Journal of Chromatography, 178 (1979) 302–306
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CHROM. 12,178

Note

Improved method for the purification of bleomycins

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(Received May 18th, 1979)

The bleomycins are a complex of glycopeptide antibiotics produced by *Streptomyces verticillus* which are used in cancer chemotherapy and tumor imaging (for reviews, see refs. 1 and 2). The major components of Blenoxane (sterile Bleomycin sulfate, Bristol Labs., Syracuse, N.Y., U.S.A.) are bleomycin A_2 ($\approx$70%) and bleomycin B_2 ($\approx$25%) with lesser amounts of other congeners (Fig. 1).

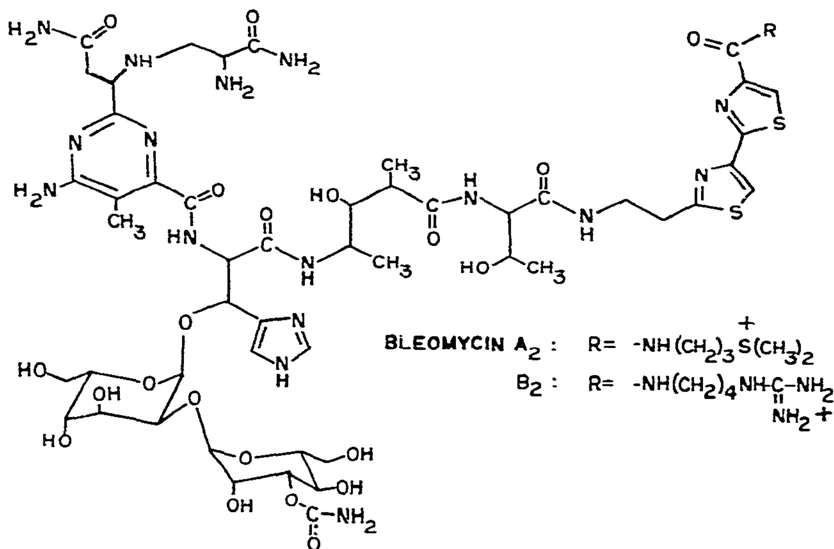

Fig. 1. Structures of bleomycin A_2 and B_2. Other bleomycins: Demethyl A_2, R = $NH(CH_2)_3SCH_3$; A_1, R = $NH(CH_2)_3S(O)CH_3$; A_5, R = $NH(CH_2)_3NH(CH_2)_4NH_2$.

Because of the problems associated with interpreting experimental data obtained with a mixture of compounds such as Blenoxane, we have attempted to purify Blenoxane on a large scale for use in our studies. The literature method of purification involves ion-exchange chromatography of Blenoxane on carboxymethyl (CM) Sephadex C-25 employing a linear gradient (0.02–1.0 *M*) of ammonium formate or sodium chloride[3,4]. We have found that total removal of ammonium formate from

the bleomycin fractions is extremely difficult, if not impossible, without damage to the integrity of the antibiotic. We have used the volatile buffer triethylammonium bicarbonate to effect the separation of up to a gram of Blenoxane. The advantages and shortcomings of this method are discussed.

MATERIALS AND METHODS

CM-Sephadex C-25 was obtained from Pharmacia (Piscataway, N.J., U.S.A.). Blenoxane was kindly provided by Drs. S. T. Crooke and W. T. Bradner, Bristol Labs. Pure samples of bleomycin A_2 and bleomycin B_2 were obtained from the National Cancer Institute. Triethylamine was purified by distillation from P_2O_5 before use. All other chemicals used were of reagent grade. The purity of the bleomycins was monitored by thin-layer chromatography on Baker IB-F silica gel plates (J. T. Baker, Phillipsburg, N.J., U.S.A.) using 10% ammonium acetate–methanol (50:50) as solvent[3,5]. The material was visualized under UV light at 254 nm and the mobilities compared to those of bleomycin A_2 and bleomycin B_2 and to literature R_F values[5]. Column effluents were monitored at 292 nm using a Beckman Model 25 spectrophotometer.

Proton magnetic resonance (proton NMR) spectra were obtained on a Bruker HX-90 spectrometer operating in the pulse Fourier-transform mode.

Preparation of triethylammonium biacrbonate (TEAB)

A mixture of distilled water and enough triethylamine to make a 1 *M* solution is cooled to 4°. A stream of CO_2 is introduced into the mixture through a fritted gas inlet tube. If solid CO_2 (dry ice) is used, the system should contain a trap for the oil found in dry ice. After a few minutes the mixture becomes homogeneous and the pH begins to drop. CO_2 is added until the pH reaches approximately 7. The TEAB solution should be used as soon as possible after preparation; however, it may be stored in a screw cap container at 4°; prolonged storage leads to loss of CO_2 and a gradual increase in pH. The pH of all stored solutions should be checked before use. Dilutions of TEAB may be made with *cold* distilled water.

