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Study of the stability of polymyxins B_1, E_1 and E_2 in aqueous solution using liquid chromatography and mass spectrometry

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Abstract

Polymyxins B_1, E_1 (colistin A) and E_2 (colistin B) were subjected to degradation in aqueous solutions of different pH values (1.4, 3.4, 5.4 and 7.4) and at different temperatures (37, 50 and 60 °C) in order to investigate the characteristics of decomposition. The progress of decomposition was followed by reversed-phase liquid chromatography on YMC-Pack Pro, C-18 stationary phase. The degradation curves showed (pseudo) first order kinetics. The pH–rate profiles indicate that colistin is more susceptible to degradation in solutions of pH above 5 and is more stable in acidic media. The degradation of polymyxin B_1 was most rapid at pH 7.4. Qualitative analysis of the degradation products by LC/MS reveals that racemization is the major mechanism of degradation in both acidic and neutral media. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Polymyxin; Stability; Liquid chromatography; Degradation products; Mass spectrometry

1. Introduction

Polymyxin B and polymyxin E (colistin) are complex mixtures of closely related polypeptides isolated from various strains of *Bacillus polymyxa* and related species and are active against Gram-negative bacteria. The general structure (Fig. 1) comprises a cyclic heptapeptide moiety attached to a tripeptide side chain with a fatty acyl residue on the N-terminus. The complex nature of these polypeptide antibiotics has been demonstrated [1–3]. Polymyxin B was subdivided into at least four components, the major component polymyxin B_1 and the polymyxins B_2, B_3 and B_4 [1]. Other minor components described so far are isoleucine-polymyxin B_1 [2] as well as two minor components recently isolated in our laboratory and named polymyxin B_5 and B_6 [4]. The major components of polymyxin E are polymyxin E_1 (colistin A) and polymyxin E_2 (colistin B). Minor

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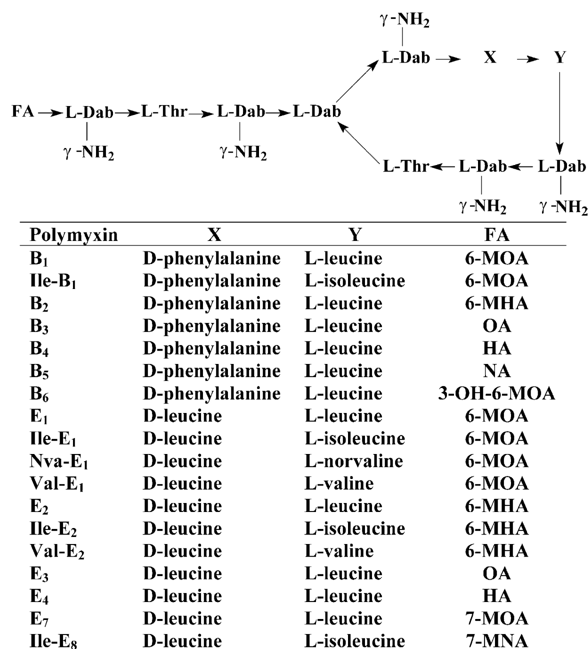
components have also been described. These include polymyxins E_3 and E_4 [1], norvaline-polymyxin E_1, valine-polymyxin E_2 [2], valine-polymyxin E_1, isoleucine-polymyxin E_1 and isoleucine-polymyxin E_2 [5]. Two minor components were recently isolated and characterized in our laboratory and the names polymyxin E_7 and isoleucine-polymyxin E_8 were proposed [6].

Aqueous solutions of polymyxins B and E sulphate are reported to be relatively stable at acid pH from 2 to 6. Above pH 6, solutions of the salts are much less stable. In dry state the salts are very stable [7]. No information has been located in literature concerning the nature of degradation of these polypeptide antibiotics in aqueous solution, except for Taylor et al. who reported a study of chemical stability of polymyxin B in aqueous solution using LC. The use of the polymyxin B mixture as starting material resulted in a very complex mixture of drug components and degradation products overlapping with each other because the LC system produced insufficient separation [8].



