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## Note

# Paired-ion high-performance liquid chromatography of bleomycins

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The bleomycins, an antibiotic complex produced by *Streptomyces verticillus*[1,2], are used in the treatment of various tumors[3,4]. The congeners are various amides of a common bleomycinic acid moiety (Fig. 1). The two components labeled $A_2$ (*ca.* 55–70%) and $B_2$ (*ca.* 25%) comprise most of the clinically used mixture of bleomycins (Blenoxane), with other derivatives present in small amounts. Because of their wide use for therapeutic purposes, a rapid method of identifying and quantitating bleomycins would be important in studying the pharmacology of these compounds.

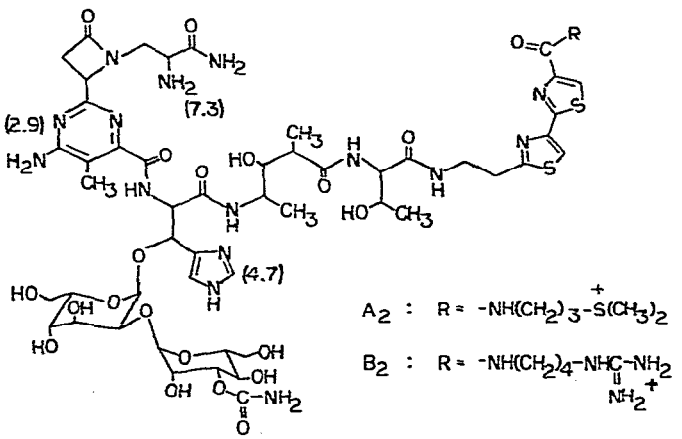


Fig. 1. Structures of bleomycin $A_2$ and $B_2$. Numbers in parentheses are the p$K_a$ values of the labeled groups.

High-performance liquid chromatography (HPLC) is widely used in the study and identification of a large number of compounds. The introduction of reversed-phase HPLC has resulted in the more rapid analyses of many drugs and this method has been used to separate bleomycins[5,7]; however, the length of time required to achieve separation of the bleomycins by reversed-phase HPLC is about 4 to 5 h. More recently, paired-ion chromatography has been applied with much success in

the rapid separation of a number of drugs[5]. Because of the highly cationic nature of the bleomycins, paired-ion chromatography is an attractive method for achieving separation of the congeners. This communication describes preliminary results on the use of paired-ion HPLC on a reversed-phase support for the rapid separation of bleomycins.

## EXPERIMENTAL

### *Apparatus*

A Waters Assoc. high-pressure liquid chromatograph Model ALC 202/6000 was used. The chromatograph was equipped with a Model 440 absorbance detector operating at 254 or 280 nm and a Model 450 variable-wavelength absorbance detector.

### *Materials and methods*

Sterile bleomycin sulfate (Blenoxane; Bristol Labs., Syracuse, N.Y., U.S.A.) and bleomycin $A_2$ and $B_2$ (obtained from the Cancer Chemotherapy National Service Center) were dissolved in distilled water and stored at +4°. Glass-distilled methanol was from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). All solutions were prepared with distilled water and filtered through 0.45-$\mu$m pore size membrane filters (Millipore, Bedford, Mass., U.S.A.) prior to use.

A Waters Assoc. $\mu$Bondapak $C_{18}$ reversed-phase column (300 × 4.6 mm I.D.) was used for the separations. Unless indicated otherwise, the mobile phase was a solution of 5 m*M* heptanesulfonic acid (Waters Assoc. PIC reagent B-7) in 50% aqueous methanol. The reagent as supplied by Waters Assoc. is buffered with acetic acid to give a pH between 3.5 and 4. For most separations reported here, the pH of the methanol solution was adjusted to 8.3 with concentrated ammonium hydroxide. Generally a flow-rate between 0.5 and 1.5 ml/min was used throughout the study. Separations were monitored by UV absorbance at 280 nm and 290 nm, the wavelength of maximum absorbance of the bleomycins.

## RESULTS AND DISCUSSION

Fig. 2 shows a separation of Blexonane obtained by paired-ion HPLC using the heptanesulfonic acid paired-ion reagent at pH 4. The peaks were assigned on the basis of their retention times which were compared with those of samples of bleomycin $A_2$ and $B_2$; however, a second pair of broad peaks is observed at retention times between 17 and 22 min after injection. In some separations, these peaks comprised the major peaks. These peaks probably are due to the complexation of heptanesulfonate with groups other than the terminal cationic group (R group in Fig. 1). There are three other dissociable groups on the bleomycin molecule: the $\alpha$-amino group of the alanine amide moiety has a $pK_a$ of 7.3 (ref. 2), the imidazole ring of the $\beta$-hydroxyhistidine has a $pK_a$ of 4.7 and the pyrimidine ring has $pK_a$ 2.9. Under the acidic conditions of the separation, the first two groups should be fully protonated (or nearly so), giving rise to two additional sites for complexation with the paired-ion reagent. Increasing the pH of the mobile phase to a value higher than the $pK_a$ of the alanine amino group should make the separation dependent only on the cationic R group. To test this, the separation was carried out using the same reagent