Preparation of column

CM-Sephadex C-25 is allowed to swell in distilled water overnight at room temperature and poured into a column (30 × 2.5 cm). All subsequent operations are carried out at 4°. The column is washed with dilute ($\approx$0.05 *M*) triethylamine until the eluent is basic (pH paper) (this alkaline wash is necessary to prevent the evolution of CO_2 in the column when TEAB is added). The column is washed with 0.5 *M* TEAB until the pH reaches 7 and then with distilled water until the pH reaches that of the inlet water. Finally, the column is equilibrated with 0.02 *M* TEAB just prior to use.

RESULTS AND DISCUSSION

A typical separation of Blenoxane on CM-Sephadex C-25 using a linear gradient (0.02–0.50 *M*) of TEAB is shown in Fig. 2a. The major fractions are labeled. The separation of a comparable amount of Blenoxane on the same size column using ammonium formate (0.02–0.80 *M*) is shown in Fig. 2b for comparison. The resolu-

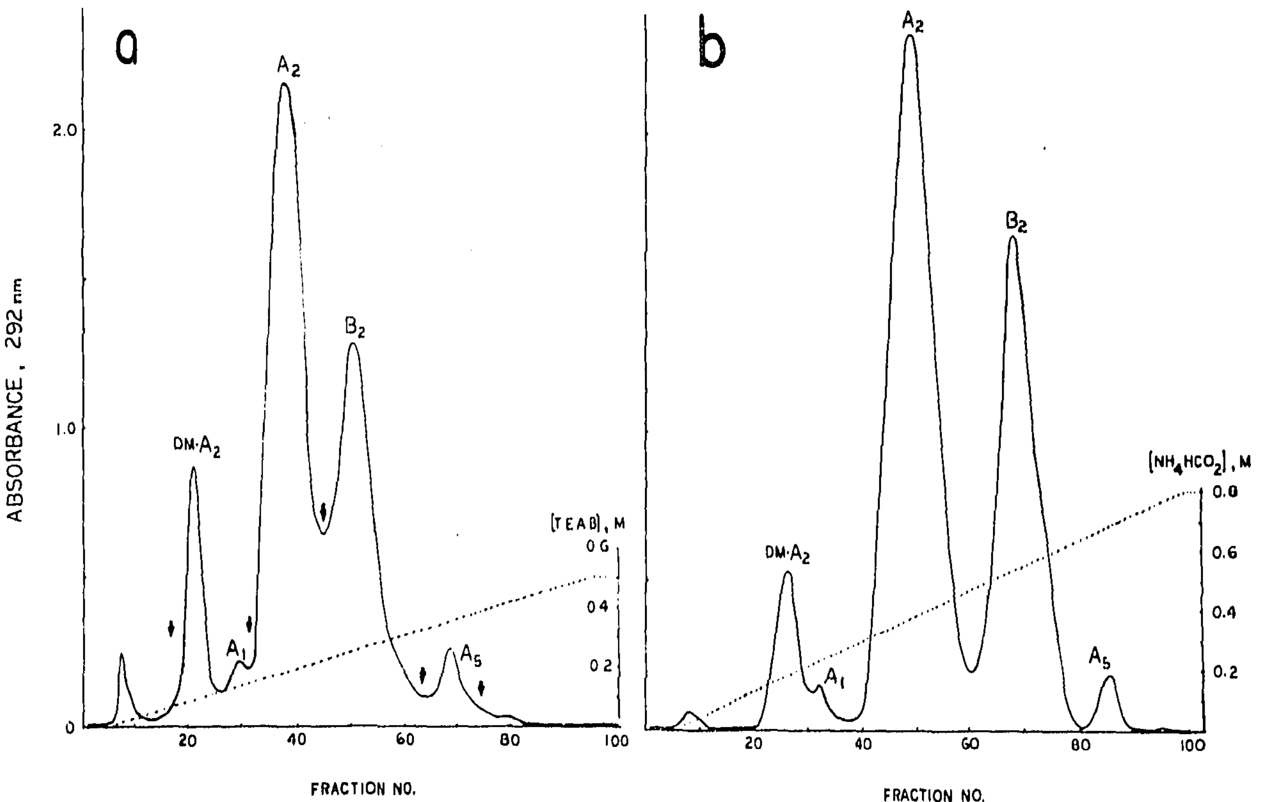

Fig. 2. (a) Elution profile of bleomycins following chromatography of Blenoxane (750 mg) on CM-Sephadex C-25 using a linear gradient (0.02 – 0.50 *M*, 1 liter total) of TEAB; column: 30 × 2.5 cm, flow-rate approximately 2 ml/min. Fractions of 10 ml were collected. Arrows indicate points at which major fractions were cut. DM-A_2: demethyl bleomycin A_2. (b) Elution profile for separation of Blenoxane (600 mg) on CM-Sephadex C-25 usin a lingear gradient (0.02 – 0.80 *M*, 1 liter total) of ammonium formate. Other conditions as in (a).

tion obtained with TEAB is less than that obtained with ammonium formate although the gradient used with TEAB is considerably more shallow. The poorer resolving power of TEAB is probably due to the higher pH at which the separation is made. Bleomycin has fewer positive charges at pH 7 (in TEAB) compared to pH 4.5 at which value the ammonium formate separations are made; however, the pH of TEAB cannot be taken very much below 7 to take advantage of the charge differences.

