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Simultaneous determination of neomycin sulfate and polymyxin B sulfate by capillary electrophoresis with indirect UV detection

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Abstract

A simple and rapid capillary electrophoresis method, with indirect UV detection, for the simultaneous determination of neomycin sulfate and polymyxin B sulfate in pharmaceutical formulations was developed. Critical parameters such as pH, buffer composition and concentration, voltage and injection time have been studied to evaluate, how they affect responses, such as resolution and migration times. Separation was performed on a fused silica capillary with 50 μm i.d. and 27 cm total length at an applied voltage of 6 kV with a 15 mM phosphate run buffer (pH 5.0) containing 40 mM *N*-(4-hydroxy-phenyl)acetamide and 50 mM tetradecylammonium bromide (TTAB). The detection wavelength was set at 280 nm. Quantitative analysis was validated by testing the reproducibility of the method, giving a relative standard deviation less than 0.4 and 2.4% for the repeatability of migration time and corrected peak area, respectively. Accuracy was tested by spiking eye–ear formulations with standards and the recoveries of neomycin sulfate and polymyxin B sulfate were found to be between 97.44–103.18% and 96.85–101.68%, respectively. Linearity of neomycin sulfate and polymyxin B sulfate were obtained in the ranges of 17–682 and 24–608 μg/mL, respectively, with r^2 values above 0.999. The established TLC–densitometric method was applied to evaluate the proposed CE method, and comparable results were obtained by using CE with much shorter analysis time and a small quantity of solvents consumed. The developed method is also the first report on the simultaneous determination of neomycin sulfate and polymyxin B sulfate in pharmaceutical preparations by CE.

Keywords: Capillary electrophoresis; Indirect UV detection; Neomycin sulfate; Polymyxin B sulfate

1. Introduction

Neomycin (Fig. 1a) is a widely used broad spectrum, water soluble aminoglycoside antibiotic, produced during the fermentation of *Streptomyces fradie*. 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 oto-and nephrotoxicity [1,2]. Polymyxin B (Fig. 1b), in contrast to neomycin, is a complex of closely related decapeptide antibiotic, which belongs to the group of polymyxin antibiotics isolated from various strains of *Bacillus polymyxa* and related species. Its sulfate salt is used for the treatment of infections caused by Gram-negative bacteria [3,4]. A combination of these antibiotics in a liquid formulation, together with other active ingredients or auxiliary substances, is used for instillation into the eye or the ear [5,6].

There have been numerous publications describing various methods for the determination of neomycin and polymyxin B in different pharmaceutical formulations individually and in combination with other active ingredients. Polymyxin B has been determined spectrophotometrically by derivative and multivariate calibration techniques [7,8], by high-performance liquid chromatography (HPLC) [3,5,9–12], by thin-layer chromatography (TLC) [13], and also by capillary electrophoresis (CE) [4,14–17]. Due to the lack of a chromophore or fluorophore in neomycin molecule, the direct detection by conventionally

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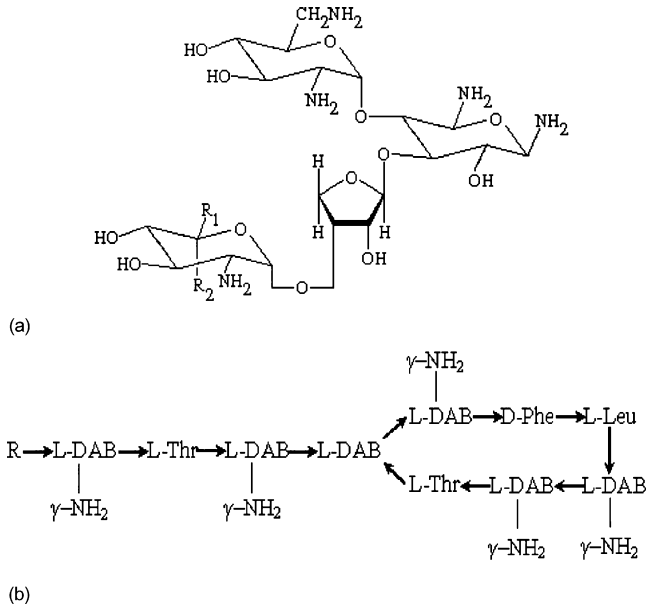