Polymyxin	X	Y	FA
B_1	D-phenylalanine	L-leucine	6-MOA
Ile-B_1	D-phenylalanine	L-isoleucine	6-MOA
B_2	D-phenylalanine	L-leucine	6-MHA
B_3	D-phenylalanine	L-leucine	OA
B_4	D-phenylalanine	L-leucine	HA
B_5	D-phenylalanine	L-leucine	NA
B_6	D-phenylalanine	L-leucine	3-OH-6-MOA
E_1	D-leucine	L-leucine	6-MOA
Ile-E_1	D-leucine	L-isoleucine	6-MOA
Nva-E_1	D-leucine	L-norvaline	6-MOA
Val-E_1	D-leucine	L-valine	6-MOA
E_2	D-leucine	L-leucine	6-MHA
Ile-E_2	D-leucine	L-isoleucine	6-MHA
Val-E_2	D-leucine	L-valine	6-MHA
E_3	D-leucine	L-leucine	OA
E_4	D-leucine	L-leucine	HA
E_7	D-leucine	L-leucine	7-MOA
Ile-E_8	D-leucine	L-isoleucine	7-MNA

Fig. 1. Structures of polymyxin B and E components. Dab, diaminobutyric acid; FA, fatty acid; 6-MOA, 6-methyloctanoic acid; 6-MHA, 6-methylheptanoic acid; OA, octanoic acid; HA, heptanoic acid; NA, nonanoic acid; 3-OH-6-MOA, 3-hydroxy-6-methyloctanoic acid; 7-MOA, 7-methyloctanoic acid; 7-MNA, 7-methylnonanoic acid.

Reaction kinetics is a powerful tool for the elucidation of mechanisms by which chemical reactions proceed. Such information permits a rational approach to the stabilization of drug products and prediction of shelf life and optimum storage conditions.

In view of the complex structure of polymyxin, it is appropriate to study the degradation by starting from purified single components. This excludes the interference of the related substances, which account for 50% of the total bulk drug. In this paper, the characteristics of degradation of purified polymyxin B_1, E_1 and E_2 in acidic and neutral media are investigated and the pH–rate profiles and temperature–rate profiles evaluated.

2. Experimental

2.1. Reference samples and chemicals

Polymyxins E_1 and E_2 were prepared from commercial colistin sulphate bulk powder (Asahi Kasei Shiraoi, Hokkaido, Japan) by preparative reversed-phase LC. Polymyxin B_1 was isolated from commercial polymyxin B sulphate powder (Ludeco, Brussels, Belgium). Isolation and characterization procedures are described elsewhere [4,6]. The chromatographic purity of these samples, determined by analytical LC, was 95% [4,6]. Chemicals used for the preparation of buffers were of analytical grade: potassium dihydrogen phosphate (Merck, Darmstadt, Germany), dipotassium hydrogen phosphate, phosphoric acid (85% m/m) and potassium chloride (Acros Organics, Geel, Belgium). Acetonitrile (HPLC grade S) was purchased from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid (TFA) was obtained from Riedel-de Haën (Seelze, Germany). Water was distilled from glass apparatus. A Milli-Q water purification system (Millipore, Bedford, MA) was used to further purify glass-distilled water necessary for LC/MS analysis.

2.2. Apparatus

The isocratic liquid chromatography system consisted of a TSP Spectra System P1000XR HPLC pump with a TSP SCM1000 vacuum degasser, a TSP Spectra Series AS100 autosampler fitted with a 100 μl loop, a TSP Spectra 100 UV detector (San Jose, CA) and an HP 3396 series III integrator (Hewlett-Packard, Avondale, PA). The column YMC-Pack Pro, C-18, 5 μm, 250 × 4.6 mm i.d. (Waters, Milford, MA) was maintained at 30 °C by means of a Julabo EM thermostat (Julabo, Seelbach, Germany).

A similar liquid chromatography system was used for coupling to the mass spectrometer. The autosampler was equipped with a 20 μl loop. The MS data were acquired with a LCQ ion trap mass spectrometer equipped with an electrospray interface operated in positive ion mode (ThermoFinnigan, San Jose, CA). Ionization source and MS parameters were tuned automatically. A voltage of 5 kV applied to the electrospray ionization needle resulted in a distinct signal. The temperature of the heated capillary was set at 210 °C. Nitrogen was used as sheath and auxiliary gas. The flow rate of the sheath and the auxiliary gas was set at 90 and 20 (arbitrary units), respectively. Helium was used as the damping and collision gas at a pressure of 0.133 Pa. Voltages across the capillary and the octapole lenses were tuned by an automated procedure to maximize the signal for the ion of interest. A narrowbore YMC-Pack Pro, C-18 column (5 μm, 250 × 2.0 mm i.d.) (Waters) was used.

