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Determination of neomycin sulfate by liquid chromatography with pulsed electrochemical detection

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Abstract

The determination of neomycin sulfate by liquid chromatography using a column packed with a poly(styrene–divinylbenzene) co-polymer and pulsed electrochemical detection on a gold electrode is described. The mobile phase consisted of an aqueous solution containing 70 g/l of sodium sulfate, 1.4 g/l of sodium 1-octanesulfonate and 50 ml/l of a 0.2 *M* phosphate buffer (pH 3.0). Sodium hydroxide was added post-column. The influence of the different chromatographic parameters on the separation was investigated. The method shows good linearity and repeatability and is stability indicating. A number of commercial samples was analyzed using this method and the results were compared with results obtained with the European Pharmacopoeia method and a previously described thin-layer chromatographic method.

Keywords: Pharmaceutical analysis; Neomycin sulfate; Antibiotics

1. Introduction

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

Neomycin sulfate is mainly composed of a mixture of neomycin B (Fig. 1) and its stereoisomer neomycin C [2]. Another minor component, neomycin A or neamine was isolated from the mixture [3] and is formed by partial hydrolysis of neomycins B or C [4,5]. Small amounts of other constituents are also present in commercial samples. Some of these impurities, formerly called neomycins D, E and F were identified as paromamine, paromomycin I and paromomycin II, respectively [6]. Neomycin LP-A and LP-B (LP=low potency), which are the mono-N-acetyl derivatives of neomycins A and B, were also isolated from the mixture [7]. 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 [8]. Neamine has no antimicrobial activity. The European Pharmacopoeia limits the amount of neomycin C to 3–15% [9]. Neomycin with a content of less than 3% neomycin C is called framycetin. The United States Pharmacopeia does not distinguish between neomycin

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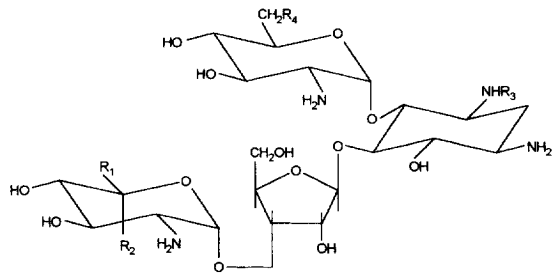
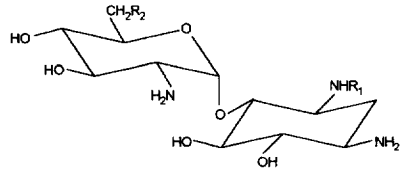


	R_1	R_2	R_3	R_4
Neomycin B	H	CH_2NH_2	H	NH_2
Neomycin C	CH_2NH_2	H	H	NH_2
Paromomycin I	H	CH_2NH_2	H	OH
Paromomycin II	CH_2NH_2	H	H	OH
LP-B	H	CH_2NH_2	Ac	NH_2



	R_1	R_2
Neamine	H	NH_2
Paromamine	H	OH
LP-A	Ac	NH_2

Fig. 1. Structures of different neomycin components.

and framycetin and therefore does not limit neomycin C in a separate test [10].

Chromatographic separation of the stereoisomers neomycins B and C is quite difficult. The detection also is problematic because neomycin has no strong UV-absorbing chromophore. Ion-exclusion liquid chromatography (IELC) with photometric ninhydrin detection has been described for the determination of neomycins B and C [11]. The IELC method was further improved by Decoster et al., but combined with refractive index detection the sensitivity was poor [12]. Thus, the method finally chosen by the European Pharmacopoeia to determine the neomycin C content combined the latter IELC method with photometric detection [9]. Gas–liquid chromatography after trimethylsilylation [13] and liquid chromatography (LC) on normal-phase after pre-column derivatization with 2,4-dinitrofluorobenzene [14–16] or 2-naphthalene-sulfonylchloride [17] have been reported. More recently, LC methods on reversed-phase combined with post-column derivatization with *ortho*-phthalaldehyde were published [18,19]. In 1991, the separation of neomycins B and C on a high-capacity anion-exchange column (CarboPac PA1) combined with pulsed electrochemical detection was described. However, this column packing is patented and rather expensive [20].

Thin-layer chromatographic separation was first described on acid-treated carbon black plates and detection was performed by bioautography [21]. Kovacs-Hadady 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 amino-glycosides migrated, while by developing with water, no migration occurred [22]. Quantitative results for different neomycins were not reported until very recently [23].