Fig. 1. Chemical structures of (a) neomycin and (b) polymyxin B. DAB, α,γ-diaminobutyric acid; Thr, threonine; Phe, phenylalanine; Leu, leucine.

spectrophotometric method is difficult [1,18]. Standard methods for neomycin analysis usually employ biologically based on detection procedures, which determine the total antibiotic activity in a sample compare to the reference standard [2,19]. Alternative methods of analysis have included HPLC and, recently, CE with either pre- or post-column derivatization or electrochemical detection [1,2,5,20]. To date, however, only a few methods have been described in the literatures for the simultaneous determination of neomycin and polymyxin B. The microbiological methods often proceeded by a chromatographic procedure to isolation the individual constituents are of great importance [6,21]. A thin-layer chromatographic–densitometric method has also been described recently [6]. However, these methods are laborious and time-consuming. Therefore, a simple, rapid and reliable analysis method is required.

Nowadays, CE is extensively utilized in pharmaceutical analysis. Efficiency, selective, speed of analysis, small volume of sample and low running cost are some of the advantages of CE [22]. Indirect photometric detection in CE is often employed for detection of compounds that lack of suitable chromophores. This detection method utilizes a light-absorbing compound as the run buffer creating a large background absorbance. When a non-absorbing solute passes through the detector, it causes a decrease in absorbance, resulting in a dip in the baseline which is the equivalence of a negative peak. The output polarity of the detector is reversed so that a positive peak is obtained [23–26].

To our knowledge, however, no CE method has been described for the simultaneous determination of neomycin sulfate and polymyxin B sulfate. In this investigation, a simple and rapid CE method with indirect UV detection was developed, optimized and validated for the simultaneous quantitative determination of neomycin sulfate and polymyxin B sulfate in eye–ear formulations. The optimized methodology was successfully applied in commercial pharmaceuticals.

2. Experimental

2.1. Chemicals and samples

The sulfate salts of neomycin and polymyxin B were purchased from Sigma–Aldrich (Poole, Dorset, UK). Pharmaceutical preparations containing the active compounds were obtained from local pharmacies. The buffer components sodium dihydrogen phosphate, disodium hydrogen phosphate, tetradecylammonium bromide (TTAB) and *N*-(4-hydroxyphenyl)acetamide were all purchased from Sigma–Aldrich (Poole, Dorset, UK). All running buffers, standard solutions and sample solutions were prepared in deionized water, produced by an Elgastat Spectrum RO (Elga Ltd., Buck, UK). Commercial eye–ear formulations were purchased from local sources in Chiang Mai, Thailand.

2.2. Preparation of running buffers

Optimized BGE consisted of 15 mmol/L phosphate buffer (pH 5.0) containing 50 mmol/L TTAB and 40 mmol/L *N*-(4-hydroxyphenyl)acetamide. Stock solutions of individual components were prepared from the salts to give 200 mmol/L dihydrogen phosphate, 200 mmol/L disodium hydrogen phosphate, 200 mmol/L TTAB, and 80 mmol/L of *N*-(4-hydroxyphenyl)acetamide. A fresh buffer was prepared daily from these stock solutions.

2.3. Preparation of standard solutions

Stock standard solutions of neomycin sulfate and polymyxin B sulfate were freshly prepared separately by dissolving an accurate 20 mg of each standard in a small amount of deionized water and then diluting to 10 mL. Before analysis, the required concentration of neomycin sulfate and polymyxin B sulfate were prepared by mixing appropriate volume of stock standard solutions and then diluting with deionized water.

2.4. Preparation of sample solutions

The contents of three bottles of each eye–ear formulation were mixed well. The sample solutions were diluted with deionized water to obtain solutions where the expected concentration of neomycin sulfate and polymyxin B sulfate were within the calibration range.