Solutions for kinetic study were stored in a Memmert oven (Schwabach, Germany) or Julabo TWB 14 water bath (Julabo). The pH was measured at room temperature with a Consort C 831 Multi-Channel Analyser pH meter (Turnhout, Belgium).

2.3. Preparation of buffers

Appropriate amounts of 0.1 M solutions of potassium dihydrogen phosphate and dipotassium hydrogen phosphate were mixed to give 0.1 M potassium phosphate buffers of pH 5.4 and 7.4. Similarly, 0.1 M buffers of pH 1.4 and 3.4 were prepared from 0.1 M solutions of potassium dihydrogen phosphate and 1.0 M phosphoric acid. A constant ionic strength (μ) of 0.2 was maintained for each buffer by adding a calculated amount of potassium chloride.

2.4. Kinetic study

Reactions were initiated by diluting stock aqueous solutions of 1.0 mg ml^{-1} polymyxin B_1, E_1 or E_2 with equal amounts of appropriate buffers to give solutions of 0.5 mg ml^{-1}. Aliquots (1.5 ml) of this solution were put in glass ampules, sealed and incubated at appropriate temperatures. The ampules were kept in sand containers inside the oven or water-bath to ensure constant temperatures throughout the study. Ampules were removed at regular intervals and immediately frozen at −20 °C till they could be analyzed as a series. The progress of decomposition was followed by LC. The development and validation of the LC methods for polymyxin B and polymyxin E has been described elsewhere [9,10]. For polymyxins E_1 and E_2 the mobile phase was composed of acetonitrile–sodium sulphate (0.7% m/v)–phosphoric acid solution (6.8% v/v dilution of phosphoric acid 85% m/m)–water (18–20:50:5:27–25, v/v/v/v). For polymyxin B_1 the mobile phase contained acetonitrile–sodium sulphate (0.7% m/v)–phosphoric acid solution (6.8% v/v dilution of phosphoric acid 85% m/m)–water (22:50:5:23, v/v/v/v). A flow rate of 1.0 ml min^{-1} was used and detection was by UV at 215 nm. The chromatographic peak area of the main peak was used to represent the sample concentration. At the concentrations used, the decomposition could readily be monitored to just over 50% decomposition. Beyond this the chromatographic profile became too complex as some degradation products also started decomposing.

2.5. MS analysis of the degradation products

LC/MS analysis was performed on 0.25 mg ml^{-1} solutions. For polymyxins E_1 and E_2, the mobile phase contained acetonitrile—0.01 M TFA (21:79, v/v). For polymyxin B_1 the mobile phase contained acetonitrile—0.01 M TFA

(23:77, v/v). A flow rate of 0.2 ml min^{-1} was used. Full MS acquisition over the mass range 500–1500 was performed on-line with UV detection at 215 nm. The doubly-charged ions of the products formed by degradation in the sample were isolated monoisotopically in the ion trap and collisionally activated with 30% CE (collision energy).

3. Results and discussion

The use of purified samples simplified the degradation pattern enabling to distinguish degradation products from impurities and related substances by reversed-phase LC. Fig. 2 shows chromatograms of partially degraded samples of polymyxin B_1 at pH 1.4; 60 °C for 6 days (Fig. 2A) and at pH 7.4; 60 °C for 6 h (Fig. 2B). The two peaks eluted after B_1 (Fig. 2) correspond to the impurities already present in the starting material polymyxin B_1. The second peak of those two peaks corresponds to polymyxin B_5 and the first peak, which is also formed upon decomposition, is of unknown identity. Chromatograms of partially degraded samples of polymyxin E_1 at pH 1.4 and 60 °C for 6 days and pH 7.4 and 60 °C for 6 h are presented in Fig. 3A and B, respectively. Fig. 4 shows chromatograms of partially degraded samples of polymyxin E_2 at pH 1.4 and 60 °C for 6 days (Fig. 4A) and at pH 7.4 and 60 °C for 6 h (Fig. 4B).

