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Chemical stability of polymyxin B in aqueous solution

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Summary

Chromatographic separations of the components of polymyxin B sulphate are reported and it is shown that the separations are such that two of the three major components detected are specific for the intact drug in the presence of chemical decomposition products. The specificity of the separations with respect to other antibacterials, propamidine, dibromopropamidine and trimethoprim is also reported. Validation of the stability indicating assay based on this separation is described. Rate constants for the decomposition of polymyxin at various temperatures between 32 and 52°C and in solutions of different pH values from 2.0 to 10.3 are determined and the t_{90} value at pH 7 and 4°C is estimated.

Introduction

Polymyxin B, a peptide antibiotic produced by a strain of *Bacillus polymyxa*, consists mainly of three components PB_1, PB_2 and PB_3 differing in the fatty acyl residue in the tripeptide side chain attached to the cyclic heptapeptide (Elverdam et al., 1981). Several different liquid chromatographic methods of separating the individual molecular species have been reported. Kimura et al. (1981) used a porous styrene-divinylbenzene copolymer packing. Fong and Kho (1979) described a separation using Hypersil ODS. Different publications have reported variable degrees of separation. Whall (1981) reported a complete separation of the three major components using what was described as an ion pairing column and an isocratic solvent of phosphate buffer and acetonitrile. In most published reports little explanation of the basis of the separation is given nor are the effects of the common chromatographic variables described in any detail. A recent separation of several polymyxin species has been demonstrated by micellar electrokinetic capillary chromatography (Kirstensen and Hanson, 1993) in which the separation mechanism is discussed. There appears, also, to be little information in the literature concerning the quantitation of polymyxins. While the paper of Fong and Kho describes the linearity and detection limit of polymyxin B, only two components are resolved and the quantitation is in terms of the sum of these peaks. The publication by Fisher and Raja (1982) estimates the relative proportions of each of the major components by peak normalisation.

No information has been located in the literature concerning the chemical stability of

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polymyxin B nor has any quantitative assay procedure been reported which is capable of undertaking such a stability study. A chromatographic method based on HPLC is preferable to separations based on capillary electrophoresis in view of the more reliable quantitation that can currently be achieved. It is intended in the present work to report methods for the reverse phase HPLC separation of the three major components of polymyxin B, to indicate the chromatographic variables affecting the separation and to validate an assay based on this separation as being stability indicating. It is also intended to report stability data for polymyxin B in aqueous solution under conditions of stressing at elevated temperatures and under extremes of pH. In addition, the specificity of the assay for polymyxin in the presence of the antibacterials trimethoprim, propamidine and dibromopropamidine is investigated. The inclusion of either of the latter two antibacterials with polymyxin is known to result in enhanced antibacterial activity against both Gram-negative and Gram-positive organisms (Richards and Xing, unpublished results). The overall purpose of this work, therefore, is to allow study of the stability of formulations, in particular those for ophthalmic use which may contain polymyxin in conjunction with other antibacterials.

Materials and Methods

The chromatographic equipment used consisted of a Jasco PU980 pump and UV975 detector. The chromatographic column was 100×4.6 mm, slurry packed in the laboratory with 3 μm ODS Hypersil. Injection was via a Rheodyne 1725 six port valve incorporating a 20 μl loop. For stability studies polymyxin solutions were stored in glass Universal Vials fitted with metal screw caps incorporating rubber seals. The vials were stored in thermostatted ovens. At specified intervals aliquots of the vial contents were withdrawn by microsyringe for analysis. Water used in the chromatography was purified by a Millipore Milli-Q system, HPLC acetonitrile was obtained from Rathburn Chemicals, and polymyxin B sulphate and trimethoprim were purchased from Sigma Chemicals. Propamidine isethionate and dibromopropamidine isethionate were supplied by Rhone Poulenc Rorer.

Results and Discussion

Assay development

The maximum in the ultraviolet spectrum of polymyxin occurs at 200 nm. This wavelength was selected since it provided maximum sensitivity and reproducibility. However, it limited the number and type of mobile phase additives which could be utilised. In particular, the use of anionic hydrophobic pairing ions such as sodium dodecyl sulphate was not possible due to the high background absorbance at 200 nm. Polymyxin is strongly basic and such pairing ions in conjunction with organic counterions such as tetrabutylammonium salts have been shown to be effective in controlling retention and modifying selectivity (Taylor et al., 1984, 1992). Variables studied in optimising the separation of polymyxin into its individual components were thus limited to concentration of acetonitrile, as organic modifier, in buffer, pH of buffer, and overall ionic strength of buffer. The effect of perchloric acid, presumably acting as a hydrophillic pairing ion, was also studied.

