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Journal of Chromatography A, 870 (2000) 237–243

JOURNAL OF CHROMATOGRAPHY A

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Liquid chromatography of polymyxin B sulphate

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Abstract

A reversed-phase liquid chromatography method for analysis of polymyxin B sulphate is described. The method uses a YMC-Pack Pro, C_{18}, 5 μm, 250×4.6 mm I.D. column maintained at 30°C. The mobile phase comprises acetonitrile–sodium sulphate (0.7%, m/v)–phosphoric acid (6.8%, v/v dilution of 85%, m/m phosphoric acid)–water (22.25:50:5:22.75) at a flow-rate of 1.0 ml/min. Detection was by UV at 215 nm. The method is able to resolve polymyxin B_1, the major component, from more than thirty other components present in the complex. Robustness was evaluated by performing a full-factorial design experiment. The method showed good selectivity, repeatability, linearity and sensitivity.

Keywords: Pharmaceutical analysis; Polymyxins; Antibiotics

1. Introduction

Polymyxin B sulphate is an antibiotic used in the treatment of infections caused by gram-negative bacteria, particularly the *Pseudomonas aeruginosa* and *Escherichia coli*. This antibiotic is a complex mixture of closely related polypeptides obtained from cultures of various strains of *Bacillus polymyxa* and related species [1]. The major constituents of polymyxin B are polymyxins B_1 and B_2. The general structure (Fig. 1) comprises a cyclic heptapeptide moiety with a straight chain tripeptide side chain. The N-terminal amino group in the side chain is acylated. The known components, B_1, B_2, B_3 and B_4 differ only in the fatty acyl moiety: B_1 contains 6-methyloctanoic acid, B_2 isooctanoic acid, B_3 octanoic acid and B_4 heptanoic acid [2]. Identification of the fatty acids has often been carried out after acid hydrolysis [3–5].

Microbiological as well as chemical methods have been described for determining polymyxin B [2,6]. These methods lack specificity and ability to demonstrate the composition of this multicomponent drug. The application of liquid chromatography (LC) has progressively demonstrated the complex nature of this antibiotic. Tsuji and Robertson separated the major components, polymyxin B_1 and B_2 by gradient elution on a μBondapak C_{18} column using a mobile phase consisting of acetonitrile and methanol in a pH 2.0 phosphate buffer. UV detection at 254 nm was used [7]. Terabe et al. developed an isocratic ion-pair reversed-phase chromatography method with a mixture of tartrate buffer (pH 3.0), acetonitrile, sodium 1-butanesulphonate and sodium sulphate as the mobile phase. This method resolved B_1, B_2 and B_3 on Nucleosil 5 C_{18} stationary phase [3]. Fong and Kho separated polymyxin B_1 and B_2 on Hypersil ODS using acetonitrile in an aqueous phase composed of tetramethylammonium chloride, sulphuric

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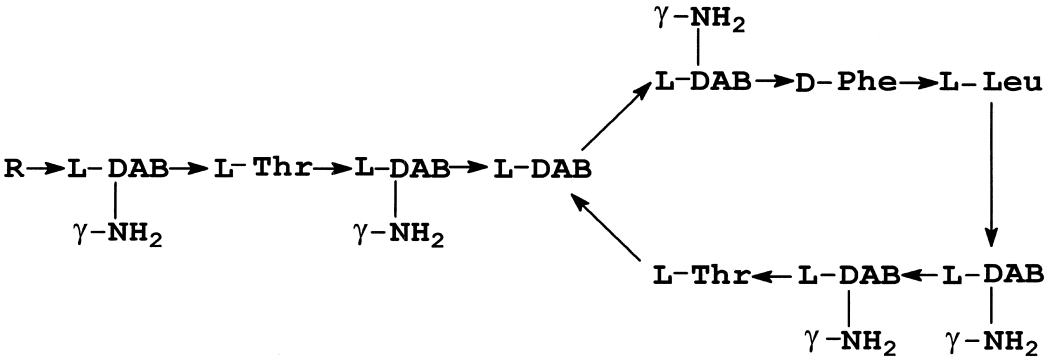
0021-9673/00/$ – see front matter
PII: S0021-9673(99)00936-X



Polymyxin B_1 R = (+)-6-methyloctanoyl
B_2 R = 6-methylheptanoyl
B_3 R = octanoyl
B_4 R = heptanoyl