The decomposition kinetics of polymyxins B_1, E_1 or E_2 at constant pH and constant temperature exhibited (pseudo) first order kinetics. A plot for polymyxin B_1 is shown in Fig. 5 as an example to demonstrate the (pseudo) first order kinetics of degradation at pH 1.4 and 60 °C. This is in agreement with the rate kinetics for polymyxin B sulphate reported in literature [8]. A typical plot illustrating the formation (peak areas) of the major decomposition products obtained from polymyxin B_1 at pH 7.4 and 60 °C is shown in Fig. 6. The peaks used in this plot are the peaks indicated in Fig. 2B with the numbers 1, 2, 3 and 4.

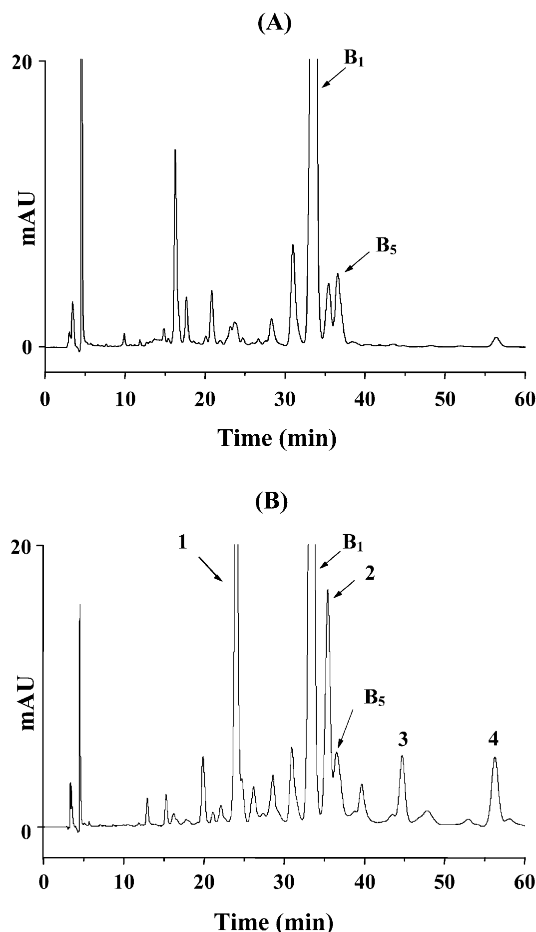


Fig. 2. Chromatograms of partially degraded polymyxin B_1 (A) at pH 1.4 and 60 °C for 6 days and (B) at pH 7.4 and 60 °C for 6 h. LC conditions: acetonitrile–sodium sulphate (0.7% m/v)–phosphoric acid solution (6.8% v/v)–water (22:50:5:23, v/v/v/v). Detection: UV at 215 nm. The peaks indicated with a number were used to construct the profile in Fig. 6.

3.1. pH–rate profiles

The decomposition kinetics of polymyxins B_1, E_1 and E_2 were studied in aqueous solutions at 37 °C over a range of pH values (1.4, 3.4, 5.4 and 7.4). The degradation exhibited (pseudo) first order kinetics. Table 1 gives the observed rate constants of degradation, k_{obs}. These k_{obs} values were calculated from the slopes of linear plots of natural logarithm of sample concentration [C] against time, t, according to the equation

$$\ln [C] = \ln [C_0] - kt$$

where $[C_0]$ is the initial concentration of the sample. Since there is a linear relationship between concentration of sample and detector response, the chromatographic peak area of the main component was used to represent the sample concentration.

The pH–k_{obs} profiles were constructed for polymyxins B_1, E_1 and E_2. The influence of pH on the rates of degradation of the polymyxins at 37 °C is shown in Fig. 7. The data indicate that these compounds are more susceptible to degradation in the neutral and basic region of the profile (pH > 5). The maximum stability occurs at pH around 3.4. Taylor et al observed a marked increase in decomposition of polymyxin B in aqueous solution above pH 7.0 while between pH value 2–7 there was little effect on decomposition [8].

