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Note

Modification of a new high-performance liquid chromatographic method for bleomycin to separate epi-, iso-, desamido-, and unmodified analogues

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This laboratory has recently developed a procedure for the separation and quantitation of many congeners of the antitumor antibiotic bleomycin. The method utilizes a linear water—methanol gradient and pentanesulfonic acid as an ion-pairing reagent [1]. We have since been successful in chemically producing definitive quantities of the epi- and iso-conformers of the common bleomycins. This communication reports a modification of our previous procedure which allows separation, identification, and quantification of epi-, iso-, desamido-, and unmodified bleomycin congeners within the same chromatogram .

MATERIALS AND METHODS

As in the previous study [1], bleomycin (as Blenoxane, lot FOX04, expiration date September, 1981) was obtained through Drs. William Bradner and Stanley Crooke as a gift from Bristol Labs. (Syracuse, NY, U.S.A.). This one lot was used throughout these studies. Milli-Q-purified (Millipore, Bedford, MA, U.S.A.) water was used throughout, as was methanol purchased from Fisher Scientific (Springfield, NJ, U.S.A.). Pentanesulfonic acid, sodium salt, was obtained from Aldrich (Milwaukee, WI, U.S.A.), while the heptanesulfonic acid salt and all other reagents were from Fisher. The structures of all modified and unmodified bleomycins are as previously given [1].

The chromatography system was as described [1]. Solvent A (water) and solvent B (methanol) each contained 5 m*M* of either the sodium salt of pentane- or heptanesulfonic acid and 0.5% glacial acetic acid. The pH of solvent A was adjusted to 4.3 with concentrated ammonium hydroxide, while solvent

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B was used without further additions. All solvents were passed through a 0.2-μm filter and sonicated for 15 min at the beginning of each day. Detection and quantification were, as before, performed using absorbance at 280 nm.

The standard procedure in our previous work [1] was a linear gradient of 28—48% solvent B in solvent A (pentanesulfonic acid) run over 45 min at a flow-rate of 1.5 ml/min through a 300 × 3.9 mm μBondapak C_{18} (Waters) column (10 μm particle size) preceded by a 23 × 3.9 mm Corasil C_{18}-filled guard column (30—38 μm particle size). In this modification, the procedure consists of a 40—50% solvent B in solvent A (heptanesulfonic acid) linear gradient run over 30 min at a flow-rate of 0.7 ml/min through a 150 × 3.9 mm Novapak C_{18} (Waters) column (4 μm particle size) preceded by the same guard column, with a resultant pressure of about 2.2 MPa (3000 p.s.i.).

The desamido-bleomycin analogues were produced using bleomycin hydrolase isolated from mouse liver by the previously described [1] modification of the method of Yoshioka et al. [2]. Epi-bleomycins [3] and iso-conformers [4] were formed exactly as described. Briefly, 100 μl of HPLC-grade water and 200 μl of 2% triethylamine were added to sterile vials (15 units each, or about 9 mg) of Blenoxane lot FOX04. One vial was allowed to react at room temperature for five days to allow carbamoyl migration from the 3-O to the 2-O position of the mannose sugar for iso-conformer formation [4]. To a second vial, 6 μl of 1.0 *M* copper sulfate were added and the vial was heated to 70°C for 6 h to form the epimers [3]. As before, the Cu(II) chelates were always chromatographed.

RESULTS AND DISCUSSION

As can be observed in Fig. 1, we have now been able to form definitive amounts of the epi- and iso-conformers of the bleomycins, but iso-bleomycins could not be separated from their respective parents with any modification of our previously described [1] procedure using pentanesulfonic acid as the ion pairing reagent. In addition, both iso- (Fig. 1B) and desamido- (data not shown) bleomycins eluted just prior to the unmodified drug. We had already performed preliminary experiments to adapt our procedure to the smaller, more efficient and economical Novapak C_{18} column. Using this column, we were also unsuccessful in separating iso- and desamido-congeners with pentanesulfonic acid, although we have been able to achieve excellent separation of the parent congeners, as before, using this salt and a linear 24—48% methanol gradient with a flow-rate of 0.6 ml/min over 35 min. This modification thus allows a saving of 10 min between runs, as well as 60% solvent reduction.