Fig. 1. Structures of known components of polymyxin B. DAB, α,γ-diaminobutyric acid; Thr, threonine; Phe, phenylalanine; Leu, leucine.

acid, dipotassium hydrogenphosphate at pH 2.7. Detection was by UV at 220 nm [8]. A porous styrene–divinylbenzene copolymer packing, Hitachi gel 3011, 10 μm column has also been used with methanol and potassium chloride–hydrochloric acid buffer, pH 2.0, as mobile phase. UV detection at 210 nm was used. Fluorescence (360 nm excitation, 450 emission) detection after derivatization was found to give better results than UV 210 nm with respect to baseline sensitivity and stability. An unknown polymyxin B_0 and polymyxins B_1 and B_2 were separated [4].

Elverdam et al. described an analytical isocratic reversed-phase chromatography method which separated polymyxin B into 12 or 13 components on Nucleosil C_{18}, 5 μm, with a mobile phase of acetonitrile, phosphoric acid, acetic acid, sodium sulphate buffer adjusted to pH 2.5 by means of triethylamine. UV detection was at 220 nm [5]. Polymyxins B_1, B_2 and B_3 were resolved on ultrasphere ion-pair, 5 μm, using acetonitrile–phosphate buffer (pH 3.0) as mobile phase and UV 200 nm detection [9]. Kalasz and Horvath separated polymyxins B_1 and B_2 on LiChrosorb RP-18, 5 μm, stationary phase and acetonitrile containing 1-butane sulfonic acid and triethanolammonium phosphate buffer (pH 2.2) as mobile phase. UV detection was at 220 nm [10]. Fisher and Raja separated B_1, B_2 and B_3 and more than ten other components on Hypersil C_{18}, 5 μm, stationary phase using water–methanol–methanesulfonic acid as the eluent with UV detection at 215 nm [11].

Micellar electrokinetic capillary chromatography was also used to separate polymyxin B into at least ten components including the major peaks B_1 and B_2 [12].

Despite the numerous efforts, complete separation of this peptide complex has not been achieved with any of the methods mentioned above. Many minor components still remain unresolved, posing a difficult challenge of developing better LC methods for quantitative determination of the relative contents of these components. In this laboratory, polymyxin B has been separated into two major components B_1 and B_2 and twenty other components on poly(styrene–divinylbenzene) (PS–DVB), 8 μm, 1000 Å stationary phase with a mobile phase of acetonitrile–sodium sulphate (0.7%, m/v)–phosphoric acid (6.8%, v/v)–water (16:50:5:29) at a flow-rate of 1 ml/min and UV 215 nm detection [13]. However, several minor components, particularly the less polar components eluting after the major peak, still remained unresolved. This prompted the improvement of this method to obtain a selective, stability indicating LC procedure useful for examining the purity and for determining the relative contents of the

components. This paper reports a robust and selective method with superior resolution of more than thirty polymyxin B components.

2. Experimental

2.1. Reagents and samples

Acetonitrile HPLC-grade S was from Rathburn (Walkerburn, UK). Anhydrous sodium sulphate and sodium hydrogen sulphate were from Merck (Darmstadt, Germany). Anhydrous sodium phosphate, dibasic was from Acros (Geel, Belgium). Phosphoric acid solution (6.8% v/v dilution) was prepared from 85% m/m phosphoric acid (Acros). Sulphuric acid solution (5.6% v/v dilution) was prepared from 98% (m/m) sulphuric acid (BDH, Poole, UK). Water was distilled twice. Polymyxin B sulphate bulk powder was from Ludeco (Brussels, Belgium). Polymyxins B_1 B_2, B_3 and B_4 reference substances were prepared in the laboratory by semi-preparative LC and their respective molecular masses determined by mass spectrometry. The test mixture was prepared by dissolving polymyxin B sulphate bulk drug in water to give a concentration of 0.5 mg/ml solution and 100 μl was injected onto the column. The reference substances were each dissolved at a concentration of 0.05 mg/ml in water and used for spiking the bulk drug in order to identify the various polymyxin B components.