2.5. Apparatus and conditions

All experiments were performed on a P/ACE model 5500 CE system, equipped with a filter carrousel UV detector and controlled by a Beckman P/ACE station version 1.1 (Beckman Coulter UK Ltd., High Wycombe, Bucks, UK). For separation of analytes, bare fused silica capillary, 27 cm total length (20 cm effective length), 50 μm i.d. × 375 μm o.d. (Composite Metal Services Ltd., Hallow, Worcs, UK) were used. The detection was performed at 280 nm in the indirect mode. The capillary, as well as the sample compartment, temperature was maintained

at 25 °C. During separation, a constant voltage of 5 kV was applied at a negative polarity (cathode at the injection end). A spectrophotometric studies were carried out with a Spekol model 1200 UV–vis spectrophotometer (Analytik Jena AG, Germany).

New capillaries were conditioned by flushing consecutively with 1.0N sodium hydroxide for 10 min, 0.1N sodium hydroxide for 10 min, deionized water for 5 min, and finally the BGE for 10 min prior to first use. At the beginning of each day, the capillary was pretreated with 0.1N sodium hydroxide for 5 min, deionized water for 1 min and then the BGE for 5 min. Between each run, the capillary was rinsed with deionized water for 2 min, 0.1N sodium hydroxide for 2 min, deionized water for 1 min, and the BGE for 3 min, successively.

3. Results and discussion

3.1. Method development

In order to propose a specific and accurate CE method for analyzing pharmaceutical products containing neomycin sulfate and polymyxin B sulfate, it is essential to find the best experimental conditions in which the analytes can be separated from each other. Because higher separation numbers can be obtained at low apparent mobilities, experiments were carried out in the anionic mode (anode at the detection side) with a reversed EOF. Since, neomycin sulfate and polymyxin B sulfate form positively charge ions in low pH buffer. One of the undesirable problems encountered in CE separation is adsorption onto the capillary wall, which can cause peak tailing and poor resolution. In this experiment, TTAB was added to the BGE in order to reverse the direction of EOF.

3.1.1. Selection of chromophoric ion

The correct choice of a chromophoric ion suitable for indirect detection that can form part of a suitably buffered electrolyte is not completing straightforward. Apart from being a chromophoric ion, this compound and the analytes should have similar electrophoretic mobilities. Indeed, when electrophoretic mobilities are mismatched, significant peak tailing or fronting might be observed. In preliminary work, the chromophoric ions biphenylamine, as well as cytosine and *N*-(4-hydroxyphenyl)acetamide were tested and, finally *N*-(4-hydroxyphenyl)acetamide was selected, since, best peak shape of analytes were obtained. In order to increase the method sensitivity, the molar absorptivity of chromophoric ion should be high and its absorption wavelength should not overlap with that of analytes. Fig. 2 shows the absorption profiles of neomycin sulfate, polymyxin B sulfate and *N*-(4-hydroxyphenyl)acetamide. *N*-(4-Hydroxyphenyl)acetamide absorbs strongly in the vicinity of 245 nm with a large molar absorptivity. Because of the availability of a 280-nm filter is a used CE instrument, also neomycin sulfate and polymyxin B sulfate have minimum absorbances at this wavelength. A detection of 280 nm was selected as a suitable compromise between strong absorption of the chromophoric ion and minimal absorption of the analytes.

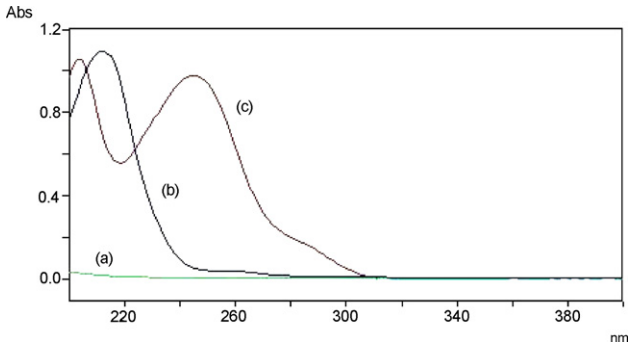

Fig. 2. UV spectra of (a) neomycin sulfate, (b) polymyxin B sulfate and (c) *N*-(4-hydroxyphenyl) acetamide.