It was found that polymyxin could be resolved into three major components and approximately seven minor components. The major components were assumed to be PB_2, PB_3 and PB_1 in increasing order of elution by analogy with the majority of previously published data. This order of elution is also consistent with the hydrophobicities of the respective fatty acid residues. The retention times were found to be very sensitive to changes in acetonitrile concentration. In acid solution, pH 3, a change in acetonitrile concentration from 20 to 24% v/v reduced the retention time of PB_2 from 37 to 7.8 min. Using a disodium hydrogen phosphate buffer adjusted with either sodium hydroxide or orthophosphoric acid the retention was studied over a range of pH values from 2.4 to 7.0. As expected, decrease in pH resulted in decreased retention times. Even at the

lowest pH studied, resolution among the major peaks was adequate. That appreciable retention and complete resolution are obtained in the fully ionised state indicates that polymyxin components are appreciably hydrophobic. This is consistent with the sensitivity of retention to organic modifier concentration. Ionic strength was found to have little effect on the retention. Doubling ionic strength resulted in only a 10% increase in retention of any of the major components detected. The addition of perchloric acid to the mobile phase at concentrations below 1% w/v was found to increase the retention of all components similar to the effect of decreasing acetonitrile concentration. Solvents containing perchloric acid were found to be less critical in routine use and to give marginally better resolution among the components of polymyxin. This was only of significance in the separation of polymyxin solutions containing other antibacterials.

On the basis of the above findings, two alternative solvent systems were optimised for the separation of polymyxin components. A specimen chromatogram of an aqueous sample containing polymyxin, propamidine, trimethoprim and dibromopropamidine is shown in Fig. 1. This was obtained using solvent A which consisted of 24:76 acetonitrile:aqueous 50 mM disodium hydrogen phosphate adjusted to pH 2.9 with orthophosphoric acid. Fig. 2 shows the corresponding chromatogram obtained using solvent B consisting of 34.5:65.5 acetonitrile:aqueous 10 mM phosphate buffer, pH 3.0, to which perchloric acid had been included at a concentration of 0.33% w/v. Either of these solvents show separation of the individual polymyxin components consistent with that demonstrated by Whall although the component PB_2 is much larger relative to the PB_1 and PB_3 peaks. This presumably reflects the different sources of the antibiotic. Also, resolution between the PB_2 and PB_3 peaks is larger than that previously reported. In both solvent systems propamidine and trimethoprim co-elute before any polymyxin peak. Solvent A, however, is not capable of resolving the dibromopropamidine from the polymyxin component PB_2. In both solvent systems dibromopropamidine is seen to have poor peak shape.This is consistent with previous

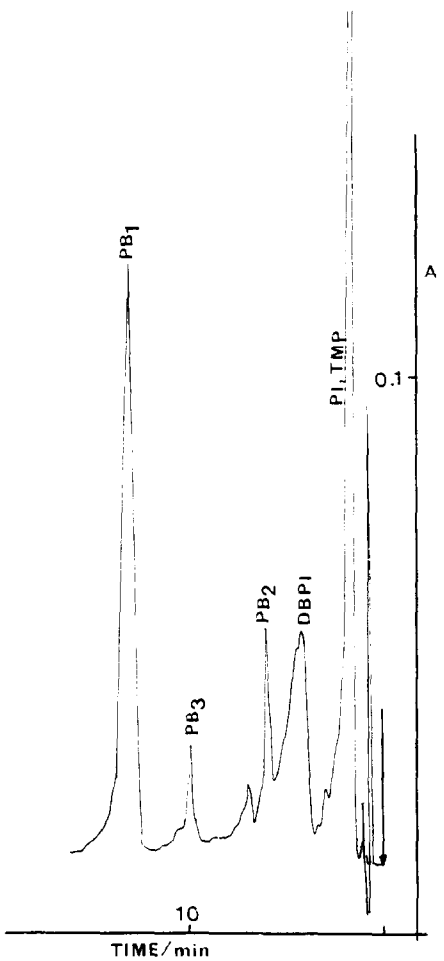

Fig. 1. Specimen chromatogram of a polymyxin sample containing also propamidine isethionate (PI) trimethoprim (TMP) and dibromopropamidine isethionate (DBPI) at concentrations of 0.0250, 0.0075, 0.0010 and 0.0114% w/v, respectively, using solvent system (A). Column, 100×4.6 mm 3 μm ODS Hypersil; flow rate, 1.5 cm^3 min^{-1}; detection, 200 nm; absorbance range, 0.16 full scale. Peak identification as indicated.

reports of the chromatography of this compound (Taylor et al., 1990).

