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Liquid chromatographic analysis of a formulation containing polymyxin, gramicidin and neomycin

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Abstract

The development of a liquid chromatographic assay system for the stability study of a formulation containing polymyxin, gramicidin and neomycin is described. For the determination of each group of antibiotics, poly(styrene–divinylbenzene) is used as the stationary phase. The mobile phase for the determination of polymyxin consists of an aqueous solution containing 7 g l^{-1} of sodium sulfate, 50 ml l^{-1} of 1 M phosphoric acid and 160 ml l^{-1} of acetonitrile. UV detection is performed at 215 nm. Gramicidin is chromatographed with a mixture of tetrahydrofuran–water (38:62) and detected by UV at 222 nm. An aqueous solution containing 70 g l^{-1} of sodium sulfate, 1.4 g l^{-1} of sodium octanesulfonate and 50 ml l^{-1} of 0.2 M phosphate buffer pH 3.0 is used as the mobile phase for the determination of neomycin. Since neomycin has no UV-absorbing chromophore, pulsed electrochemical detection is chosen to determine neomycin.

For each method, the influence of the different chromatographic parameters on the separation, the selectivity towards the other active compounds and the excipients, the repeatability and the linearity were investigated. The stability of the formulation was examined at 0, 6 and 12 months.

Keywords: Assay; Gramicidin; Liquid chromatography; Neomycin; Polymyxin B

1 Introduction

A combination of three antibiotics in a liquid formulation is used for instillation into the eye or the ear. 1 ml contains 1 mg of polymyxin B sulfate, 0.02 mg of gramicidin and 5 mg of neomycin sulfate. This mixture possesses broad spectrum activity against a wide range of gram-positive and gram-negative bacteria.

Polymyxin B is a complex of closely related decapeptide antibiotics. They are composed of a cyclic heptapeptide moiety and a N-acylated tripeptide side-chain. The complex composition of these products has been demonstrated by thin-layer chromatography (TLC) [1,2] and by liquid chromatography (LC) [3–6]. Microbiological assays showed a difference in the potency of the

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main components polymyxin B_1 and B_2 [7]. In the literature, no degradation products are mentioned for polymyxin.

Gramicidin is a linear N-formylated pentadecapeptide–ethanolamide complex. It is a fairly constant mixture of about 75% gramicidin A, 8% gramicidin B and 17% gramicidin C. In each gramicidin, the ratio of the major component valine gramicidin, [Val]Gdin, to the minor component isoleucine gramicidin, [Ile]Gdin, is about 85:15 [8]. Secogramicidin or desformylgramicidin is the major degradation product.

Neomycin is an aminoglycoside antibiotic. It is mainly composed of neomycin B and its stereoisomer neomycin C. The antimicrobial potency of neomycin C is lower than that of neomycin B. The European Pharmacopoeia limits the amount of neomycin C to 3–15% [9]. Small amounts of other constituents, such as neamine, paromamine, LP-A, LP-B (LP = low potency), paromomycin I and paromomycin II may also be present in commercial samples. Chromatographic separation of the stereoisomers neomycin B and C is quite difficult and the detection is also problematic because neomycin has no UV-absorbing chromophore. Gas–liquid chromatography after trimethylsilylation [10], LC combined with pre- or post-column derivatization [11–13] and TLC with scanning [14] have been reported as separation methods.

This report presents three LC methods, one for each group of antibiotics, all using poly(styrene–divinylbenzene) as the stationary phase. Selectivity towards the other active compounds and the excipients was achieved by adapting the composition of the mobile phase, the column temperature and the detection system. The methods can be used for assay and for stability control.

