afforded the highest response. By immersing the plate for 1 s in a hexane solution of liquid paraffin, the fluorescence was enhanced by a factor of about 1.5 [25]. When stored in the dark and protected from dust the fluorescence remained stable for several months.

3.5. Linearity and detection limits

A calibration curve was prepared from mixtures containing 1% neomycin C up to 50% neomycin C. The linearity was examined and the following results were found: $y = -0.10860 + 1.007699x$; $r = 0.9988$; $S_{y,x} = 0.1247$, where $y =$

ratio neomycins C/B prepared; x = ratio neomycins C/B found; r = coefficient of correlation; $S_{y,x}$ = standard error of estimate; number of concentrations examined = 11; number of scans per concentration = 3. These experiments were performed on one single plate. In the assay of commercial samples, a calibration curve was not used, but standard mixtures, applied on the same plate, were used in a single-point calibration.

For an application of 4 μg of the sample to the plate, the limit of detection was about 1% (m/m) (0.04 μg), which is similar to that obtained with the official European Pharmacopoeia method.

3.6. Assay of commercial samples

Several samples of neomycin and framycetin were assayed using the described method. A typical chromatogram is shown in Fig. 2. To calculate the percentage of neomycin C, the nearest chromatogram of a standard mixture was used. The C/B ratio of the standard mixtures were adapted to the ratio present in the samples. The results are shown in Table 2, which also contains figures calculated by normalisation and figures which were mentioned previously [13] for a number of the neomycin samples, using the European Pharmacopoeia method: IELC, detection with ninhydrin and calculation by normalization. For the TLC method, the values obtained by normalisation are smaller than those obtained by calculation against a standard. The latter correspond well with the figures obtained with the official method. This would suggest that the derivatization reaction on the plate is less complete for neomycin C than for neomycin B. The amount of neomycin C in older neomycin samples varies from 9.4 to 39.9%. In more recent neomycin samples the neomycin C content is between 7 and 13%. The relative standard deviation (R.S.D.) for n = 10 is 6–8%. In experiments with the official method [8] the R.S.D. for a framycetin sample containing 1.4% of neomycin C was 16% (n = 3). The neomycin samples comply with the European Pharmacopoeia limits (3–15% C). The framycetin samples examined

Fig. 2. Fluorescence scan of a typical chromatogram of commercial neomycin sulphate.

also comply with European Pharmacopoeia limits (<3% C).

4. Conclusions

Neomycins B and C can be separated by TLC on Whatman plates. The method allows to determine the composition of commercial samples. The results show that the qualitative composition may vary over a wide range. The results confirm the need for a limit test for the composition in official texts such as pharmacopoeias. The proposed method can be used as an alternative to IELC followed by ninhydrin colorimetry, which is the official method prescribed by the European Pharmacopoeia.

Table 2
Relative amounts of neomycin C in commercial samples

Sample	Method		
	TLC–fluorescence detection		IELC–ninhydrin detection [13]
	With standards	With normalization	
Old neomycin samples			
U.XZ-336	10.3	7.8	9.8
S. 52001	39.9	30.9	37.8
U. TRO-32	9.4	6.9	9.6
R. 7S-1251	10.2	7.9	10.0
R. 9S0581	15.0	11.6	15.3
St. Nat.	29.8	23.1	27.0
G. 58510	18.5	14.1	19.1
G. S.51009	32.4	25.1	30.0
T. H5XHTN4	15.4	11.9	16.9
Recent neomycin samples			
04/0393 A	8.3	6.4	
30/062 A	10.6	9.0	
939006 A	10.4	10.0	
939005 A	8.7	8.3	
40/418 A	11.3	8.9	
30/042 A	12.2	10.7	
Framycetin samples			
93/0420 A	1.8	1.1	
940/370 A	1.8	1.9	
7SO2224 B	1.3	0.7	
9SO238	1.1	0.6	
9SO425	1.1	0.6	

Acknowledgements

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