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Development of a liquid chromatographic method for ear drops containing neomycin sulphate, polymyxin B sulphate and dexamethasone sodium phosphate

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Abstract

Two liquid chromatographic methods were developed to analyse ear drops containing neomycin sulphate, polymyxin B sulphate and dexamethasone sodium phosphate. This formulation will be described in the Belgian National Formulary. Since neomycin, an aminoglycoside antibiotic, has no UV chromophore and pre or post column derivatization is complicated, pulsed electrochemical detection on a gold electrode was chosen to determine neomycin. Polymyxin B sulphate and dexamethasone sodium phosphate do have a UV chromophore. So, a single LC method with UV detection was developed for the determination of polymyxin B sulphate and dexamethasone sodium phosphate. The sample pretreatment is simply done by diluting the formulation with water. For each method, the influence of the different chromatographic parameters on the separation, the interference of other active compounds and excipients, the repeatability and the linearity were investigated. Finally, the content of the actives in the formulation was studied at 0, 2, 4, 6, and 8 weeks.

Keywords: Liquid chromatography; Ear drops; Neomycin sulphate; Polymyxin B sulphate; Dexamethasone sodium phosphate; Stability

1. Introduction

The Belgian National Formulary intends to describe a preparation of ear drops consisting of neomycin sulphate (250 mg/50.0 ml), polymyxin B sulphate 500.000 I.E (69.1 mg/50.0 ml) and dexamethasone sodium phosphate (36 mg/50.0 ml) as active ingredients and propylene glycol, citric acid, sodium citrate, sodium edetate and water as excipients [1]. It is therefore necessary to develop an analytical method for this preparation and to follow its stability over a period of two months.

Neomycin is an aminoglycoside antibiotic that is produced during the fermentation of *Streptomyces fradiae* [2]. It is mainly composed of the two isomeric components neomycins B and C (Fig. 1). In neomycin, three sugars are attached to the central 2-deoxystreptamine ring. Small amounts of other components are also found in commercial samples: neamine, paromomycin I and II, paromamine and the low-potency neomycins A and B (LP-A and LP-B). Neamine is formed by partial hydrolysis of neomycins B and C. LP-A and LP-B are the mono-*N*-acetyl derivatives of neamine and neomycin B, respectively. Neomycin C has less anti-bacterial activity than neomycin B and is limited in neomycin to 3–15%. When less than 3% of neomycin C is present, the substance is called framycetin [3].

Polymyxins are a group of closely related antibiotic substances isolated from strains of *Bacillus polymyxa*. The general structure of polymyxin B comprises a cyclic heptapeptide moiety with a straight tripeptide side chain. The *N*-terminal amino group of the side chain is acylated with a fatty acid [4]. Polymyxin contains characteristic constituents such as α,γ-diaminobutyric acid (DAB), L-threonine and a fatty acid. They differ by the presence or absence of additional amino

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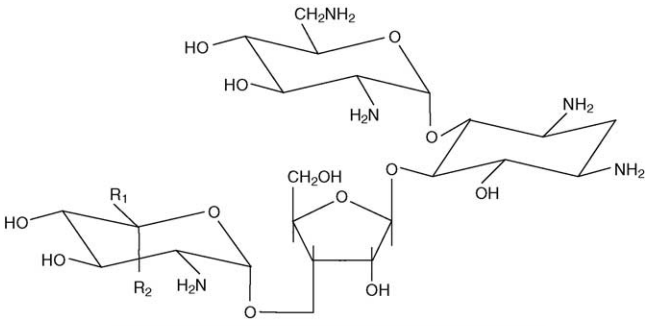
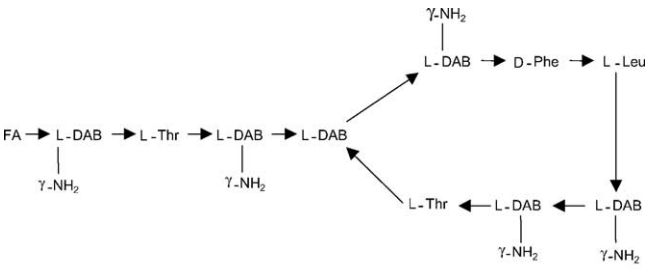