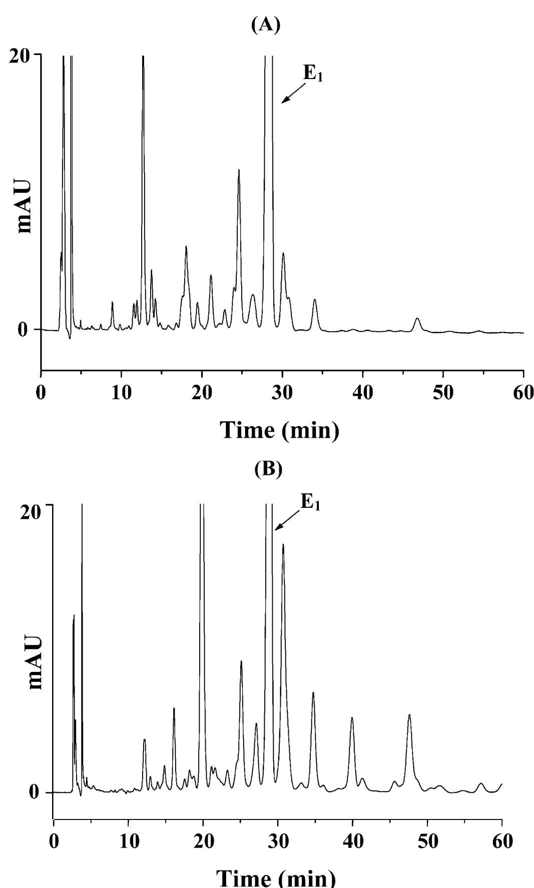


Fig. 3. Chromatograms of partially degraded polymyxin E_1 (A) at pH 1.4 and 60 °C for 6 days and (B) at pH 7.4 and 60 °C for 6 h. LC conditions: acetonitrile–sodium sulphate (0.7% m/v)–phosphoric acid solution (6.8% v/v)–water (20:50:5:25, v/v/v/v). Detection: UV at 215 nm.

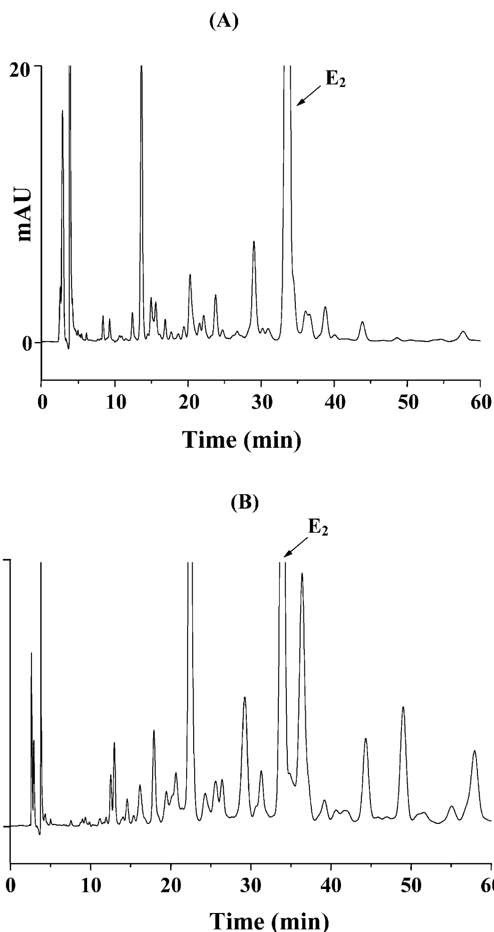


Fig. 4. Chromatograms of partially degraded polymyxin E_2 (A) at pH 1.4 and 60 °C for 6 days and (B) at pH 7.4 and 60 °C for 6 h. LC conditions: acetonitrile–sodium sulphate (0.7% m/v)–phosphoric acid solution (6.8% v/v)–water (18:50:5:27, v/v/v/v). Detection: UV at 215 nm.

3.2. Temperature–rate profiles

For determination of temperature dependence of the kinetics, the degradation procedure described above was repeated at 50 and 60 °C for

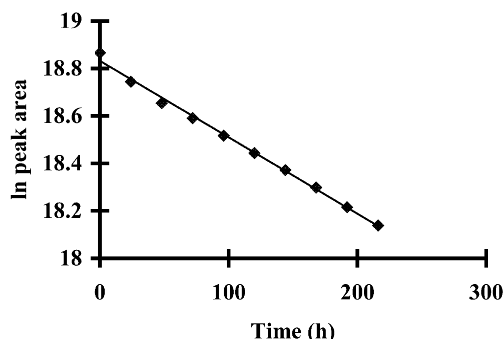


Fig. 5. Plot showing first order kinetics of degradation of polymyxin B_1 at pH 1.4 and 60 °C. ln peak area = natural logarithm of polymyxin B_1 peak area remaining as a function of time.