2. Experimental

2.1. Reagents and samples

Water was distilled twice from glass apparatus. Grade S acetonitrile and PA grade tetrahydrofuran (THF) were from Rathburn Chemicals (Walkerburn, UK). Phosphoric acid solution (1 M) was prepared from 85% m/m phosphoric acid (Acros Chimica, Geel, Belgium). The buffer solution was made by mixing 0.2 M phosphoric acid and 0.2 M potassium dihydrogen phosphate (Acros Chimica) until a pH of 3.0 was reached. Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany), 98% sodium 1-octanesulfonate monohydrate was from Acros Chimica and helium was from Air Liquide (Machelen, Belgium). The 0.5 M sodium hydroxide solution was made by diluting 50% m/m sodium hydroxide (Baker, Deventer, The Netherlands).

Polymyxin B sulfate (Pfizer, New York), gramicidin (Apothekernes Laboratorium, Oslo, Norway) and neomycin sulfate (Upjohn, Kalamazoo, MI), used during the preparation of the formulation, were also used here as the reference substances. Besides the active components, the formulation consists of polyethylene glycol, polysorbate 20, propylene glycol, ethanol, sodium chloride, sodium hydroxide and purified water.

Secogramicidin was prepared as described by Ishii and Witkop [15]. Neomycin B, neomycin C, neamine, paromamine, LP-A and LP-B reference substances were prepared in the laboratory from commercial samples as described [16]. A mixture of paromomycin I and paromomycin II was obtained from Carlo Erba (Milan, Italy).

2.2. Apparatus

The chromatographic procedures were carried out using a L-6200 Intelligent Pump (Merck–Hitachi, Darmstadt, Germany), a Marathon autosampler (Spark, Emmen, The Netherlands), an electronic integrator HP 3393A or HP 3396 Series II (Hewlett-Packard, Avondale, PA), and a L 4000 UV detector (Merck–Hitachi) or a PED-1 pulsed electrochemical detector (Dionex Corporation, Sunnyvale, CA), equipped with a gold working electrode, a Ag/AgCl reference electrode and a stainless-steel counter electrode. The cell of the pulsed electrochemical detector was placed in a laboratory-made hot-air oven to keep the temperature at 35°C. The columns (250 mm × 4.6 mm i.d.) were packed with poly(styrene-divinylbenzene) PLRP-S 1000 Å, 8 μm (Polymer Laborato-

Table 1
Chromatographic conditions for determining polymyxin B, gramicidin and neomycin

Chromatographic condition	Polymyxin B	Gramicidin	Neomycin
Injection volume (μl)	100	100	20
Mobile phase	7 g of sodium sufate, 50 ml of 1 M phosphoric acid, 160 ml of acetonitrile, water up to 1000 ml	380 ml of THF, water up to 1000 ml	70 g of sodium sulfate, 1.4 g of sodium octanesulfonate, 50 ml of 0.2 M phosphate buffer pH 3.0, water up to 1000 ml
Flow rate (ml min^{-1})	1.0	1.0	1.0
Column temperature (°C)	60	65	35
Detection	UV at 215 nm	UV at 222 nm	Pulsed electrochemical detection

ries, Church Stretton, Shropshire, UK). The columns were immersed in a water bath with a heater (Julabo, Seelbach, Germany). For the determination of neomycin, sodium hydroxide was added post-column, using a helium-pressurized laboratory-made pneumatic device.

2.3. Chromatography

An overview of the final chromatographic conditions chosen is given in Table 1. For determination of polymyxin B, 3.0 ml of sample was diluted with 3.0 ml of water. The reference substance polymyxin B sulfate was dissolved in water at a concentration of 0.5 mg ml^{-1}. Polymyxin B in the mobile phase has an absorption maximum at 196 nm, but a wavelength of 215 nm was used because the sensitivity at this wavelength is still good and a more stable baseline is obtained

To determine gramicidin, 3.0 ml of the sample was diluted with 3.0 ml of THF–water (35:65 v/v) 25.0 mg of the gramicidin reference substance was dissolved in 25.0 ml of THF. After complete dissolution, water was added to give a final volume of 50.0 ml. This solution was further diluted with THF–water (35:65 v/v) to obtain a solution of 10.0 μg ml^{-1}.