	R_1	R_2
Neomycin B	H	CH_2NH_2
Neomycin C	CH_2NH_2	H

Fig. 1. Chemical structure of neomycins B and C.

acids as well as the nature of the fatty acid. Polymyxin B is divided into four major components in which polymyxins B1 and B2 are the main active components (Fig. 2). They differ from each other in the fatty acyl moiety: polymyxin B1 contains 6-methyl octanoic acid (6-MOA), B2 6-methyl heptanoic acid (6-MHA), B3 octanoic acid (OA) and B4 heptanoic acid (HA) [5]. Dexamethasone sodium phosphate belongs to the glucocorticoids [6]. It typically consists of three six-carbon rings and a five-carbon ring. Its structure can be found in the European Pharmacopoeia [7].

There are several methods described for the determination of neomycin: microbiological assay, colorimetric determination, thin layer chromatography (TLC), gas chromatography (GC) and liquid chromatography (LC) [8]. A thin layer chromatography (TLC)-densitometric method for simultaneous identification and quantitative determination of neomycin sulphate, polymyxin B sulphate, zinc bacitracin and methyl and propyl hydroxyl benzoate in ophthalmic ointment was described by Krzek et al. [9]. In a comparative study between microbiology and LC, the results proved that LC is more reliable and advantageous than the microbiological method [10]. Apffel et al. described the analysis of neomycins A–C using reversed-phase ion-pair LC. Detection was performed by post column derivatization of the analytes with *ortho*-phthaladehyde (OPA) in the presence of mercaptoethanol [11]. Since neomycin has no UV absorbing chromophore, most of the described methods use pre or post column derivatization. However, these derivatization procedures are difficult to perform and give problems during quantification. A reversed phase liquid chromatographic method using poly(styrene-divinylbenzene) (PSDVB) as stationary phase with pulsed electrochemical detection (PED) was developed for the analysis of neomycin bulk samples. The mobile phase consisted of an aqueous solution containing sodium sulphate, sodium octane sulphonate, and 0.2 M potassium dihydrogen phosphate [3]. The same method was applied previously to a formulation, which contained neomycin sulphate, polymyxin B sulphate, and gramicidin [12].



Polymyxin	FA
B1	6-MOA
B2	6-MHA
B3	OA
B4	HA

Fig. 2. Structure of polymyxins B1, B2, B3 and B4. FA: fatty acid; 6-MOA: 6-methyloctanoic acid; 6-MHA: 6-methylheptanoic acid; OA: octanoic acid; HA: heptanoic acid.

Thomas et al. described a microbiological and chemical analysis method for polymyxins B and E [13]. Tsuji et al. used LC with linear gradient elution to separate the polymyxin B1 and B2 components [14]. Fong et al. published an isocratic reversed phase LC method for the analysis of polymyxins B1 and B2, but it could not separate the other components [15]. An analytical method on a micellar electrokinetic capillary chromatography was developed by Kristensen et al. This method achieved complete separation of polymyxins B2 and B3, but the latter was not well separated from other components [16]. Capillary zone electrophoresis with cyclodextrin as additive was applied by Kang et al. This method could separate polymyxins B1, B2, B3, and B4 [17]. Polymyxins B1, B2, and 20 other components could be separated on a poly (styrene-divinylbenzene) stationary phase [18]. More recently, an improved method was developed on a reversed phase C18 column (YMC pack pro) using 0.7% sodium sulphate–acetonitrile–6.8% phosphoric acid–water (50:20:5:25, v/v/v/v) as mobile phase. UV detection was performed at 215 nm. This method could separate polymyxins B1 and B2 from 25 other impurities [19].