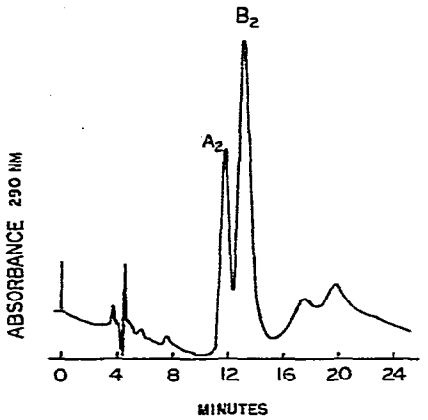


Fig. 2. Paired-ion HPLC of 10 $\mu$g Blenoxane (Lot G7X04 March 1979) on $\mu$Bondapak $C_{18}$ (300 × 4.6 mm I.D.) at 20°. Flow-rate, 1.0 ml/min; pressure, 2100 p.s.i.; mobile phase, 5 m*M* 1-heptanesulfonic acid (Waters PIC reagent 7A) in 50% aqueous methanol, pH 4.

at pH 8.3. A typical elution profile is shown in Fig. 3a). Clearly, these conditions provide a much better separation of the bleomycins; increasing the flow-rate permits essentially the same separation in less than 15 min (Fig. 3b). The identification of the minor peaks has not been made; the most common minor constituents are bleomycin $A_1$ [R = $NH(CH_2)_3\,SOCH_3$] and demethyl $A_2$ [R = $NH(CH_2)_3SCH_3$][2,7]. Fig. 4 shows the ability of this method to resolve number of contaminants and breakdown products in a sample of bleomycin $B_2$; the broad trailing edge probably contains a number of unresolved peaks.

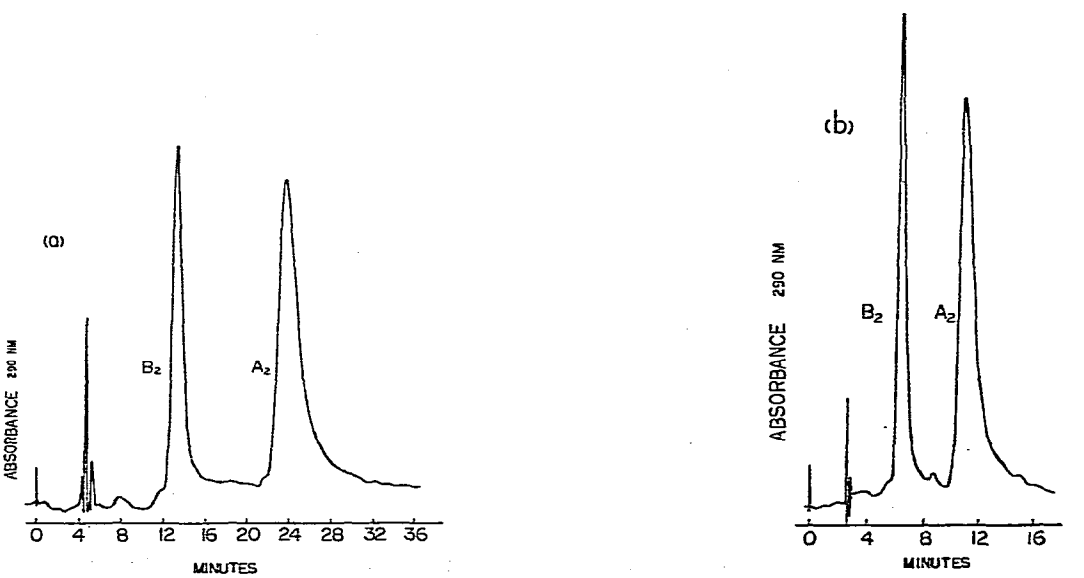


Fig. 3. (a) Paired-ion HPLC of 10 $\mu$g Blenoxane (Lot E7X15 February 1979) on $\mu$Bondapak $C_{18}$. Flow-rate 0.7 ml/min; pressure, 1800 p.s.i.; mobile phase, 5 m*M* 1-heptanesulfonic acid in 50% aqueous methanol, pH 8.3. (b) same as (a), except: flow-rate, 1.5 ml/min; pressure, 3000 p.s.i.

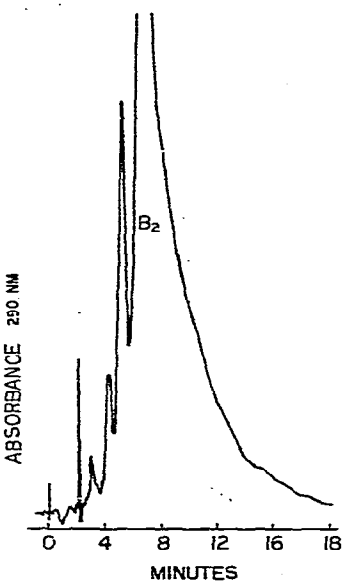


Fig. 4. Paired-ion HPLC of sample of bleomycin $A_2$, 5 $\mu$g. Conditions as described in Fig. 3b.

Comparable elution profiles are obtained if column effluents are monitored at 280 nm, however, observation at 290 nm allowed slightly better resolution of overlapped peaks or shoulders. It is estimated that 10 pmoles of a bleomycin derivative can be seen easily using available detectors. The use of a fluorescence detector to take advantage of the fluorescence of the bithiazole moiety[8] should permit at least a tenfold increase in sensitivity.

Although identification of some of the peaks present in a chromatogram of Blenoxane has not been made, the resolution of the major peaks and the speed with which it is achieved clearly indicate that paired-ion HPLC should be developed for use in the study of the bleomycins.

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