For the determination of neomycin, 2.0 ml of sample was diluted to 20.0 ml with water. The reference substance neomycin sulfate was dissolved in water to a concentration of 0.5 mg ml^{-1}. To allow pulsed electrochemical detection sodium hydroxide was added post-column at a flow rate of 0.3 ml min^{-1}. The detector settings are shown in Table 2.

3. Results and discussion

3.1. Determination of polymyxin B

A typical chromatogram of the formulation obtained under conditions to determine polymyxin B is shown in Fig. 1. The identity of the two main peaks of polymyxin B sulfate was assigned based on the relative amounts of polymyxin B_1 and B_2. Polymyxin B_1 is known to be the major component [6]. The elution order is in accordance with that found on a C_{18}-bonded stationary phase [3–6].

The influence of the different chromatographic parameters on the separation of polymyxin B_1 and B_2 was evaluated using the retention times. The influence of the amount of acetonitrile in the mobile phase was examined between 14% and 20%. The retention times decrease on in-

Table 2
Settings for the pulsed electrochemical detector

Time (s)	Potential (V)	Integration
0.00	0.05	
0.20	0.05	Begin
0.40	0.05	End
0.41	0.75	
0.60	0.75	
0.61	−0.15	
1.00	−0.15	

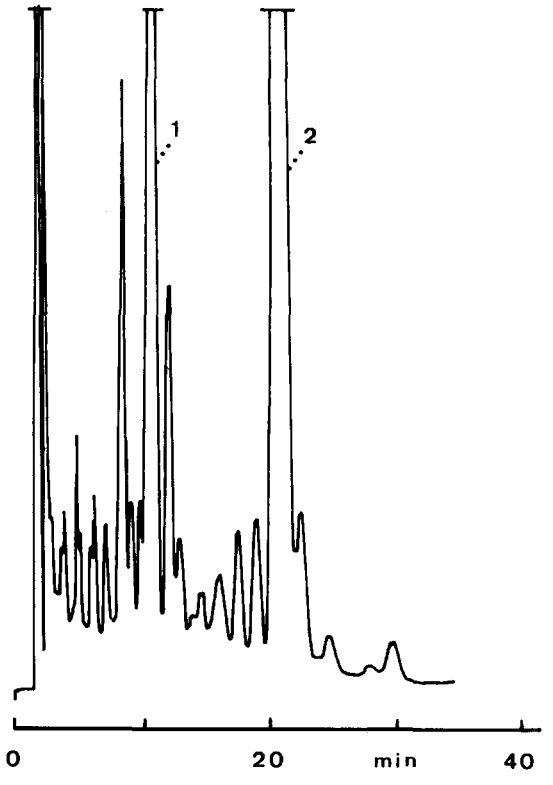

Fig. 1. Chromatogram of the formulation under conditions to determine polymyxin B: (1) = polymyxin B_2; (2) = polymyxin B_1.

creasing the amount of acetonitrile and small differences in the acetonitrile content have significant consequences. To check the influence of the amount of phosphoric acid, the volume of 1 M phosphoric acid in 1000 ml of mobile phase was varied and the following volumes were used: 25, 50, 75 and 100 ml. In the range examined, the amount of phosphoric acid in the mobile phase has no significant effect on the retention times. The influence of the amount of sodium sulfate, investigated by using mobile phases containing 5, 7 and 10g l^{-1} of sodium sulfate, is also not significant. The column temperature was varied between 55 and 70°C. As expected, the retention times of polymyxin B_1 and B_2 decrease on increasing the temperature. The same separation was obtained with different batches of poly(styrene–divinylbenzene), 1000 Å, 8 μm.

No interference from the other antibiotics or the excipients was observed. The excipients and neomycin have no UV-absorbing chromophore and gramicidin is eluted near the solvent peak.

The linearity in the range 50–200% of the theoretical value (0.5 mg ml^{-1}) was examined for polymyxin B_1 and B_2. The results are shown in Table 3.