Quantitative determination of dexamethasone and dexamethasone sodium phosphate in pharmaceutical dosage forms by LC was described by Gupta [20]. Gagne et al. also described a LC method for the analysis of dexamethasone sodium phosphate in formulations such as ophthalmic solutions, injectable preparations and inhalant formulations [21]. For the simultaneous determination of trimethoprim, dexamethasone sodium phosphate and polymyxin B sulphate, a method using micellar electrokinetic capillary chromatography was developed by Gallego and Perez Arroyo [22].

2. Experimental

2.1. Instrumentation

The chromatographic system for neomycin sulphate was equipped with a LC pump (L-6200, Merck–Hitachi,

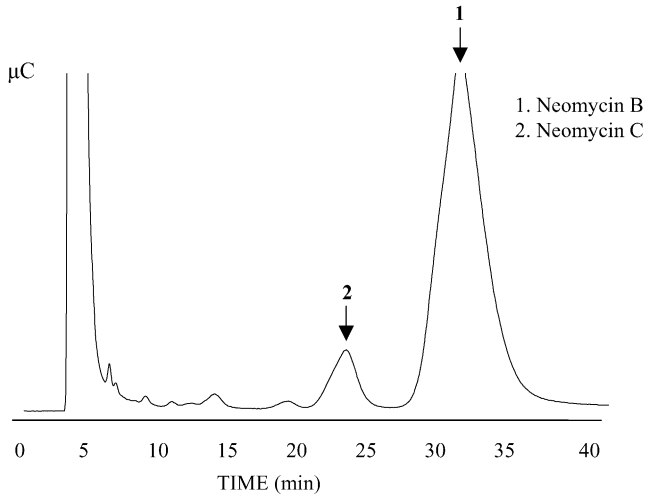


Fig. 3. Chromatogram of the formulation in conditions to determine neomycin sulphate. Chromatographic conditions: Mobile phase: anhydrous sodium sulphate (70 g/L), sodium 1-octanesulphonate (1.4 g/L), potassium dihydrogen phosphate buffer (pH 3.0, 0.2 M) (50.0 ml/L); column: poly (styrene-divinylbenzene) 1000 Å, 8 μm, 250 mm × 4.6 mm; flow rate: 1.0 ml/min, injection volume: 20 μl, column temperature: 35 °C; post column solution: sodium hydroxide (0.5 M), 0.3 ml/min; detection: pulsed electrochemical detection; PED parameters: E1: +0.05 V (0–0.40 s), E2: +0.75 V (0.41–0.60 s), E3: −0.15 V (0.61–1.00 s), integration: 0.20–0.40 s.

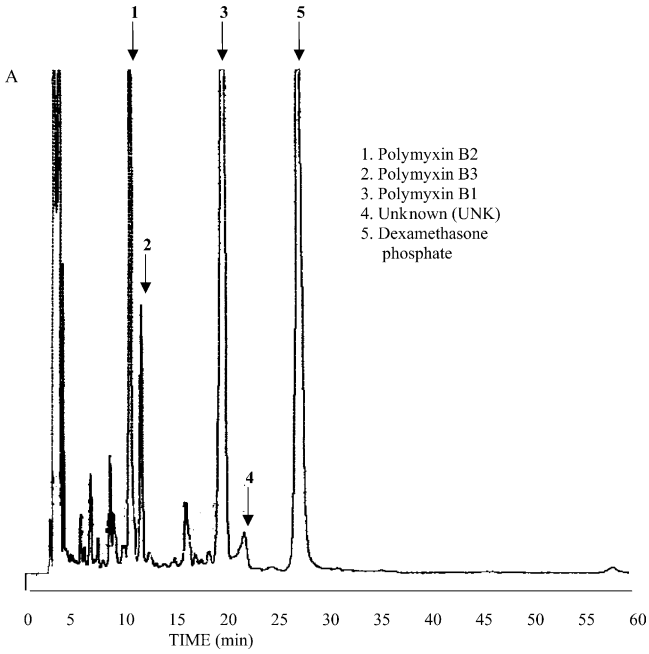


Fig. 4. Typical chromatogram of the formulation in conditions to determine polymyxin B sulphate and dexamethasone sodium phosphate. Chromatographic conditions: mobile phase: sodium sulphate 0.7% (w/v) (560 ml), acetonitrile (240 ml), 6.8% (v/v) phosphoric acid (56 ml), distilled water up to 1000 ml; column: YMC pack pro C_{18}120 Å, 5 μm, 250 mm × 4.6 mm; column temperature: 40 °C, injection volume: 50 μl, flow rate: 1.0 ml/min, detection: UV at 210 nm.