pH 1.4 and 7.4. The rate constants of degradation of the polymyxins B_1, E_1 and E_2 at pH 1.4 and 7.4 and at different temperatures are given in Table 1. Regression analysis was performed on these data in accordance with the natural logarithmic form of the Arrhenius equation

$$\ln k_{obs} = \ln A - \frac{E_a}{RT}$$

where k_{obs} is the observed pseudo-first order reaction rate constant, A is the pre-exponential factor and is a constant, E_a is the activation energy, T is the absolute temperature and R is the universal gas constant. The Arrhenius relationships and E_a so obtained are given in Table 2. The activation energies obtained are within the usual range of activation energy for many reactions, that is, between 50 and 100 kJ mol^{-1} [11]. For polymyxin B_1 this value is comparable to a previous study which reported E_a for polymyxin B at pH 6.0 as 64.8 kJ mol^{-1} [8].

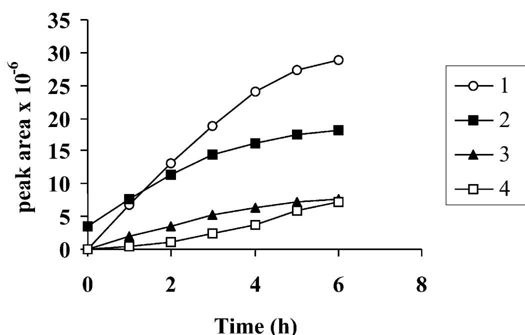


Fig. 6. Time course for major degradation products, peak numbers 1, 2, 3 and 4 (Fig. 2B) of polymyxin B_1 at pH 7.4 (60 °C), obtained by measurement of peak areas as a function of time.

The enthalpy and entropy of activation, $\Delta H^{\#}$ and $\Delta S^{\#}$ were calculated from the equations below and are given in Table 2.

$$\Delta H^{\#} = E_a - RT$$

$$\Delta S^{\#} = \frac{\Delta H^{\#}}{T} - R \ln\frac{T}{k} - R \ln\frac{\kappa}{h}$$

where κ is the Boltzmann constant (the gas constant per molecule) and h is Planck's constant. The entropy change for a process is a measure of the change in disorder and a decrease in entropy reflects a decrease in disorder whereas entropy increase indicates an increase in disorder. $\Delta S^{\#}$ for a unimolecular reaction is expected to be positive because there is no change in the number of molecules i.e. one particle in the initial state is transformed to one particle in the transition state.

In a bimolecular reaction, two reactant particles must surrender the freedom they normally have to assume many possible arrangements in space and adapt only that one that leads to a reaction. Thus a considerable loss in entropy is involved so $\Delta S^{\#}$ is expected to become negative.

The positive $\Delta S^{\#}$ values obtained indicate that the mechanism of degradation, in the rate determining step, of polymyxin B_1 in both acidic and neutral media is of unimolecular nature. The positive $\Delta S^{\#}$ values obtained in neutral media for the colistins indicate that in neutral media the mechanism of degradation, in the rate determining step, of the polymyxins E_1 and E_2 is of unimolecular nature. Furthermore for colistin it was observed that the mechanism of degradation in acidic media shows small negative values of $\Delta S^{\#}$; the nature of degradation in the rate-determining step is therefore not conclusive as all bimolecular reactions have large negative entropies.

Table 1
Pseudo-first order rate constants (±standard error) observed for degradation of polymyxins B_1, E_1 and E_2 at different pH values and temperatures

Temperature (°C)	pH	k_{obs} (h^{-1}) × 10^3			$t_{1/2}$ (h)		
		B_1	E_1	E_2	B_1	E_1	E_2
37	1.4	0.37 (±0.02)	0.38 (±0.04)	0.42 (±0.01)	1870	1820	1650
37	3.4	0.11 (±0.01)	0.11 (±0.01)	0.11 (±0.01)	6300[a]	6300[a]	6300[a]
37	5.4	0.36 (±0.03)	0.36 (±0.03)	0.30 (±0.03)	1930[a]	1930[a]	2310[a]
37	7.4	8.26 (±0.32)	10.00 (±0.23)	9.79 (±0.03)	84	69	71
50	1.4	1.14 (±0.02)	1.12 (±0.06)	1.10 (±0.03)	610	620	630
50	7.4	49.60 (±0.65)	53.46 (±0.96)	43.73 (±0.9)	14	13	16
60	1.4	3.22 (±0.07)	2.80 (±0.15)	2.66 (±0.05)	220	250	260
60	7.4	112.80 (±8.06)	114.28 (±11.19)	105.42 (±6.8)	6	6	7

[a] Determined with less than one half-life.