In this work an LC method is described using a column packed with a poly(styrene–divinylbenzene) co-polymer. Ion-pair LC combined with pulsed electrochemical detection was investigated. The composition of the mobile phase was based on those described by Apffel et al. [18]. The method has been used to analyze a number of commercial samples.

2. Experimental

2.1. Reagents and reference substances

Water was distilled twice from glass apparatus. The buffer solution was made by mixing a 0.2 *M* solution of phosphoric acid and a 0.2 *M* solution of potassium hydrogenphosphate till a pH of 3.0 was achieved. These solutions were prepared with phosphoric acid 85% (m/m) (Acros Chimica, Geel, Belgium) and potassium dihydrogenphosphate (Acros Chimica). Sodium sulfate anhydrous was obtained from Merck (Darmstadt, Germany); sodium 1-octanesulfonate, monohydrate 98% from Acros Chimica and helium from Air Liquide (Machelen, Belgium). The 0.5 *M* sodium hydroxide solution was made using 50% sodium hydroxide (m/m), aqueous solution (Baker, Deventer, Netherlands). Neomycin

B, neomycin C, LP-B, neamine, paromamine and LP-A reference substances were prepared in the laboratory from commercial samples as described [24]. A mixture of paromomycin I and paromomycin II was obtained from Carlo Erba (Milan, Italy). Mixtures of the neomycins B and C laboratory standards ranging from 3 to 40% (m/m) neomycin C in neomycin B were prepared. To obtain a homogeneous mixture the two substances were dissolved in water, after which the water was evaporated under vacuum. Commercial samples were obtained from Sifa (Paris, France), Roussel-Uclaf (Romainville, France), Upjohn (Kalamazoo, MI, USA) and Takeda (Osaka, Japan).

A house standard of neomycin B base was available in the laboratory. The purity of this standard was 92.7% (m/m), expressed on the substance as is. The base content of this standard was determined by a non-aqueous potentiometric titration of the base with 0.1 *M* perchloric acid using glacial acetic acid as the solvent. The water content was determined by Karl–Fischer titration and amounted to 6.74%. The total mass explained by titration and water determination was 99.58%. The percentage of neomycin C, determined by LC, was 0.12%.

2.2. Apparatus

The chromatographic procedure was carried out using a L-6200 Intelligent Pump (Merck–Hitachi, Darmstadt, Germany), a Marathon autosampler (Spark, Emmen, Netherlands) with a fixed loop of 20 μl, a laboratory-made pneumatic device, allowing pulse-free post-column addition of sodium hydroxide solution and an electronic integrator HP 3393A (Hewlett-Packard, Avondale, PA, USA). The column (250×4.6 mm I.D.) was packed with poly(styrene–divinylbenzene) co-polymer PLRP-S 1000 Å, 8 μm (Polymer Laboratories, Shropshire, UK). The temperature of the column was maintained at 35°C using a water bath with a circulator (Julabo, Seelbach, Germany). The PED-1 pulsed electrochemical detector from Dionex (Sunnyvale, CA, USA) was equipped with a gold working electrode with a diameter of 3 mm, a Ag/AgCl reference electrode and a stainless-steel counter electrode. The detector was put in a laboratory-made hot-air oven to keep the temperature at 35°C.

2.3. Chromatography

All substances to be analyzed were dissolved in water. The mobile phase, consisting of an aqueous solution containing 70 g/l of sodium sulfate, 1.4 g/l of sodium 1-octanesulfonate and 50 ml/l of a 0.2 *M* phosphate buffer (pH 3.0), was sonicated before use. The flow-rate was 1 ml/min. Through a mixing-tee, 0.5 *M* NaOH was added post-column from a helium-pressurized reservoir (1.6 bar) and mixed in a packed reaction coil (1.2 m, 500 μl) from Dionex which was linked to the electrochemical cell. The post-column addition of the base must be pulse-free and it is necessary to raise the pH of the mobile phase to approximately 13 to improve the sensitivity of the detection [25]. The base was added at a flow-rate of 0.3 ml/min. Although the flow-rate is not critical, it should be reproducible between runs. The NaOH solution was made starting from a 50% (m/m) aqueous solution which was pipetted into helium degassed water because it is very important to avoid carbonates that foul the electrodes. For this reason it is advisable to pipette the NaOH solution from the center of the bottle and to use only two-thirds of the bottle [26].