3.1.2. Effect of pH

The buffer pH plays an important role in CE separation because it affects both of the overall charge of analytes and their electrophoretic mobilities, as well as the sensitivity in indirect UV detection. To obtain the positively charged analytes, the buffer pH chosen must be low enough to guarantee the substantial amount of analytes in the ionized forms. The effect of buffer pH was investigated within the range of 4.0–7.0 at a fixed buffer concentration. Under these conditions the analytes are protonated and migrate away from the cathodic end. At the same pH polymyxin B sulfate reaches the detector before neomycin sulfate.

Considering that this separation order corresponds to an acid pH, one can analyze if the order matches the mass/charge ratio. The pK_a's and molecular weights of the sulfate salts of neomycin and polymyxin B are 8.3 (908.9 g/mol) [27] and 8.9 (1532 g/mol) [28], respectively. The most charged species is neomycin ion, which could move against EOF with the stronger force than polymyxin B ion. The effect of pH (4.0–7.0) on the migration time of neomycin sulfate and polymyxin B sulfate is shown in Fig. 3. The migration times of cationic compounds decreased significantly with increased pH from 4.0 to 7.0 in the anionic mode with a reversed EOF. However, it was found that the peak of polymyxin B sulfate was not developed at pH 7.0. This suggest that polymyxin B sulfate is largely unprotonated and therefore migrates unseparated with the EOF. Fig. 4 shows the separa-

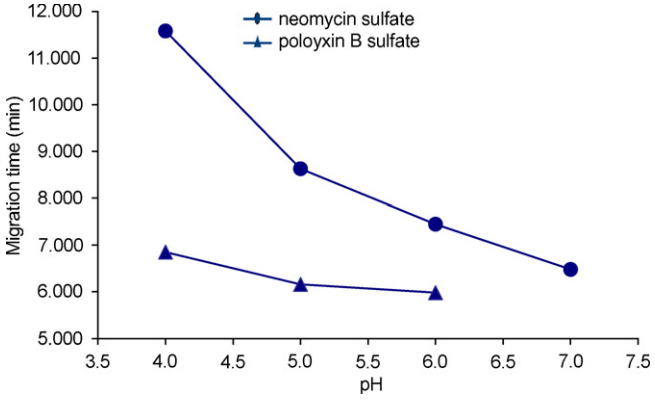

Fig. 3. Effect of buffer pH on migration time (t_m). Conditions: 27 cm (20 cm effective length) × 50 μm i.d. capillary, 10 mM phosphate, 50 mM TTAB, 10 mM *N*-(4-hydroxyphenyl)acetamide; −5 kV, 25 °C, 280 nM.