3.3. Degradation products

The chemical and physical instability of peptides in general was reviewed by Reubsaet et al. [12,13]. Frequently described chemical reactions responsible for instability of peptides include deamination, hydrolysis and racemization of amino acids [14,15]. For all examined polymyxins, the degradation products formed in acidic media were mainly more polar compounds, eluted prior to the parent compound on reversed-phase LC. At pH 7.4, in addition to polar components, some late eluted compounds were also formed (Figs. 2–4). The degradation products formed in acidic media (pH 1.4 and 3.4) were different from those formed in neutral media (pH 5.4 and 7.4). This was noted by the different retention times obtained on the chromatograms. Except for an impurity eluted immediately after B_1 and after E_1 (Figs. 2 Aand B and 3A and B) which increased on degradation at pH 7.4, the other degradation products were eluted at different retention times compared to the normal impurities found in the bulk drug. Attempts to isolate degradation products by preparative LC were unsuccessful because the degradation products underwent further degradation during isolation.

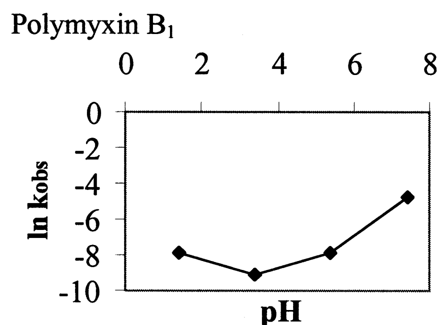
Qualitative analysis of the degradation products was therefore carried out by LC/MS analysis. Important features in combining LC and MS are the incompatibility with MS of non-volatile mobile phase additives and the incompatibility of high flow rate with the electrospray interface. For this reason the degradation products were separated on a narrowbore YMC-Pack Pro, C-18 allowing a flow rate of 0.2 ml min^{-1} with a volatile mobile phase of acetonitrile—0.01 M TFA. MS analysis was performed with the electrospray interface operated in the positive ion mode. Based on the previous observations that the degradation products formed in acidic media (pH 1.4 and 3.4) were different from those formed in neutral media (pH 5.4 and 7.4), it was decided to analyze the partially degraded samples of the investigated polymyxins at pH 1.4 and 60 °C after 6 days and at pH 7.4 and 60 °C after 6 h. Most of the products formed in acidic and neutral media were found to possess the same mass/charge ratio (m/z) and MS/MS spectrum as the parent compounds (polymyxin B_1, $[M+H]^+$, m/z 1203; $[M+2H]^{2+}$, m/z 602; polymyxin E_1, $[M+H]^+$, m/z 1169; $[M+2H]^{2+}$, m/z 585; polymyxin E_2, $[M+H]^+$, m/z 1155; $[M+2H]^{2+}$, m/z 578) but had different retention times in reversed-phase LC. This indicates the formation of isomers. Additionally a limited number of products were formed by loss of water and by hydrolysis (ring opening and water addition). MS/MS fragmentations did not offer clear information about the position of the water loss or the ring opening. Racemization of different amino acids present in the polymyxin structure is accepted to be the major mechanism of degradation in acidic and neutral media.

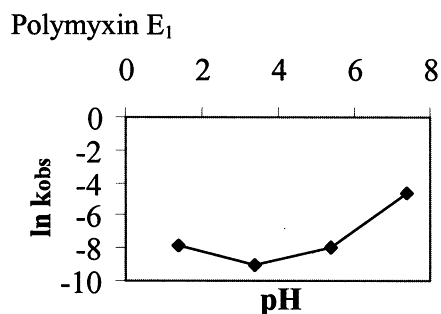
3.4. Half-life estimation

The half-life, $t_{1/2}$, which is described as the time taken for 50% decomposition to occur, was calculated according to equation

$$\ln\left(\frac{C_0}{2}\right) = \ln C_0 - k_{obs}t_{1/2}$$

where C_0 is the initial concentration. The values obtained are shown in Table 1.