Darmstadt, Germany) delivering the mobile phase at a flow rate of 1 ml/min. An automatic injector (Gilson, Villiers-le-Bel, France) with a loop of 20 μl was used to inject the samples. The stationary phase PLRP-S, 1000 Å, 8 μm, (Polymer Labs, Shropshire, UK) was packed in a stainless steel column (250 mm × 4.6 mm). To maintain the temperature of the column, a water bath with a heating immersion circulator (Julabo, Seelbach, Germany) was used. For PED detection of aminoglycosides, at least pH 12 is necessary. Since the mobile phase has a lower pH, 0.5 M sodium hydroxide was added post column to raise the pH using a helium-pressurized reservoir. Addition of the post column solution should be pulse-free to avoid disturbances in base line. The post column solution was added at a flow rate of 0.3 ml/min. After mixing both solutions in a mixing coil (1.2 m, 500 μl) (Dionex, Sunnyvale, CA, USA), the resulting solution entered into the electrochemical cell (PED-1, Dionex). The electrochemical cell was kept in a hot air oven to keep the temperature constant at 35 °C. Data acquisition software (Chromeleon, Dionex) was used to record the signal.

The liquid chromatographic system for polymyxin B sulphate and dexamethasone sodium phosphate consisted of a Spectra Physics P4000 pump connected to an auto injector, Spectra Physics AS 3000 (TSP, San Jose, CA, USA). The column was immersed in a water bath and temperature was maintained using a Julabo EM heating circulator. A YMC pack pro C18 (YMC Inc., Milford, MA, USA) column was used for method development. A Spectra 1000 UV detector (TSP) was used for detection. A Hewlett Packard (Avondale, PA, USA) 3396 series 3 integrator was connected to the detector to record the signals.

2.2. Reagents and samples

Anhydrous sodium sulphate extra pure and sodium 1-octanesulphonate 98% were obtained from Merck (Darmstadt, Germany) and Acros Organics (Geel, Belgium), respectively. Helium was from Messer (Machelen, Belgium). The buffer was prepared by adding 0.2 M phosphoric acid to 0.2 M potassium dihydrogen phosphate till pH 3.0 was achieved. Phosphoric acid 85%, potassium dihydrogen phosphate and HPLC-grade acetonitrile were obtained from Acros Organics (Geel, Belgium). The 0.5 M sodium hydroxide solution was prepared starting from a 50% (m/m) aqueous solution (Baker, Deventer, The Netherlands) that was added to previously with helium degassed (10 min) distilled water. The mixture was degassed again for 10 min.

Neomycin sulfate was obtained from Chemiphar (Brugge, Belgium) and polymyxin B sulphate and dexamethasone sodium phosphate from Ludeco (Brussels, Belgium). Reference substances of polymyxins B1, B2, and B3 were available in the laboratory [23].

Sample preparation for analysis: 2.0 ml of sample is diluted to 20.0 ml with distilled water for the analysis of neomycin sulphate and for polymyxin B sulphate–dexamethasone sodium phosphate, 3.0 ml is diluted to 10.0 ml with distilled water.

