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Note

Reversed-phase high-performance liquid chromatography of bleomycin

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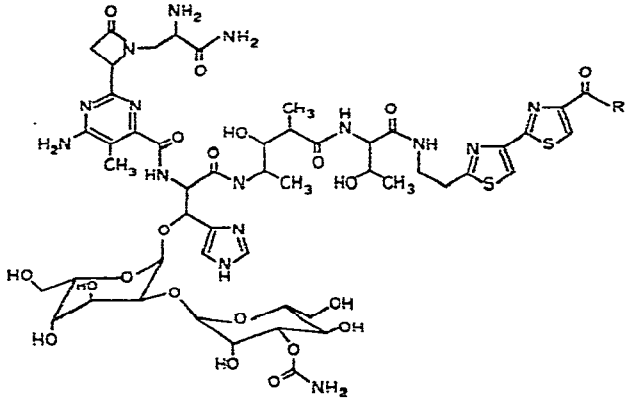
The bleomycin complex (Fig. 1) produced by *Streptomyces verticillus* (ATCC 15003) is a mixture of closely related, water-soluble basic glycopeptide antibiotics which differ only in the limited area of their terminal amine[1,2]. The structures of the two major components A_2 and B_2 and nine minor components in the natural complex



Bleomycin	*R*	*% in complex*[2]
A_1	$NH-CH_2-CH_2-CH_2-SO-CH_3$	9.3
BM-A_2	$NH-CH_2-CH_2-CH_2-S-CH_3$	2.4
A_2	$NH-CH_2-CH_2-CH_2-\overset{+}{S}=(CH_3)_2$	54.5
A_2-a	$NH-CH_2-CH_2-CH_2-CH_2-NH_2$	3.6
A_2-b	$NH-CH_2-CH_2-CH_2-NH_2$	
A_2-c	$NH-CH_2-CH_2-$ (imidazole)	
B_1	NH_2	trace
B_2	$NH-(CH_2)_4-NH-C(=NH)-NH_2$	26.7
B_4	$NH-(CH_2)_4-NH-C(=NH)-NH-(CH_2)_4-NH-C(=NH)-NH_2$	2.4
A_5	$NH-(CH_2)_3-NH-(CH_2)_4-NH_2$	1.3
A_6	$NH-(CH_2)_3-NH-(CH_2)_2-NH-(CH_2)_3-NH_2$	trace

Fig. 1. Structure of bleomycin.

have been identified[3,4]. The antitumor and chelating properties of the bleomycin complex have been investigated and utilized for therapeutic[5,6] and diagnostic[7,8] purposes.

The first reported separation of the bleomycin complex in its copper form was achieved by ion-exchange chromatography on CM-Sephadex C-25[9] using a linear gradient of ammonium formate (0.05–1.0 *M*). This provided a rough separation of major components[3]. The repeated chromatography of the coarse fractions in different systems like pyridine–acetate buffer on Dowex 50W-X4 or sodium bicarbonate on CM-Sephadex C-25 was required to obtain pure fractions. In our attempt to evaluate the tumor uptake of ^{57}Co-labeled individual bleomycins[10] we have successfully employed high-performance liquid chromatography (HPLC) of copper-free bleomycin using a silica gel packing (Porasil A or Partisil 20), 0.3% ammonium formate–methanol (1:1) as the mobile phase, and a flow gradient (Fig. 2).

Successful applications of the reversed-phase HPLC for the separation of various antibiotics have been reported[11–13]. This communication describes the satisfactory use of a reversed-phase system in the separation of the bleomycin complex.

EXPERIMENTAL

Apparatus

A Waters Associates high-pressure liquid chromatograph Model ALC 202/6000 was used throughout this study. The additional equipment consisted of a second Type M600A pump with a triple inlet manifold, a Type 660 solvent programmer, and a Type U6K septumless injector. The chromatograph was equipped with a UV detector operating at 254 mm. Narrow-bore analytical columns with an I.D. of 2.3 mm were dry packed with Bondapak C_{18}/Corasil and Bondapak Phenyl/Corasil by adding small amounts of packing at a time and gently tapping. Bondapak C_{18}/Porasil B and μBondapak C_{18} columns were custom-packed by Waters Associates.

Materials and methods

Sterile bleomycin sulfate (Blenoxane) and bleomycin A_2 sulfate were prepared in water solutions (1%) and stored at +4° prior to use in sealed serum vials.

Ammonium formate (Certified; Fisher Scientific, Pittsburgh, Pa., U.S.A.), methanol (Spectranalyzed; Fisher Scientific) and freshly distilled water were used for the preparation of the mobile phases.

The following columns were used: (i) 1830 mm × 2.3 mm I.D., Bondapak C_{18}/Corasil; (ii) 1830 mm × 2.3 mm I.D., Bondapak Phenyl/Corasil; (iii) 1220 mm × 9.5 mm I.D., Bondapak C_{18}/Porasil B; (iv) 300 mm × 4.6 mm I.D., μBondapak C_{18}.

After use the columns were flushed with distilled water and stored filled with methanol.

RESULTS AND DISCUSSION

Of the four column packings investigated, three (i–iii) provided a separation pattern similar to that obtained with the silica gel type of support. This is probably due to insufficient coating of the silica with the nonpolar monolayer. Only μBondapak C_{18} packing performed as a true reversed-phase packing.