The advantage to the TEAB method lies in the easy removal of the buffer from the fractions after chromatography. Lyophilization from water (2 or 3 times) effects removal of nearly all the TEAB. This easy removal of buffer permits pooling of major peaks from valley to valley (Fig. 2a) and rechromatography in much less time than that required for the removal of buffer from ammonium formate fractions. Fig. 3 shows an example of a bleomycin A_2 fraction which has been rechromatographed using TEAB. The presence of a small amount of bleomycin B_2 is due to the slight overlap of the peaks in the initial separation and the fact that the peaks were taken from valley to valley. The small amounts of bleomycin A_1 and demethyl bleomycin A_2 are characteristic of the instability of bleomycin A_2 to demethylation and oxidation in solution[5,6]. The bleomycin A_2 fraction can be pooled and lyophilized to give samples which are >95–97% pure by thin-layer chromatography. Bleomycin B_2 can be purified to homogeneity (as determined by thin-layer chromatography) in the same manner.

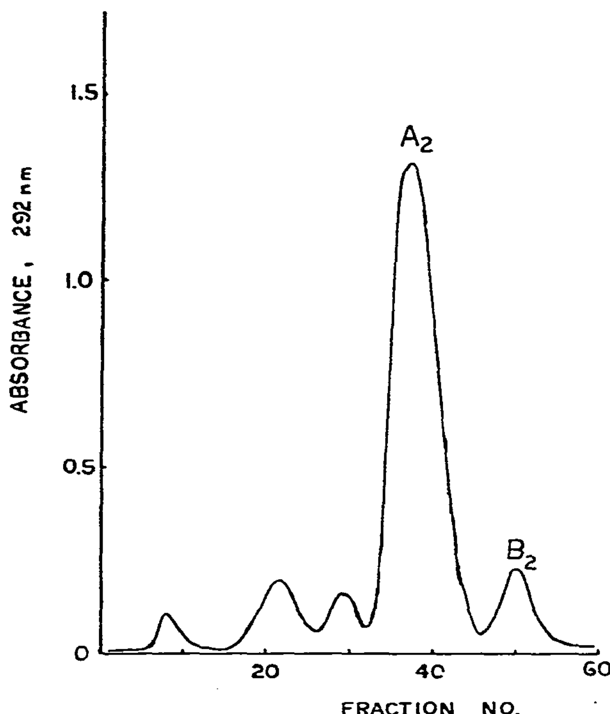

Fig. 3. Elution profile of bulk bleomycin A_2 fraction from Fig. 2a following re-chromatography under the same conditions as described in Fig. 2a.

Proton NMR spectra of samples of bleomycin A_2 and bleomycin B_2 after two or three lyophilizations from water indicated the presence of less than 1 mole of triethylamine per mole of bleomycin. This estimate is made by assigning a value of 1 proton to the integrated intensity of any of the four aromatic single proton resonances of bleomycin and comparing this value with one-fifteenth the integrated intensity of the triethylamine ethyl resonances[8]. Bleomycin A_2 and B_2 separated using the TEAB method retain all the UV, fluorescence[7] and NMR[8] spectral characteristics of bleomycins prepared by the ammonium formate method. In addition, the bleomycins separated using the TEAB method are fully active in their ability to degrade DNA as measured by the DNA–ethidium bromide fluorescence assay[9]. There is no evidence for the isomerization of the compounds via acyl migration which is known to occur in base[10].

The purified bleomycin bicarbonates can be converted to the desired anion form by titrating an aqueous solution of the bleomycin with slightly more than one equivalent of the conjugate acid of the anion desired. Bleomycin concentrations can be determined by UV spectroscopy using a molar absorptivity of $1.5 \cdot 10^4$ M^{-1} cm^{-1}. In the case of volatile acids such as HCl, the solution may be evaporated directly to give the bleomycin as the chloride salt. With less volatile acids such as H_2SO_4 or H_3PO_4, gel filtration of the solution on Sephadex G-10 using water permits ready separation of the bleomycin salt (in the void volume) from excess acid. In addition, this step removes any residual triethylamine.

The methods described here should be applicable to the separation and purification of any ionic compounds which might exhibit sensitivity to separation and/or desalting procedures.

ACKNOWLEDGEMENTS

We thank Drs. S. T. Crooke and W. T. Bradner of Bristol Laboratories for supplying Blenoxane and Dr. Harry B. Wood, Jr. of the National Cancer Institute for samples of pure bleomycin A_2 and bleomycin B_2. This work was supported by USPHS Grant CA-24411 (to J. D. Glickson and T.T.S.). We acknowledge the assistance of the Cancer Center NMR Core Facility which is supported by USPHS Grant CA-13148 (to J. R. Durant and J. D. Glickson).

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