The time and voltage parameters for the detector were provided by Dionex [20] and were set as follows: E_1, E_2 and E_3 were respectively +0.05 V, +0.75 V and −0.15 V with the assigned pulse durations t_1: 0–0.40 s, t_2: 0.41–0.60 s and t_3: 0.61–1.00 s. Integration of the signal occurred between 0.2 and 0.4 s. Although theoretically the continuous sequence of the potentials cleans the electrode surface, it is necessary to polish the gold working electrode after about 60 analyses to obtain a good repeatability. When the electrode is cleaned with a fine polishing compound it is sonicated in water for 10 min. After installation of the polished electrode, it takes about 1 h to obtain a stable baseline.

3. Results and discussion

3.1. Development of the chromatographic method

Poly(styrene–divinylbenzene) was chosen as the stationary phase because of the remarkable stability of this material. Optimal conditions were determined

by analysing a mixture of the different neomycin components dissolved in water at concentrations of 0.30 mg/ml for neomycin B sulfate, 0.04 mg/ml for neomycin C sulfate, 0.03 mg/ml for LP-B, 0.03 mg/ml for a mixture of paromomycins I and II, 0.01 mg/ml for neamine hydrochloride, 0.01 mg/ml for LP-A and 0.01 mg/ml for paromamine. The influence of the different chromatographic parameters on the separation of the neomycin components was evaluated using the capacity factors (k'). Methanol was used to determine t_0. For every parameter examined, the mixture of reference compounds was injected three times. For the calculation of the k' value of each compound, the average of the retention times was used. The relative standard deviation (R.S.D.) for the retention time of neomycin B was not greater than 0.3%. Each time, only one parameter was changed and the others were kept constant.

The influence of the pH of the mobile phase on the k' values of the neomycin components is illustrated in Fig. 2. As can be seen, there are little changes between pH 2.0 and pH 5.0. By further increase of the pH, the retention times decrease since less amino groups are protonated and the interaction with octanesulfonate decreases.

The influence of the buffer type was also examined. When an acetate buffer of pH 3.0 was used instead of a phosphate buffer of pH 3.0, the baseline was less stable and the peak symmetry was less good.

The influence of the column temperature was examined at 30, 35, 40 and 45°C. As expected, the k' values of the components decrease when the column temperature is increased.

Sodium octanesulfonate acts as an ion-pairing agent. It is added to retain the neomycin molecules, which are positively charged at pH 3.0. Varying the sodium octanesulfonate concentration of the mobile phase in the range 1.1 to 1.6 g/l revealed an optimal separation at a concentration of 1.4 g/l. As expected, the capacity factors decrease by decreasing the sodium octanesulfonate concentration. Below 1.3 g/l the separation between LP-B and neomycin C is insufficient (resolution <1.25).

The influence of the sodium sulfate concentration of the mobile phase on the retention times of the different neomycin components was examined in the range 65 to 75 g/l. An increase of the sodium sulfate concentration results in a decrease of the k' values. Sodium sulfate has been used in ion-pairing mobile phases to shorten retention times as the sulfate anions are more hydrophillic than the anions of the ion-pairing agent [27]. When the concentration of sulfate anions in the mobile phase is not sufficient, the chromatographic peaks are distorted.

Using the developed mobile phase, the separation on PLRP-S, 8 μm, 1000 Å was much better than those obtained on PLRP-S having smaller pore sizes of 100 and 300 Å.

A typical chromatogram of a commercial sample

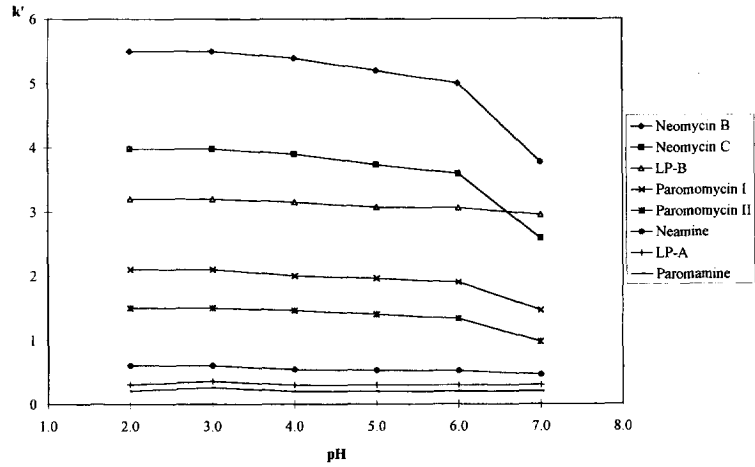


Fig. 2. Influence of the pH of the mobile phase on the k' values.