2.2. LC apparatus and operating conditions

The isocratic LC system consisted of a Spectra System P1000XR LC pump with a SCM1000 vacuum degasser, a Spectra Series AS100 autosampler, a Spectra 100 UV detector (TSP, San Jose, CA, USA) and an HP 3396 series III integrator (Hewlett-Packard, Avondale, PA, USA) The columns (250× 4.6 mm I.D.), YMC-Pack ODS-AQ, 5 μm (YMC, Wilmington, NC, USA), YMC-Pack Pro, 5 μm (YMC), Supelcosil LC-ABZ C_{18}, 5 μm (Supelco, Bellefonte, PA, USA), Hypersil BDS C_{18}, 5 μm (Shandon, Runcorn, UK) and Chromspher B C_{18}, 5 μm (Merck), were obtained pre-packed. Other columns (250×4.6 mm I.D.) were packed in the laboratory with Hypersil C_{18}, 5 μm (Shandon) PLRP-S, 8 μm, 1000 Å (Polymer Labs., Church Stretton, UK) Bakerbond C_{18}, 5 μm (Baker, Phillipsburg, USA) or Bio-Sil C_{18} LL, 5 μm (Alltech, Laarne, Belgium). The column temperature was maintained at 30°C by means of a Julabo EM thermostat (Julabo, Seelbach, Germany).

3. Results and discussion

3.1. Development of the chromatographic method

A LC method using PS–DVB had been developed earlier in this laboratory for analysis of a formulation containing polymyxin B [13]. However, the resolution between polymyxin B_2 and B_3 and that between other polymyxin B components was incomplete. Starting with the LC conditions described previously with the PS–DVB column, the effect of varying the pH of the mobile phase was first investigated at pH values 0.8, 1.0, 2.0, 3.0 and 4.0. The chromatographic results remained the same up to pH 2.0. Further increase in the pH of the mobile phase resulted in decreased chromatographic performance: the resolution between polymyxin B_2 and B_3 and the column efficiency decreased with an increase in pH. The asymmetry of the major peak also increased with increase in mobile phase pH (Table 1). The effect of using different acids in the mobile phase was also investigated. However, sulphuric acid (5.6%, v/v dilution; pH 0.1) or sodium hydrogensulphate (12.0%, m/v; pH 0.8) in the mobile phase produced similar chromatographic performance to phosphoric acid (Table 1). The use of phosphoric acid (6.8%, v/v dilution) in the mobile phase was retained.

The selectivity on other stationary phases was examined. Classical silica-based reversed-phase materials examined showed poor chromatographic performance as that obtained with PS–DVB. Base deactivated columns improved the chromatographic performance compared to the classical silica reversed-phase materials. Optimum selectivity was obtained with YMC-Pack Pro C_{18} stationary phase with perfect end capping. YMC-Pack ODS-AQ stationary phase with hydrophilic end capping produced the same good selectivity. The sequence of elution of polymyxin B components on different

Table 1
Influence of mobile phase pH on chromatographic parameters[d]

Acid used in mobile phase (x)	pH	Symmetry[a]	Resolution[b]	Theoretical plates[a]	k'^{a}
Phosphoric acid (6.8%, v/v)	0.8	1.5	1.2	2330	7.6
Phosphoric acid (6.8%, v/v)	1.0[c]	1.5	1.2	2330	8.0
Phosphoric acid (6.8%, v/v)	2.0[c]	1.5	1.2	2200	7.9
Phosphoric acid (6.8%, v/v)	3.0[c]	2.2	0.8	1580	7.4
Phosphoric acid (6.8%, v/v)	4.0[c]	3.2	0.8	850	8.6
Sulphuric acid (5.6%, v/v)	0.1	1.3	1.3	2570	8.1
Sodium hydrogen sulphate (12.0%, m/v)	0.8	1.5	1.2	2550	7.7

[a] Calculated for polymyxin B_1 peak.
[b] Resolution between polymyxin B_2 and B_3.
[c] pH adjusted with disodium hydrogen phosphate (14.2%, m/v).
[d] LC conditions: column: PLRP-S, 8 μm, 1000 Å. Mobile phase: acetonitrile–sodium sulphate (0.7% m/v)–x–water (22.25:50:5:22.75) at a flow-rate of 1.0 ml/min. Detection: UV at 215 nm.

columns seemed to be the same, at least for the known components. YMC-Pack Pro C_{18} stationary phase was used for further work because it gave the best symmetry for the major peak and also the best selectivity (Table 2). Chromatography was optimized by varying the composition of acetonitrile, of phosphoric acid and of sodium sulphate in the mobile phase. Fig. 2 shows a chromatogram of 0.5 mg/ml solution of polymyxin B sulphate commercial sample, using optimized conditions. The major component, polymyxin B_1 was separated from polymyxins B_2, B_3, B_4 and several other minor components of unknown identity. The elution order is in accordance with that described in other publications [3,5,7,11]. The chromatographic peaks with areas below 0.5% after normalization are not labeled on the chromatogram in Fig. 2.