3.2. Determination of gramicidin

A typical chromatogram of the formulation under conditions to determine gramicidin is shown in Fig. 2 . The elution sequence of the components of the gramicidin complex is deduced from the ratio of the components and their lipophilicity. The elution order is comparable with that found on a C_{18}-bonded stationary phase [8]. The influence of the different chromatographic parameters was evaluated using the retention times of [Val]Gdin A, [Val]Gdin B, [Val]Gdin C and [Ile]Gdin A. The influence of the amount of THF in the mobile phase was investigated in the range 37–40%. The retention times decrease drastically with a small increase in the THF content. The column temperature was varied between 55 and 75°C. As expected, the retention times of the gramicidin components decrease when the column temperature is increased. The same separation was obtained with several batches of poly(styrene–divinylbenzene), 1000 Å, 8 μm.

There is no interference from the other active compounds or the excipients. The latter, and neomycin, have no UV-absorbing chromophore and polymyxin B is eluted near the front peak. Secogramicidin is eluted after the other gramicidin components at about 28 min.

The linearity in the range 50–150% of the theoretical value (10 μg ml^{-1}) was examined for [Val]Gdin A. The results are shown in Table 3.

3.3. Determination of neomycin

A typical chromatogram of the formulation under conditions to determine neomycin is shown in Fig. 3 . The influence of the different chromatographic parameters on the separation of the different neomycin components was evaluated using

Table 3
Linearity of the methods

Parameter[a]	Polymyxin		Gramicidin	Neomycin	
	B_1	B_2		B	C
$y/1000$	$84916x + 3426$	$55680x + 2159$	$969x + 195$	$280211x + 1686$	$361271x - 430$
r	0.9999	0.9999	0.9964	0.9989	0.9975
$S_{y.x}$	202	89	375	3195	630

[a] y = peak area, x = concentration (mg ml^{-1}, r = coefficient of correlation and $S_{y.x}$ = standard error of estimate.

the capacity factors (k'). The influence of the pH of the mobile phase examined using buffer solutions with a pH between 2.0 and 7.0. Little change is observed between pH 2.0 and 5.0. With further increase in the pH, the retention times decrease. Therefore, an acidic mobile phase is necessary because the neomycin molecules must be positively charged to interact with the anioic octanesulfonate. The influence of the amount of sodium octanesulfonate was investigated in the range 1.1–1.6 g l^{-1}. As expected, the capacity factors increase on increasing the sodium octanesulfonate concentration. The concentration of sodium sul-

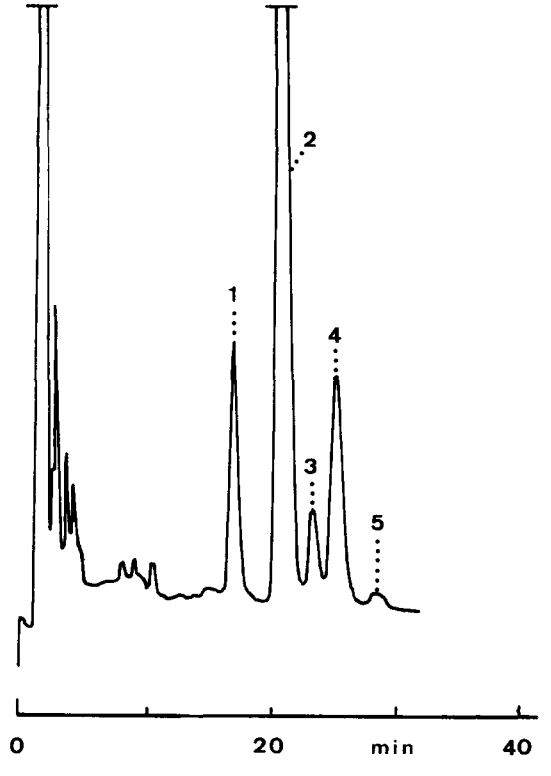

Fig. 2. Chromatogram of the formulation under conditions to determine gramicidin: (1) = [Val]Gdin C; (2) = [Val]Gdin A; (3) = [Val]Gdin B; (4) = [Ile]Gdin A; (5) = [Ile]Gdin B.