Table 1
Chromatographic parameter settings in the central composite design used for the robustness study of the neomycin sulphate separation

	Lower value (−1)	Central value (0)	Upper value (+1)
Sodium octanesulphonate (g/l) (Oct)	1.35	1.40	1.45
Sodium sulphate (g/l) (Sod)	69	70	71
pH	2.8	3	3.2
Temperature (°C) (Temp)	32	35	38

3. Results and discussion

3.1. Chromatographic method for neomycin sulphate

A method for the analysis of neomycin sulphate bulk samples was previously developed in our laboratory [3]. The same method was applied for the analysis of neomycin sulphate in a pharmaceutical preparation, which contained neomycin, polymyxin and gramicidin [12]. Here, the same method was tested for this formulation. It was observed that there was interference neither from the other active substances nor from the excipients. Polymyxin B sulphate and dexamethasone sodium phosphate were not detected and excipients were eluted in the front of the chromatogram along with the solvent peak. This was confirmed by injecting excipient, polymyxin B sulphate and dexamethasone sodium phosphate individually. Impurities like neamine, paromomycin I and II, paromamine, LP-A and LP-B were separated from neomycins B and C. In the formulation, the content of neomycin C was approximately 10% compared to neomycin B. Fig. 3 shows a typical chromatogram. The chromatographic conditions are mentioned in the legend.

3.2. Chromatographic method for polymyxin B sulphate and dexamethasone sodium phosphate

The method was developed by optimizing the conditions on the YMC pack pro C18 column used for the analysis of polymyxin. This column has shown better selectivity and sensitivity than PSDVB since it has more theoretical plates [19]. Therefore, the same type of column was chosen to develop a method for the simultaneous determination of polymyxin B sulphate and dexamethasone sodium phosphate. To improve the sensitivity, a wavelength of 210 nm was selected. It was observed that dexamethasone sodium phosphate was eluted at the end of the chromatogram, well separated from all the polymyxin peaks but excessively increasing the analysis time. The mobile phase was optimized in order to reduce the total analysis time and to maintain the separation between the most important polymyxin components. Optimization of the mobile phase was started by varying the concentration of acetonitrile (ACN). Using less than 240 ml/L of ACN, the sensitivity of the system was low and with concentrations above 240 ml/L the system was very fast and too many peaks were coeluted. The effect of sodium sulphate was tested by varying the sodium sulphate solution (0.7%, w/v) from 500 to 570 ml/L with increments of 10 ml/L. At each increment the temperature was tested at 30, 35, 40, and 45 °C.

Table 2
Chromatographic parameter settings in the central composite design used for the robustness test of the method to determine polymyxin B sulphate and dexamethasone sodium phosphate

	Lower limit (−1)	Central value (0)	Upper value (+1)
0.7% sodium phosphate (ml) (Sod)	550	560	570
6.8% phosphoric acid (ml) (Phosp)	54	56	58
Acetonitrile (ml) (ACN)	234	240	246
Temperature (°C) (Temp)	37	40	43