3.2. Robustness

The robustness of the method was evaluated by performing a full-fraction factorial design experi-

Table 2
Chromatographic performance on different stationary phases

Stationary phase (250×4.6 mm I.D.)	CH_3CN (%)	Symmetry[a]	Resolution[b]	Theoretical plates[a]	k'^{a}
PLRP-S, 1000 Å 8 μm	18.00	1.9	1.3	1700	6.2
PLRP-S, 1000 Å 8 μm	18.25	1.9	1.5	2490	5.7
Bio-Sil LL,C_{18}, 5 μm	23.75	2.6	1.3	2770	5.6
Bakerbond C_{18}, 5 μm	23.50	4.5	1.8	5620	6.2
Hypersil ODS, 5 μm	25.50	3.0	1.7	5030	9.0
Hypersil BDS, 5 μm	23.25	1.7	1.8	7930	8.1
Chromspher B C_{18}, 5 μm	22.50	1.0	2.2	6840	8.5
Supelcosil LC ABZ C_{12-18}, 5 μm	21.25	1.3	1.8	7130	6.2
Supelcosil LC ABZ, 5 μm	22.00	1.6	2.2	10 440	5.4
YMC-Pack ODS-AQ, 5 μm	25.00	1.4	2.6	12 060	4.4
YMC-Pack Pro, 5 μm	23.75	0.9	3.3	19 450	5.4

[a] Calculated for polymyxin B_1 peak.
[b] Resolution between polymyxin B_2 and B_3.

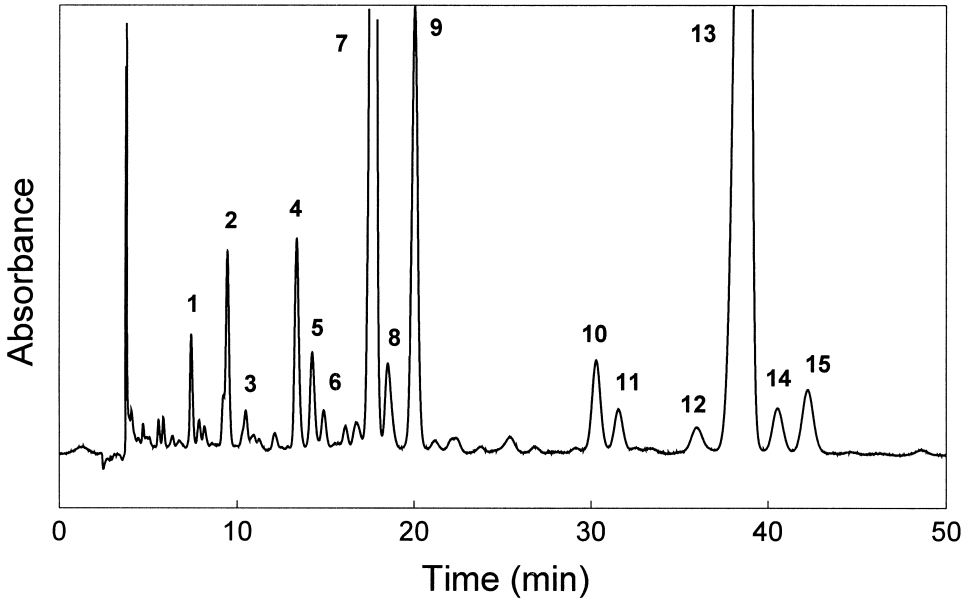


Fig. 2. Chromatogram of a 0.5 mg/ml solution of polymyxin B sulphate commercial sample. Column: YMC-Pack Pro, 5 μm, 250×4.6 mm I.D. maintained at 30°C. Mobile phase: acetonitrile–sodium sulphate (0.7%, m/v)–phosphoric acid (6.8%, v/v)–water (22.25:50:5:22.75) at a flow-rate of 1.0 ml/min. Detection: UV at 215 nm. Peaks: 2=polymyxin B_4 (2%); 4=polymyxin B_0 (2%); 7=polymyxin B_2 (22%); 9=polymyxin B_3 (7%); 13=polymyxin B_1 (48%); the other peaks are of unknown identity.