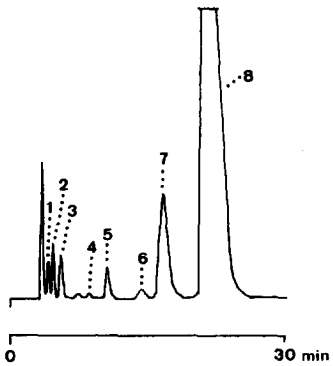


Fig. 3. Typical chromatogram of a commercial sample of neomycin sulfate: 1=paromamine, 2=LP-A, 3=neamine, 4=paromomycin II, 5=paromomycin I, 6=LP-B, 7=neomycin C, 8=neomycin B.

of neomycin sulfate, obtained with the selected chromatographic conditions, is shown in Fig. 3.

3.2. Robustness

By means of a 4-factorial design, the importance of the individual chromatographic parameters and parameter interactions of this LC method was studied. The set-up of the applied factorial design was supported by the statistical graphics software system, Statgraphics version 6 (Manugistics, Rockville, MD, USA). The chromatographic parameters examined as variables were: the concentration of sodium sulfate, the sodium octanesulfonate concentration, the pH of the mobile phase and the column temperature. The measured response variables were the retention times of paromamine, LP-A, neamine, paromomycins I and II, LP-B, neomycin C and neomycin B. The values used in the design are shown in Table 1. Two blocks of 17 experiments were done. The results show that, under the examined conditions, the main peak was always separated from its impurities and that the LC system is principally influenced by the sodium sulfate concentration. The second and third most important chromatographic parameter, by which the retention times of paromamine, LP-A and neamine are influenced are the sodium octanesulfonate concentration and the column temperature, respectively. For the retention times of the other neomycin components, the column temperature is more important than the concentration of the ion-pairing agent. Investigation of the resolution between LP-B and neomycin C indicates that it was also mostly influenced by the three parameters mentioned above. In the examined range, the pH has no significant effect on the retention times and no significant interactions between the parameters were observed. With the most significant chromatographic parameters as independent variables and the retention times as response variables, a regression model was calculated. From the found regression model, a response surface plot was constructed to help optimizing the parameters, but no further improvement could be made.

3.3. Quantitative aspects of the LC method

The sample load capacity of the column was found to be 100 μg. For quantitative analysis of neomycin sulfate an amount of 10 μg was used. For this quantity, the limit of detection for neomycin C was 0.05% (m/m) (5 ng), determined at a signal-to-noise ratio of 3. The limit of quantitation was 0.15% (m/m) (R.S.D.=6.9% for n=4).

The repeatability was checked by analysing, six times, a solution of a mixture of neomycin B and neomycin C (90:10) which was dissolved in water at a concentration of 0.5 mg/ml. The R.S.D. values on the areas of the neomycin B and neomycin C peaks were 1.3 and 1.5%, respectively. The linearity of a mixture of neomycins B and C (90:10) in the range

Table 1
Factorial analysis: nominal values corresponding to −1, 0 and +1

Chromatographic parameter	Low value (−1)	Central value (0)	High value (+1)
Sodium sulfate (g/l)	65	70	75
Sodium octanesulfonate (g/l)	1.2	1.4	1.6
pH of the mobile phase	2.0	3.0	4.0
Column temperature (°C)	30	35	40

from 5 to 160% of the theoretical value (0.5 mg/ml) was examined. The following results were found for neomycin B: $y=280211x+1686$; $r=0.9989$ and $S_{y,x}=3195$, and for neomycin C: $y=361271x-430$; $r=0.9975$ and $S_{y,x}=630$ where y=peak area/1000; x=concentration in mg/ml; r=coefficient of correlation and $S_{y,x}$=standard error of estimate.

When a solution of neomycin reference compounds was analyzed repeatedly with different mobile phases containing increasing amounts of sodium sulfate, the retention times decreased as expected, but the peak areas also decreased, probably due to overloading of the electrode.