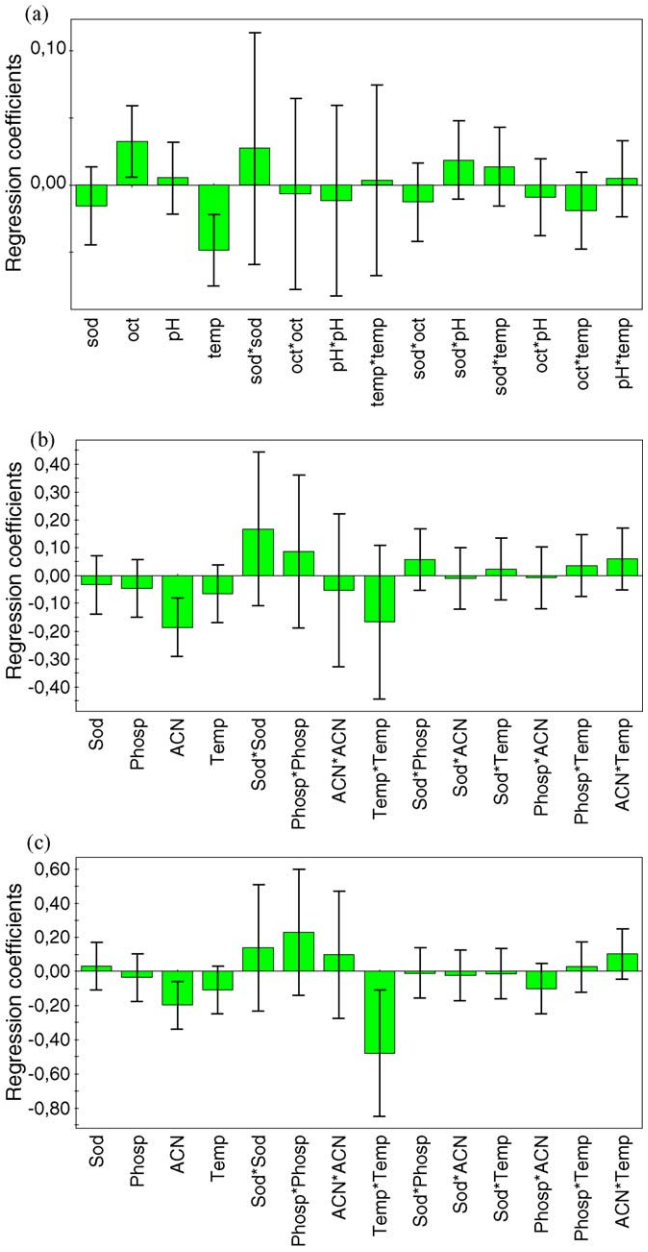


Fig. 5. Regression coefficients plots for the resolution between (a) neomycins B and C, (b) polymyxin B1-UNK and (c) polymyxins B2 and B3 (Abbreviations: see Tables 1 and 2).