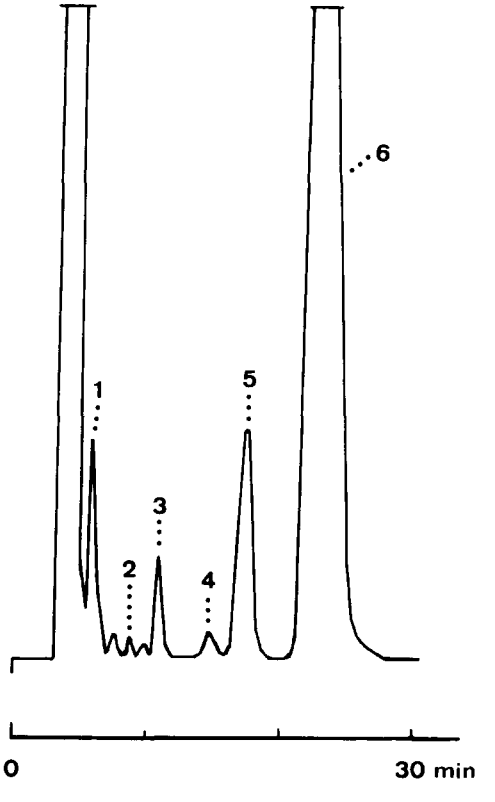

Fig. 3. Chromatogram of the formulation under conditions to determine neomycin: (1) = neamine; (2) = paromomycin II; (3) = paromomycin I; (4) = LP-B; (5) = neomycin C; (6) = neomycin B.

Table 4
Stability of the formulation : content (%) of polymyxin B_1 and B_2, gramicidin and neomycin B and C at 0, 6 and 12 months[a]

Time (months)	Polymyxin		Gramicidin	Neomycin	
	B_1	B_2		B	C
0	98.1 (0.1)	98.7 (0.1)	103.0 (1.6)	101.4 (1.3)	99.6 (1.3)
6	96.0 (0.4)	95.8 (0.4)	98.5 (0.6)	101.8 (0.4)	100.1 (1.2)
12	93.3 (0.7)	94.5 (1.2)	90.7 (1.4)	101.8 (0.3)	98.5 (0.9)

[a] The R.S.D value (%) for six analyses is given in parentheses after each content.

fate was varied between 65 and 75 g l^{-1}. An increase in the sodium sulfate concentration results in a decrease of the k' values. Different batches of poly(styrene–divinylbenzene), 1000 Å, 8 μm, gave the same separation.

Neomycin B is separated from its related substances: neomycin C, LP-B, paromomycin I and II, neamine, LP-A and paromamine. The latter two coelute with the excipients and gramicidin near the solvent peak. Polymyxin was not detected.

The linearity of a mixture of neomycins B and C (90:10) in the range 5–160% of the theoretical value (0.5 mg ml^{-1}) was examined. The results are shown in Table 3.

3.4. Stability of the formulation

The stability of the formulation was examined by determining the content of each of the antibiotics at 0, 6 and 12 months. The results are shown in Table 4. The content of polymyxin B_1 and B_2, the total content of gramicidin, the content of [Val]Gdin A and the content of neomycins B and C were calculated with respect to the reference substances. For each antibiotic, the test solution and the reference solution were injected alternately. The percentages of neomycin B and neomycin C were calculated using the areas of the reference solution injected just before and after the test solution.

4. Conclusions

The methods developed for the analysis of polymyxin, gramicidin and neomycin in a formulation are robust and free from interference from the other constituents. The sample pretreatment consists simply of diluting the formulation with an appropriate solvent. The methods show good repeatability, selectivity and linearity.

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