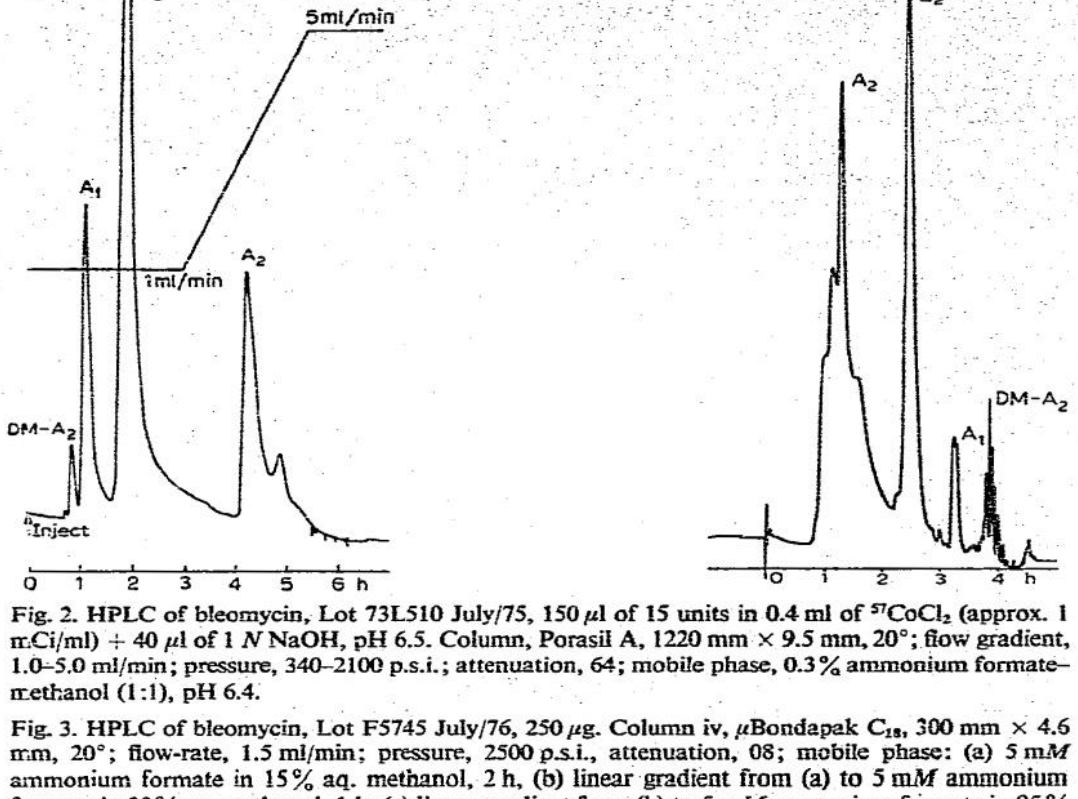


Fig. 2. HPLC of bleomycin, Lot 73L510 July/75, 150 μl of 15 units in 0.4 ml of $^{57}CoCl_2$ (approx. 1 mCi/ml) + 40 μl of 1 *N* NaOH, pH 6.5. Column, Porasil A, 1220 mm × 9.5 mm, 20°; flow gradient, 1.0–5.0 ml/min; pressure, 340–2100 p.s.i.; attenuation, 64; mobile phase, 0.3% ammonium formate–methanol (1:1), pH 6.4.

Fig. 3. HPLC of bleomycin, Lot F5745 July/76, 250 μg. Column iv, μBondapak C_{18}, 300 mm × 4.6 mm, 20°; flow-rate, 1.5 ml/min; pressure, 2500 p.s.i., attenuation, 08; mobile phase: (a) 5 m*M* ammonium formate in 15% aq. methanol, 2 h, (b) linear gradient from (a) to 5 m*M* ammonium formate in 30% aq. methanol, 1 h, (c) linear gradient from (b) to 5 m*M* ammonium formate in 95% aq. methanol, 1.5 h.

The presence of ammonium formate in the mobile phase is critical for the elution and resolution of bleomycin complex. Ammonium formate may act as an ionization suppressant and increase the polarity of the mobile phase. At the same time a high concentration of ammonium formate makes the recovery of the collected fractions difficult and, like high temperature and elevated or lowered pH, may lead to alteration or destruction of the antibiotic[10]. All our separations were performed at pH 6.4 ± 0.1.

The separations displayed (Fig. 2) were obtained at 5×10^{-3} *M* concentration of ammonium ion, the lowest possible concentration at which elution and separation are achieved. The only difficulty we have encountered is the behavior of bleomycin A_2, which, because of its structure (Fig. 1), is permanently in a cationic form and whose ionization cannot be suppressed by the presence of ammonium ions.

Contrary to conflicting reports, the manipulation of the methanol concentration in the mobile phase greatly enhances the resolution and allows the separation of a basic antibiotic complex by a reversed-phase technique[14]. The major fractions were collected, freeze-dried with simultaneous removal of volatile ammonium formate, and identified by their corresponding values on silica gel thin-layer chromatography[9].

The reported technique represents the most rapid method thus far reported for the complete separation of individual bleomycin components from the natural complex mixture without inducing structure damage or alteration.

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