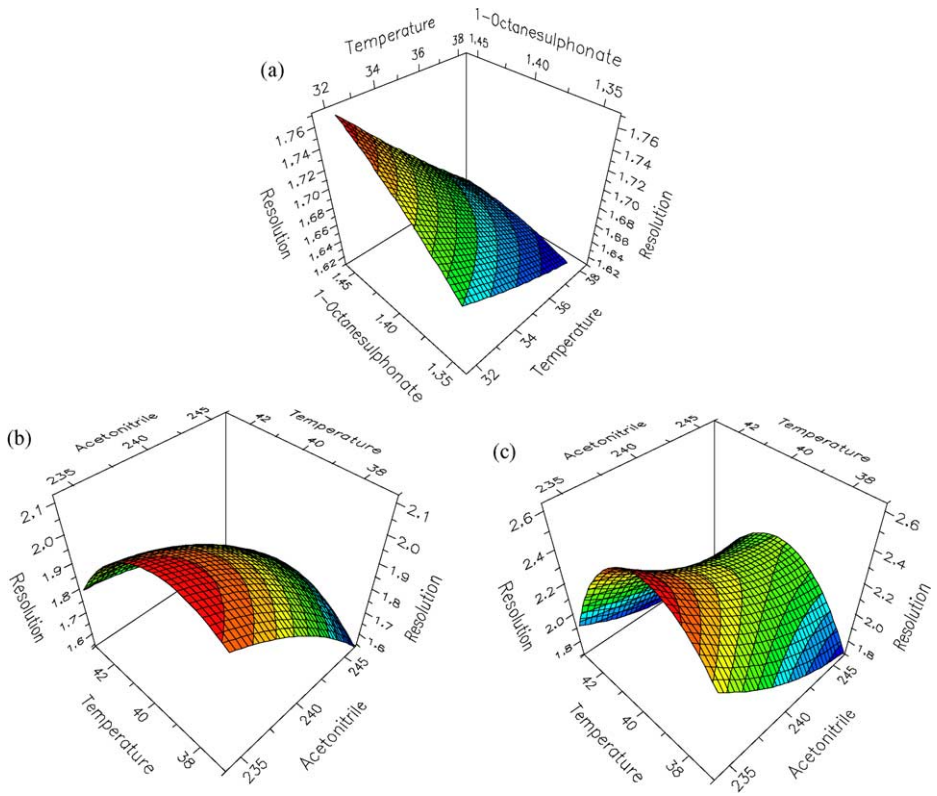


Fig. 6. Response surface plots for the effects of the parameters on the resolution between (a) neomycins B and C as a function of temperature and octanesulphonate concentration at constant values of sodium sulphate concentration and the pH, (b) polymyxin B1-UNK as a function of temperature and acetonitrile concentration, (c) polymyxins B2 and B3 as a function of temperature and acetonitrile concentration. The phosphoric acid and sodium sulphate concentration were kept constant for (b) and (c).

Using 560 ml/L of the sodium sulphate solution, polymyxin B1 was well separated from its neighboring impurities at 40 and 45 °C, but the separation between polymyxins B2 and B3 was less good at 45 °C. At 30 and 35 °C the run-time was unnecessary high. Therefore, 560 ml/L of 0.7% (w/v) sodium sulphate and a temperature of 40 °C were chosen. The effect of 6.8% (v/v) phosphoric acid was tested from 50 to 62 ml/L with increments of 3 ml/L. The best separation was obtained at a value of 56 ml/L. With higher and lower values for the concentration of phosphoric acid, a small impurity was coeluted with polymyxin B1. A typical chromatogram is shown in Fig. 4. Polymyxins B1 and B2 and dexamethasone sodium phosphate were identified by injecting the individual components. Neomycin was not detected due to lack of a UV-absorbing chromophore.

3.3. Robustness

The effects and interactions between the chromatographic parameters were examined using a central composite design. Modde 5.0 Software (Umetrics AB, Umea, Sweden) was used to investigate the responses. For both the chromatographic system to determine neomycin sulphate and this to determine polymyxin-dexamethasone sodium phosphate, the influence of four chromatographic parameters on the selectivity was investigated. $2^k + 2k + n$ experiments were carried out where k and n were the number of factors ($k = 4$) and central points ($n = 3$), respectively. So, in each case 27 experiments were carried out. Tables 1 and 2 show the lower (−1), central (0) and higher (+1) values for the chromatographic parameters studied in the two systems, respectively.

The coefficients of the model calculated by the software, represent the relationship between the response variables measured and the factors studied. Single coefficients describe the quantitative effect of a factor, cross-products the interaction between factors and squared coefficients the non-linear effects. The effect of a factor is denoted by a bar and the 95% confidence limits by an error line. A regression coefficient smaller than the error line interval shows that the variation in the response caused by changing that variable is smaller than the experimental error.

For neomycin, the resolution between neomycins B and C was taken as response variable. The regression coefficient plot of Fig. 5(a) shows that the concentration of sodium octanesulphonate and the temperature have a slightly positive and negative effect, respectively. A positive effect means that an increase of the factor value also increases the response studied. A negative effect means that an increase of the factor value causes a decrease of the response studied. The remaining factors and their interactions had no significant influence. Fig. 6(a) shows the response surface plot for the resolution between neomycins B and C.

Table 3
Linearity and repeatability for neomycin sulphate, polymyxin B sulphate and dexamethasone sodium phosphate

	Repeatability at 100% (n = 6) R.S.D. (%)	Linearity			
		Range (%) (n = 3)	R^2	y	$S_{y,x}$
Neomycin B	1.0	25–150	0.9971	$2852x - 2696$	806
Neomycin C	0.6	25–150	0.9987	$293x - 373$	557
Polymyxin B1	0.2	25–150	0.9998	$535x + 958$	465
Polymyxin B2	0.9	25–150	0.9990	$252x + 732$	240
Dexamethasone sodium phosphate	0.3	25–150	0.9995	$500x + 288$	644

R.S.D.: relative standard deviation; range: percentage range studied; n: number of injections per concentration; R^2: coefficient of determination; y: peak area; x: concentration (%); $S_{y,x}$: standard error of estimate.