ment. The set-up of the full-fraction factorial design, together with the analysis of the measured response variable and multivariate regression calculation were supported by the statistical graphic software system STATGRAPHICS Version 6.0 (Manugistics, Rockville, MD, USA). The influence of each of the three chromatographic parameters that governed the separation most was examined by applying a full factorial design at two levels. This involved $2^3=8$ different experimental measurements, combining the three parameters examined at two previously fixed extreme levels of each parameter. One central level was included in the design and so nine measurements were performed as well as duplicate experiments. The chromatographic parameters examined as variables were the concentrations of acetonitrile, of phosphoric acid and of sodium sulphate in the mobile phase. The values of the design are given in Table 3. The estimated effects of the three chromatographic parameters with their second order interactions on the selectivity between peaks 7 (polymyxin B_2) and 8, peaks 8 and 9 (polymyxin B_3), peaks 12 and 13 (polymyxin B_1) and peaks 13 and 14 are given in Table 4. The critical *t*-value for an α of 0.05

Table 3
Nominal values corresponding to low (−1), central (0) and high (+1) levels of the design

Chromatographic variable (%)	Low value (−1)	Central value (0)	High value (+1)
Acetonitrile	22.00	22.25	22.50
Phosphoric acid (6.8%, v/v)	3.00	5.00	7.00
Sodium sulphate (0.7%, m/v)	45.00	50.00	55.00

Table 4
Estimated effects of parameters (ABC) and parameter interactions (AB, AC, BC) on selectivities α_{7-8}, α_{8-9}, α_{12-13} and α_{13-14}[a]

Parameter	α_{7-8}	α_{8-9}	α_{12-13}	α_{13-14}
(A) Acetonitrile	−0.52	0.47	1.07	−0.37
(B) Sodium sulphate	1.56	−0.94	−1.07	−1.10
(C) Phosphoric acid	0.00	−0.47	0.36	1.83
AB	1.57	−1.17	−1.79	−1.10
AC	1.04	−1.64	−0.36	0.37
BC	−0.52	0.70	0.36	−0.37

[a] The critical *t*-value for an α of 0.05 is 2.23.

is 2.23. Estimated effects of which the values are smaller than the critical *t*-value are considered as not significant. Effects may be positive or negative. A positive effect means an increase in the selectivity with an increase in the chromatographic parameter while a negative effect means a decrease in the selectivity with an increase in the chromatographic parameter. Table 4 shows that the chromatographic parameters and the parameter interactions within the range examined had no significant effect on the selectivities between the examined polymyxin B components, indicating the robustness of the method.

Response surface plots were constructed with capacity factors as a function of the chromatographic interactions that had the largest effects. Response surface plots constructed for peaks 7, 8 and 9 with the capacity factors as a function of the concentrations of (a) acetonitrile and sodium sulphate and (b) acetonitrile and phosphoric acid are presented in Fig. 3a and b, respectively. Fig. 3c shows response