Quantitation and validation for stability studies

The most relevant analytical parameters for validation of the method for use in stability studies are specificity, linearity of detector response and overall precision. In the assay of such a multicomponent drug it is not easy to quantitate

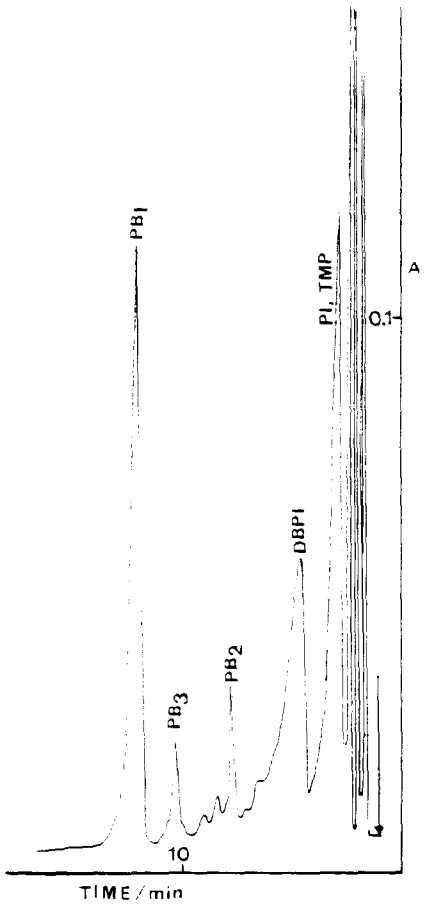

Fig. 2. Specimen chromatogram of a polymyxin sample containing also propamidine isethionate (PI), trimethoprim (TMP) and dibromopropamidine isethionate (DBPI) using solvent system (B). Conditions and analyte concentrations as in Fig. 1

individual constituents, since standard compounds are not readily available. In this work, quantitation was on the basis of the percentage peak height remaining during decomposition relative to the original peak height produced by a freshly made up solution. This was adequate for the purpose of establishing rate constants since the decomposition, under the conditions studied, followed first order kinetics. To allow for normal variations in performance of the chromatographic system over the period of the accelerated stability test, fresh polymyxin solution was used as a standard at each time point measured and the peak heights normalised with respect to the original concentration of total drug used at the start of the stability run.

The specificities of solvent systems A and B have been discussed above with respect to propamidine, trimethoprim and dibromopropamidine. The specificity with respect to decomposition products was established by examining chromatograms of extensively decomposed polymyxin solution. In such samples no peaks are present at retention times corresponding to PB_1 or PB_2. In these samples additional chromatographic peaks were seen close to the solvent front and one of these appeared to overlap with the PB_3 retention time. This was confirmed during decomposition experiments when PB_3 was distorted due to overlap of decomposition product peaks. Furthermore, first order rate constants calculated using PB_1 and PB_2 were in good agreement while the corresponding values calculated using PB_3 were always appreciably smaller. In the present study solvent system A was used.

To determine the linearity of detector response with concentration for the individual major components detected, four aqueous fresh polymyxin solutions were prepared ranging in concentration from 125 to 500 μg cm^{-3}. The peak absorbances of each component were measured at 0.1 AUFS following injection of 20 μl of each concentration. Least-squares regression of peak absorbance on total polymyxin sulphate concentration (C) yielded the following equations.

Peak PB_1: peak height (mm)

$$= 3.904C\ (\mu g\ cm^{-3}) + 32\ (r^2 = 0.9974)$$

Peak PB_2: peak height (mm)

$$= 0.9712C\ (\mu g\ cm^{-3}) - 2.8\ (r^2 = 0.9997)$$

Peak PB_3: peak height (mm)

$$= 0.7344C\ (\mu g\ cm^{-3}) - 0.80\ (r^2 = 0.9998)$$

While these equations do not allow absolute quantitation of the individual polymyxin species, the high correlation coefficients and the small relative standard error of the slopes (0.7–2.8%)

show the rectilinear relationship between the peak heights of any of the major components and the total polymyxin concentration. Peak PB_1 is obviously the most sensitive indicator while PB_3 is not adequately specific in the presence of decomposition products. The combined precision of retention and peak height determination was assessed by replicate injections of a standard aqueous polymyxin sample. The relative standard deviations for PB_1 and PB_2 were 5.2 and 4.7%, respectively. Limits of detection were not established as these were considered irrelevant since, at the concentrations used, the decomposition could readily be monitored over 70% decomposition.