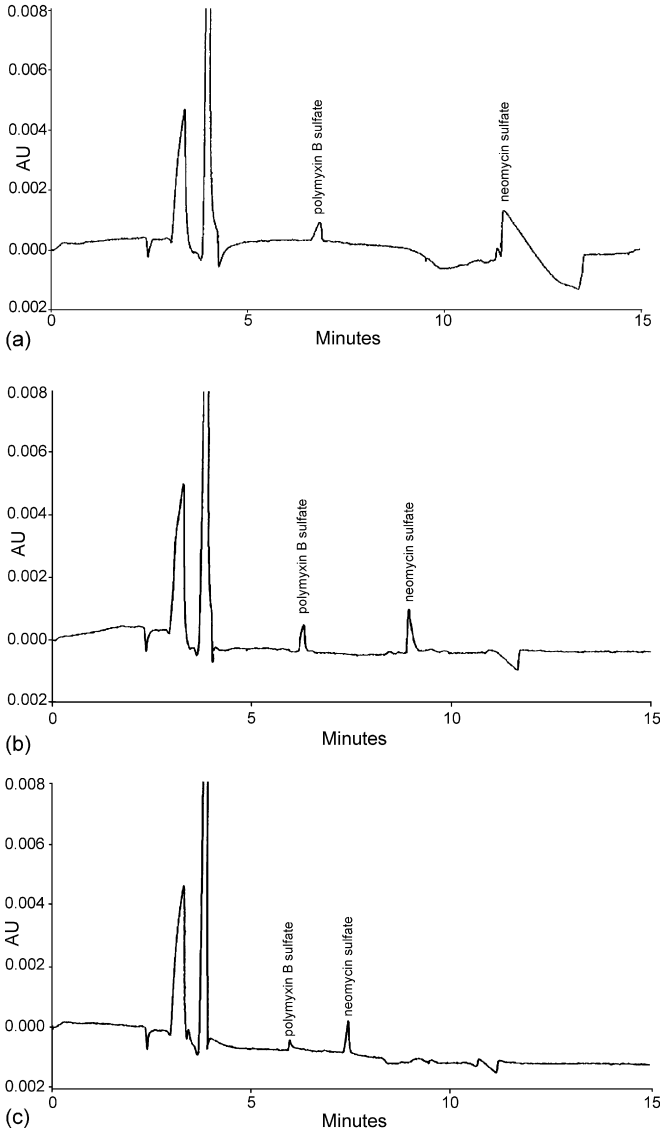

Fig. 4. Comparison of separation between neomycin sulfate and polymyxin B sulfate among different pH values: (a) pH 4.0, (b) pH 5.0 and (c) pH 6.0 under conditions proposed in Fig. 3.

tion of neomycin sulfate and polymyxin B sulfate at various pH (4.0–6.0). As it can be seen, the peak of neomycin sulfate was very poorly developed at pH 4.0. At lower pH the degree of protonation of both analytes are higher, resulted in their longer migration times corresponded to the higher positively charge. The peak sensitivity of polymyxin B is lower than neomycin in all pH values. This suggest that the electrophoretic mobility of chromophoric ion match that of neomycin ion more than polymyxin B ion (especially at a pH of 5.0), and hence, the higher possible sensitivity obtained. A pH of 5.0 was selected as optimum, because at this pH value provides the maximum sensitivity (peak height) and well-defined peak.

3.1.3. Effect of buffer ionic strength

Buffer concentration has also a significant effect on the separation performance through its influence on the EOF and the current produced in the capillary. The optimal ionic strength of the BGE must be chosen to provide a balance between an acceptable low current (to minimize the noise) and a good peak efficiency. A series of buffer concentrations (10, 15, 20 and 30 mM phosphate) at constant pH of 5.0 were evaluated for the effect on the migration time of the compounds. Buffer concentrations from 10 to 30 mM resulted in similar resolution for neomycin and polymyxin B with a gradual decrease in migration times. Since, a concentration of 15 mM phosphate buffer provides the maximum sensitivity and maintains well-defined peak, this value was selected for further studies.

3.1.4. Effect of TTAB concentration

TTAB was added to the BGE in order to suppress the attractive forces between the positively charged analytes and the negatively charge Si–O^- groups on the inner capillary wall, as well as to reverse the direction of EOF. As expected, without adding TTAB to the BGE both peaks of compounds were not developed. As the concentrations of TTAB increased from 5 to 100 mM, the resolution and the migration times of analytes were increased. We suggest that, such an effect may be related to the lower EOF resulting from a decrease in the zeta potential at the capillary wall–solution interface. The maximum sensitivity was obtained at the TTAB concentrations of 25 and 50 mM for neomycin and polymyxin B, respectively. At 50 mM TTAB, the sensitivity of neomycin decreased by 25% from the maximum but it was sufficient for practical use. Also, considering the efficiency, resolution and peak shape, a TTAB concentration of 50 mM was optimal.