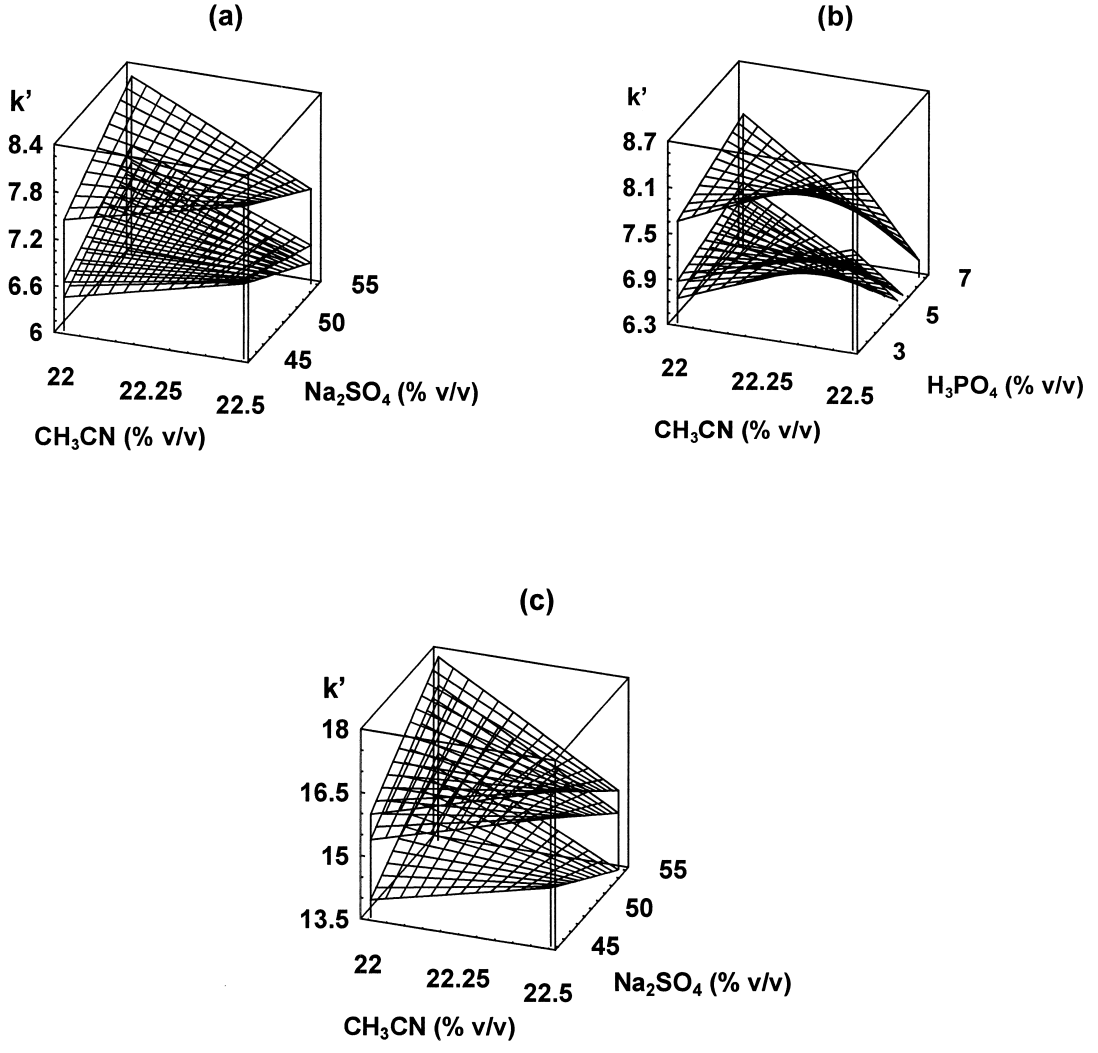


Fig. 3. Estimated response surface plots for peak 7 (lower plane), peak 8 (middle plane) and peak 9 (upper plane) constructed with capacity factors as a function of the concentrations of (a) acetonitrile and sodium sulphate, (b) acetonitrile and phosphoric acid and (c) estimated response surface plots for peak 12 (lower plane), peak 13 (middle plane) and peak 14 (upper plane) constructed with capacity factors as a function of the concentrations of acetonitrile and sodium sulphate in the mobile phase.

surface plots constructed for peaks 12, 13 and 14 with the capacity factors as a function of the concentrations of acetonitrile and sodium sulphate in the mobile phase. Although the selectivity between peaks 7 and 8 is reduced with increase in acetonitrile concentration and a decrease in sodium sulphate concentration (Fig. 3a), all the response surface plots show no overlapping, confirming the robustness of the method.

3.3. Repeatability, linearity and detection limits

The precision of the method was assessed using six replicate injections of a 0.5 mg/ml solution of polymyxin B sulphate. The relative standard deviation (RSD) of the peak area of the main component (48% of total area) was 1.4%. The calibration curve obtained by replicate analysis ($n=3$) of a series of analyte concentrations corresponding to 10, 20, 30, 40, 50, 60, 80, 100, 120 and 140% of the nominal content was subjected to linear regression analysis: $y = 193.16\,x + 1.27$, where y = peak area $\times 10^{-6}$, x = concentration in mg/ml; correlation coefficient $r = 0.9993$, standard error of estimate $S_{y,x} = 1.2$. The limit of quantification was 0.2% of 0.5 mg/ml solution, i.e., 0.10 μg injected mass ($n=6$; RSD = 12%). The limit of detection with a signal-to-noise ratio of 3 was 0.05%, i.e., 0.03 μg injected mass.

4. Conclusion

A selective and robust liquid chromatographic method capable of separating more than 30 chromatographic peaks has been developed for polymyxin B sulphate. This method shows good repeatability, linearity and sensitivity.

Acknowledgements

J.A.O. thanks the ABOS of the Belgian Government for financial support.

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