Table 4
Results for the stability of the formulation, taking the percentage at time 0 as 100%

Compound	Batch No	Time 0 weeks % (R.S.D.)	Time 2 weeks % (R.S.D.)	Time 4 weeks % (R.S.D.)	Time 6 weeks % (R.S.D.)	Time 8 weeks % (R.S.D.)
Neomycin B sulphate	1	100.0 (0.6)	100.2 (2.2)	98.9 (1.4)	99.8 (0.4)	99.9 (1.3)
	2	100.0 (2.1)	98.0 (2.2)	98.6 (0.9)	99.9 (0.8)	99.0 (1.7)
Neomycin C sulphate	1	100.0 (1.9)	101.1 (2.7)	100.8 (2.7)	99.8 (1.7)	100.8 (3.9)
	2	100.0 (2.0)	99.1 (4.2)	100.3 (0.8)	99.7 (2.0)	100.9 (2.7)
Polymyxin B1 sulphate	1	100.0 (1.1)	96.3 (1.2)	93.4 (0.6)	89.7 (2.0)	92.1 (1.2)
	2	100.0 (0.8)	95.8 (1.2)	95.5 (0.8)	90.9 (0.7)	91.9 (1.5)
Polymyxin B2 sulphate	1	100.0 (0.9)	95.2 (1.1)	92.3 (0.8)	90.9 (1.0)	90.8 (1.5)
	2	100.0 (0.9)	95.3 (1.3)	94.4 (1.1)	90.4 (0.9)	90.4 (1.6)
Dexamethasone sodium phosphate	1	100.0 (0.6)	98.6 (1.5)	96.8 (0.5)	98.0 (0.6)	95.0 (1.0)
	2	100.0 (0.9)	97.6 (0.6)	99.7 (0.5)	96.8 (0.7)	93.3 (0.6)

For polymyxin B Fig. 5(b) and (c) show the effects of the factors on the resolutions between polymyxin B1-UNK (UNK is the unknown peak as indicated in Fig. 4) and polymyxins B2-B3, respectively. Only ACN has a slightly negative effect on both resolutions. No important interactions were noticed. The temperature shows a non-linear effect for the pair polymyxin B2-B3. The influence of the parameters is estimated more easily in the response surface plots in Fig. 6(b) and (c). The other parameters were kept constant at the central value. As can be seen, in the range studied sufficient resolution was obtained for both pairs.

3.4. Quantitative aspects

After sample preparation, a concentration of 0.50 mg/ml of neomycin sulphate, 0.41 mg/ml of polymyxin B sulphate and 0.22 mg/ml of dexamethasone sodium phosphate is obtained. These concentrations were considered as 100%. For repeatability, the sample solution was injected six times. The linearity of the method was tested at 25, 50, 100, 125, and 150%. For each concentration (%), three analyses were performed. The results are shown in Table 3.

3.5. Stability study

To study the stability of the formulation, two batches of the formulation were prepared. Each batch was stored at room temperature and analysed at 0, 2, 4, 6, and 8 weeks. The active substances used in the formulation were also used as reference substances. At each time point and for each batch two separately prepared sample solutions were analysed against two separately prepared reference solutions. Each sample and each reference solution were injected three times. In Table 4, it can be seen that after eight weeks the neomycin content remains invariable, whereas the polymyxin content and, to a lesser extent, the dexamethasone sodium phosphate content decrease to content between 90% and 95%. It may be decided to overdose the polymyxin and dexamethasone contents by 5% in the formulation to reach a final concentration above 95% after two months, which is considered an acceptable lifetime for such a preparation.

4. Conclusion

The active components were well separated from the impurities and the excipients in both LC-UV and LC-PED methods. The LC-UV method allows simultaneous determination of polymyxin B sulphate and dexamethasone sodium phosphate. Due to lack of a UV-absorbing chromophore, neomycin sulphate was not detected with this method and is analysed by a separate LC-PED method. Both methods were proven to be linear, repeatable and robust. By using these two LC methods, the stability of the formulation was followed during a period of eight weeks.

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