3.1.5. Effect of chromophoric ion concentration

To enhance the sensitivity of indirect detection, the concentration of chromophoric ion should be as low as possible so that the background noise is minimise. However, decreasing the concentration of the chromophoric ion decreases the linear dynamic range and causes the peak shape to become asymmetric and broad. The concentration of chromophoric ion is chosen to provide a balance between signal-to-noise and linear dynamic range [23–26]. *N*-(4-Hydroxyphenyl)acetamide concentration between 10 and 50 mM was investigated. The result

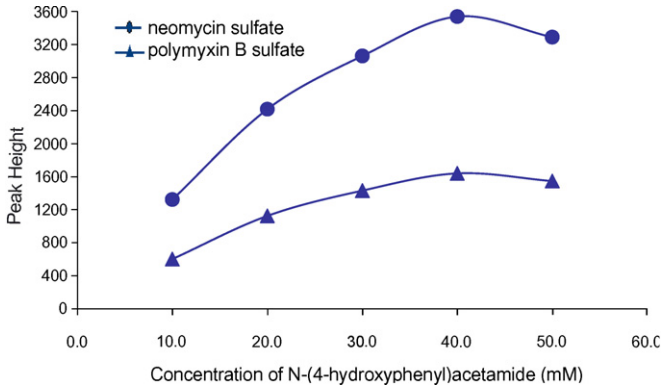

Fig. 5. Effect of concentration of *N*-(4-hydroxyphenyl)acetamide on peak height of neomycin and polymyxin B. Conditions: 27 cm (20 cm effective length) × 50 μm i.d. capillary, 15 mM phosphate, pH 5.0, 50 mM TTAB; −5 kV, 25 °C, 280 nM.

Table 1
Precision of CE migration times and the corrected peak area

	Neomycin sulfate (%R.S.D.)	Polymyxin B sulfate (%R.S.D.)
Repeatability		
Migration time	0.24	0.38
Corrected area	2.33	1.68
Intra-day precision		
Migration time	0.57	0.95
Corrected area	3.12	4.30

is shown in Fig. 5. Highest peak response was found at 40 mM *N*-(4-hydroxyphenyl)acetamide, where symmetrical peaks were obtained.

3.1.6. Optimization of sample loading

Injection time was varied in an attempt to optimize sample loading. Sample solutions were injected into the capillary by pressure while the injection time was varied from 1 to 10 s (steps of 1 s). As expected, when the injection time increased the corrected peak area of both compounds also increased. However, at longer injection time the capillary overload resulted the poor peak shape. The injection time selected was 5 s, where the suitable peak response and the well-defined peak were obtained.

3.2. Method performance

3.2.1. Ranges and linearity

In order to study the range of the CE response, stock solution of 2 g/L of neomycin sulfate and polymyxin B sulfate were prepared. Dilutions of 10–852 and 24–608 mg/L in deionized water were made for neomycin sulfate and polymyxin B sulfate, respectively. Each solution was injected in triplicate using the previously described CE method. Regression curves were obtained by plotting the corrected peak area versus concentration, using the least square method. Regression lines were $y = 15.212x - 160.27$, $r^2 = 0.9992$ and $y = 6.7683x - 68.548$, $r^2 = 0.9993$ over the concentration ranges of 17–682 and 24–608 mg/L for neomycin sulfate and polymyxin B sulfate, respectively.

3.2.2. Reproducibility

Method reproducibility was determined by measuring repeatability and intermediate precision (intra-day precision) of migration time and the corrected peak areas for each component. In order to determine the repeatability of the method, replicate injections ($n = 6$) of a standard solution containing 168 mg/mL neomycin sulfate and 300 mg/mL polymyxin B sulfate were carried out in the optimum experimental conditions as described previously. The precision was calculated as a percentage of relative standard deviation (%R.S.D.) of migration times and the corrected peak areas. As can be seen in Table 1, the repeatability was better than 0.4% for the migration time and 2.4% for the corrected peak area.