Stability study

Solutions of polymyxin B sulphate (260 μg cm^{-3}) were made in disodium hydrogen phosphate buffer (50 mM, pH 6.0). Samples (50 cm^3) were sealed in Universal glass vials and stored in thermostatted ovens at 32, 37, 43 and 52°C for a period of approx. 30 days. The temperature range over which the reaction could be studied was limited since preliminary measurements at higher temperatures, in the region of 80–90°C, caused changes in the decomposition mechanism shown by complete loss of the main peaks on the chromatographic separation. Also, rate constants measured at these temperatures in a preliminary study yielded a poor fit in the Arrhenius equation. Samples were withdrawn at intervals of 3 days and the percentage of each of the three major components determined by comparison with a freshly prepared standard of polymyxin as described above. A corresponding set of polymixin solutions were prepared in phosphate buffers of pH 2.0, 3.5, 5.0, 7.0, 8.0 and 10.3. These were stored at 37°C and the decomposition with time followed.

TABLE 1

First order rate constants and associated standard deviations for polymyxin decomposition at different pH values and temperatures obtained by measurement of polymyxin peaks PB_1 and PB_2

Temperature (°C)	pH	Rate constant ($h^{-1} \times 10^4$) (SD $\times 10^4$)			
		PB_1		PB_2	
32	6.0	4.3	(0.42)	4.6	(0.74)
37	6.0	6.3	(0.77)	6.1	(0.71)
43	6.0	13.0	(0.70)	13.3	(0.61)
52	6.0	19.9	(2.7)	20.2	(2.9)
37	2.0	5.3	(1.8)	5.4	(2.3)
37	3.5	5.9	(2.4)	6.2	(2.9)
37	5.0	8.7	(2.6)	9.3	(3.5)
37	7.0	15.7	(2.8)	15.0	(3.3)
37	8.0	55.6	(7.6)	47.5	(7.4)
37	10.3	90.1	(29.0)	86.0	(37)

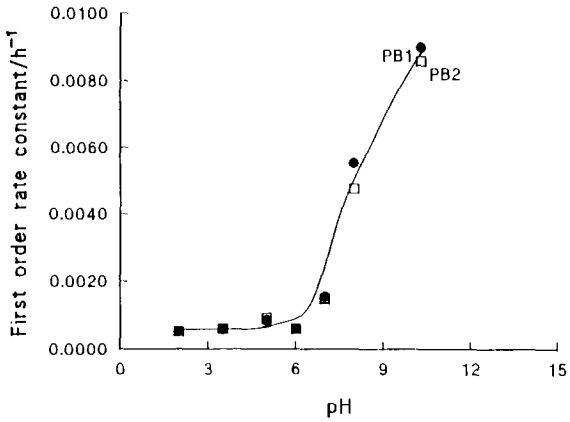

Fig. 3. Plot of first order rate constants against pH for polymyxin decomposition as measured from PB_1 and PB_2.

It was found that in all cases the decomposition followed first order kinetics. Table 1 shows the first order rate constants calculated at the different temperatures and pH values studied. The values obtained by monitoring polymyxin peaks PB_1 and PB_2 are shown separately. The rate constants derived at the different temperatures studied were fitted to the Arrhenius equation ($r^2 = 0.9650$, RSD of slope = 7.7%) and yielded an activation energy of 64.8 kJ mol^{-1}. The variation of rate constant with pH is represented graphically in Fig. 3. It is evident from this that below pH 7 pH has little effect upon the decomposition but above this value, increase of pH markedly accelerates the reaction.

Conclusions

The chromatographic methods outlined produce separation of polymyxin into its individual

components comparable to those previously reported employing HPLC and by CE. Alternative solvent systems may be used. Solvent (A) consisting of acetonitrile:phosphate buffer provides adequate resolution for the study of polymyxin alone in solution. The inclusion of perchloric acid would allow improved resolution and consequent specificity in the presence of co-formulated antibacterial drugs.

On the basis of the brief stability study carried out, polymyxin alone, at pH 6.0, has a t_{90} value in the region of 130 days at 4°C based on extrapolation from the above measurements at elevated temperatures. It will be the subject of a future publication to report the correspondence between the chemical stability and the microbiological activity.

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