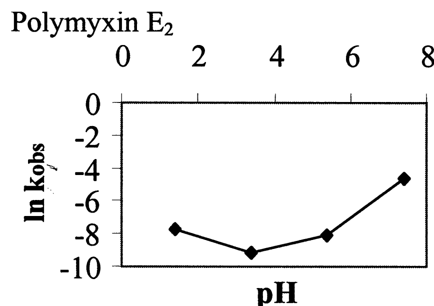


Fig. 7. pH–rate profiles of polymyxins B_1, E_1 and E_2 at 37 °C.

3.5. Shelf life estimation

Of more pharmaceutical concern is the shelf life, which may be attributed to a formulated product. This parameter indicates the period of storage to which a product may be subjected without serious loss of potency, provided no untoward physico-chemical modifications are apparent or no toxic degradation products are produced. Under these conditions the shelf life is normally described as the time taken for 10% decomposition to occur. The rate constants at 25 °C for the examined polymyxins in buffer pH 1.4 or 7.4 were calculated according to equation

$$\ln k_{25} = \ln A - \frac{E_a}{RT}$$

The shelf life at 25 °C was then estimated from equation

$$t_{10\%} = \frac{0.105}{k_{25}}$$

The rate constants at 25 °C for the polymyxins in buffer pH 3.4, the most stable condition, was estimated using the average estimate of Q_{10} value of 3. The quantity Q_{10} is defined as

$$Q_{10} = \frac{k_{(T+10)}}{k_T}$$

and is a factor by which the rate constant increases for a 10 °C temperature increase [11]. The estimated shelf lives for the investigated polymyxins in solutions buffered at pH 1.7, 3.4 and 7.4 are listed in Table 3, together with the estimated half-lives. The approximate shelf life for solutions buffered at pH 3.4 was estimated to be 3570 h.

4. Conclusion

The study of the stability of polymyxins B_1, E_1 and E_2 in solution has been successfully accomplished using a selective LC method. This analytical LC method separates the polymyxins B_1, E_1 and E_2 from many decomposition products formed. The results obtained shows that the inves-

Table 2
Arrhenius relationships and activation parameters of polymyxins B_1, E_1 and E_2 in 0.1 M potassium phosphate buffer pH 1.4 or 7.4 and ionic strength 0.2

Polymyxin	pH	Arrhenius relationship	E_a (kJ mol^{-1})	$\Delta H^{\neq}$ (kJ mol^{-1})	$\Delta S^{\neq}$ (e.u.)
B_1	1.4	$\ln k_{obs} = 23.19 - 9653(1/T)$ $r = 0.9974$	80.25	77.66	+7.57
	7.4	$\ln k_{obs} = 33.57 - 11876(1/T)$ $r = 0.9933$	98.75	96.15	+93.06
E_1	1.4	$\ln k_{obs} = 20.90 - 8932(1/T)$ $r = 0.9988$	74.27	71.68	−11.46
	7.4	$\ln k_{obs} = 31.16 - 11070(1/T)$ $r = 0.9930$	90.05	89.46	+73.01
E_2	1.4	$\ln k_{obs} = 18.75 - 8237(1/T)$ $r = 0.9975$	68.50	65.90	−29.29
	7.4	$\ln k_{obs} = 30.01 - 10732(1/T)$ $r = 0.9985$	89.21	86.61	+63.77

e.u.: entropy units in J mol^{-1} K^{-1}.

tigated polymyxins exhibit (pseudo) first order kinetics and are more susceptible to decomposition above pH 5.4. The maximum stability occurs in solutions buffered at pH 3.4. LC/MS analysis of the degradation products revealed that racemization is the major mechanism of decomposition of the examined polymyxins both at pH 1.4 and 7.4.

Table 3
Estimated shelf life of polymyxins B_1, E_1 and E_2 at room temperature (25 °C) in 0.1 M potassium phosphate buffer pH 1.4, 3.4 or 7.4 and ionic strength 0.2

Polymyxin	Ph	k_{25} (h^{-1}) × 10^3	$t_{1/2}$ (h)	$t_{10\%}$ (h)
B_1	1.4	0.10	6800	1030
	3.4	0.03	23500	3570
	7.4	1.91	360	55
E_1	1.4	0.12	5950	900
	3.4	0.03	23500	3570
	7.4	2.55	270	41
E_2	1.4	0.14	4960	750
	3.4	0.03	23500	3570
	7.4	2.51	280	42

Estimated from experimental values for pH 1.4 and 7.4 or using Q_{10} value for pH 3.4.

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