The intermediate precision was also evaluated over 3 days by performing six successive injections each day. The results

Table 2
Comparison of the results obtained by CE and TLC–densitometric method for the assays of eye–ear preparations containing neomycin and/or polymyxin B ($n = 3$)

Sample	Amount of neomycin found (mg/mL)		Amount of polymyxin B found (mg/mL)	
	CE method	TLC–densitometric method	CE method	TLC–densitometric method
(A) Eye drops (3.5 mg/mL neomycin and 1 mg/mL dexamethasone sodium phosphate)	3.49 ± 0.19 ($t_{calculated} = 1.65$)	3.30 ± 0.07 ($t_{calculated} = 1.65$)		
(B) Eye drops (3.5 mg/mL neomycin and 1 mg/mL dexamethasone sodium phosphate)	3.59 ± 0.06 ($t_{calculated} = 1.90$)	3.40 ± 0.16 ($t_{calculated} = 1.90$)		
(C) Eye–ear drops (1.75 mg neomycin/mL, 5000 units/mL polymyxin B[a] and 0.025 mg/mL gramicidine)	1.72 ± 0.09 ($t_{calculated} = 0.83$)	1.76 ± 0.02 ($t_{calculated} = 0.83$)	0.469 ± 0.017 ($t_{calculated} = 0.21$)	0.466 ± 0.012 ($t_{calculated} = 0.21$)
(D) Eye–ear drops (2.0 mg neomycin/mL, 5000 units/mL polymyxin B[a] and 0.025 mg/mL gramicidine)	1.89 ± 0.07 ($t_{calculated} = 1.32$)	2.00 ± 0.12 ($t_{calculated} = 1.32$)	0.456 ± 0.016 ($t_{calculated} = 1.12$)	0.467 ± 0.008 ($t_{calculated} = 1.12$)

[a] Polymyxin B, 1 mg = 10,000 units [29].

are summarized in Table 1. This performance suggests that the proposed CE method presents acceptable reproducibility.

3.2.3. Accuracy

Accuracy of the method was assessed with recovery. Three known concentration levels corresponding to 80, 100 and 120% of targeted working concentration were added into the solutions prepared from a commercial eye–ear drops. All samples were injected in three replicates for each concentration. The concentration found was calculated against the concentration added. The recoveries of neomycin sulfate and polymyxin B sulfate were found to be between 97.44–103.18 and 96.85–101.68%, respectively. It is important to consider that additives and excipients did not interfere in the determination of the two active ingredients since the samples used to evaluate recovery were prepared with those additives and excipients.

3.2.4. Limit of detection (LOD) and limit of quantification (LOQ)

The limits of detection and quantification were calculated by measuring the noise in different blanks, and taking into account a factor of 3 and 10 for limits of detection and limits of quantification, respectively, and by using the signal obtained from standard solutions in order to convert to concentration units.

The limits of detection were 3.7 and 14.7 μg/mL and the limits of quantification were 12.5 and 49.7 μg/mL for neomycin sulfate and polymyxin B sulfate, respectively.

3.2.5. Application of the methods

The contents of neomycin and polymyxin B in four commercial eye–ear preparations manufactured by different pharmaceutical companies were determined by the developed method. Each solution was analyzed three independent determinations and each series were injected three times. Results generated by the developed CE method were compared with those expected by the labelled claim. The calculated *t*-values (in Table 2) were less than the table list *t*-value (2.78) [30] at the 95% confidence with four degree of freedom. Therefore, these two methods are not significantly different at the 95% confidence level.

4. Conclusions

A CE method with indirect UV detection has been developed for the determination of neomycin and polymyxin B in pharmaceutical formulations. The present indirect photometric detection does not require any derivatization and extraction for the assay of such compounds. The electrophoretic method has been validated for the analysis of four eye–ear preparations without any excipient in the formulations interfere, giving results comparable to those reported by the manufacturers. The CE method with indirect UV detection has proven to be rapid, cheap and easy to use. Therefore, it offers a good alternative to the published methods for the quality control of bulk drugs and their pharmaceutical preparations.

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