3.4. Analysis of commercial samples

Several samples of neomycin and framycetin were assayed using the described method. The relative amounts of neomycin C in commercial samples were calculated by normalization and standardization using the peak areas. The C/B ratio of the standard mixtures was adapted to the ratio present in the samples. The results are shown in Table 2, which also contains figures which were reported previously, and which were obtained using a TLC method with fluorescence detection [23] and the European Pharmacopoeia method (IELC with ninhydrin detection) [12]. The values correspond well with the figures obtained with the other two methods. In contrast to the TLC method, the differences between normalization and standardization are small for the HPLC–PED method. Nevertheless, it is advisable to use standards, since the peak area also depends on the retention time. The amount of neomycin C in older neomycin samples varies from 9 to 37%. In more recent neomycin samples the neomycin C content is between 8 and 14%. The R.S.D. for neomycin samples is 0.5–1.3% ($n=3$) and for framycetin samples 1.5–2.6% ($n=3$). In experiments with the official method [9], the R.S.D. for a framycetin sample containing 1.4% of neomycin C was 16% ($n=3$) and with the TLC method 8% ($n=3$).

Table 3 shows the results for the total base content of the commercial samples, calculated with

Table 2
Amounts of neomycin C (%, m/m) in commercial samples, relative to the sum of neomycins B and C

Sample	HPLC–PED		TLC–fluorescence detection [23]	IELC–ninhydrin detecton [12]
	Normal.	Stand.		
Old neomycin samples				
U. XZ-336	10.8	10.6	10.3	9.8
S. 52001	34.9	37.0	36.4	37.8
U. TRO-32	9.9	9.6	9.6	9.6
R. 7S-1251	10.8	10.4	10.2	10.0
R. 9S0581	15.7	15.4	16.6	15.3
St. Nat.	26.6	27.8	27.7	27.0
G. 58510	18.0	17.7	16.8	19.1
G. S.51009	29.2	30.6	29.3	30.0
T. H5XHTN4	14.8	14.4	14.4	16.9
Recent neomycin samples				
04/0393 A	8.5	8.3	8.2	
30/062 A	11.1	11.0	10.8	
939006 A	13.8	13.4	12.6	
939005 A	11.6	11.2	10.9	
40/418 A	11.8	11.3	11.2	
30/042 A	13.2	12.8	12.9	
Framycetin samples				
93/0420 A	2.2	2.1	2.2	
940/370 A	2.2	2.1	1.8	
7S02224 B	2.0	2.0	1.8	
9S0238	3.3	3.1	3.0	
9S0425	2.6	2.4	2.5	

Table 3
Composition of commercial samples of neomycin and framycetin sulfate

Sample	% B base	% C base	% Other	% Total base
Old neomycin samples				
U. XZ-336	49.9	6.0	2.0	57.9
S. 52001	39.1	21.0	1.7	61.8
U. TRO-32	48.8	5.4	6.9	61.1
R. 7S-1251	49.4	6.0	4.8	60.2
R. 9S0581	48.0	8.9	4.1	61.0
St. Nat.	47.3	17.2	3.0	67.5
G. 58510	45.7	10.0	3.8	59.5
G. S.51009	42.2	17.6	1.6	61.4
T. H5XHTN4	46.8	8.1	3.5	58.4
Recent neomycin samples				
04/0393 A	56.1	5.2	3.2	64.5
30/062 A	51.2	6.5	3.7	61.4
939006 A	51.5	8.2	3.8	63.5
939005 A	53.1	7.0	2.4	62.5
40/418 A	54.4	7.3	4.4	66.1
30/042 A	49.8	7.6	3.9	61.3
Framycetin samples				
93/0420 A	65.7	1.4	0.9	68.0
940/370 A	61.0	1.4	2.0	64.4
7S02224 B	59.0	1.2	0.8	61.0
9S0238	58.9	2.1	0.9	61.9
9S0425	60.0	1.6	1.2	62.8

reference to the neomycin B house standard (92.7%, m/m, as is). Since the European Pharmacopoeia limits the amount of sulfate to 27–31%, calculated to the dried substance, and the water content to 8% in neomycin [9] and framycetin sulfate samples [28], the total base content should be at least 61%. The old neomycin samples contain 39–50% of neomycin B base, 5–21% of neomycin C and 1–7% of other substances, all expressed as neomycin B base. The contents of neomycin B, neomycin C and other substances, all expressed as neomycin B base, in recent neomycin samples are: 49–57%, 5–9% and 2–5%, respectively. For the framycetin samples, the following base contents were found: 58–66% of neomycin B, 1–2% of neomycin C and 0–2% of other substances.

4. Conclusion

This method, using a poly(styrene–divinylbenzene) co-polymer as the stationary phase, allows to separate all the known components of neomycin. The total time of analysis is not more than 30 min. Pulsed electrochemical detection suffers from some stability problems and experience is required to obtain reproducible results, but compared to previously published methods, this method allows sensitive detection of neomycin and shows good repeatability and linearity.

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