We than attempted to effect separation of these conformers, using the Novapak column and heptanesulfonic acid as the paired ion, additionally hoping to distinguish the desamido-analogues. This resulted in the development of the conditions indicated in Fig. 2. That is, a shallow gradient of 40—50% methanol with 5 m*M* heptanesulfonic acid, pH 4.3, was begun at time zero and run over 30 min. As can be seen in Fig. 2, these conditions afforded separation of the iso- and epi-conformers from their respective unmodified bleomycins. Epimerization (*R*, instead of *S*, at the α-methine carbon of the pyrimidine moiety) has drastically affected retention (Fig. 2C) of all the bleomycin con-

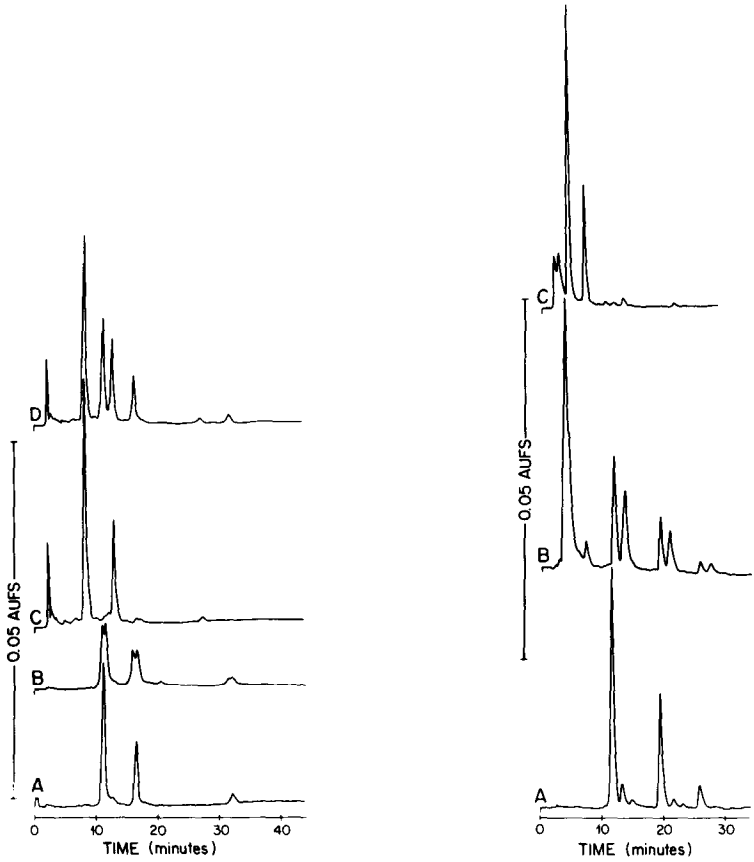

Fig. 1. Standard procedure [1] for the separation of bleomycin congeners using a 300 × 3.9 mm μBondapak C_{18} with 5 m*M* pentanesulfonic acid, as described in Materials and methods. (A) Blenoxane lot FOX04; order of elution: A_2 (about 12 min), B_2 (17 min), and DM-A_2 (33 min); (B) about 50% conversion of FOX04 to the iso-conformer; the first peak in each doublet is the respective iso-bleomycin; (C) essentially 100% conversion to the epi-conformers; order of elution: epi-A_2 (about 8.5 min), epi-B_2 (13.5 min), and epi-DM-A_2 (28 min); (D) a mixture of epi- and unmodified Blenoxane lot FOX04 (70:30 mol ratio). AUFS = absorbance units full scale.

Fig. 2. Modified procedure for detecting and separating epi-, iso-, and unmodified conformers using a 150 × 3.9 mm Novapak C_{18} with 5 m*M* heptanesulfonic acid, as described in Materials and methods. (A) Blenoxane lot FOX04, the order of elution is the same as in Fig. 1; (B) about 50% conversion of this lot to the iso-conformers, which elute after the ummodified congeners (at about 14, 21.5, and 28 min for iso-A_2, B_2 and DM-A_2, respectively); (C) essentially 100% conversion of FOX04 to the epi-conformers (4.5, 7.5, and 22.5 min for epi-A_2, B_2, and DM-A_2, respectively). AUFS = absorbance units full scale.

geners. These epimers are reported to have equal or greater in vitro DNA cleaving ability than the respective parent drug [5], although their antibacterial activity and cytotoxicity are reduced [3, 5]. These phenomena could all be due to more efficient metal binding, although this has not been studied.

On the other hand, iso-bleomycins are retained slightly longer than the umodified drugs (Fig. 2B), induce significantly less DNA strand scission in vitro [5], but retain considerable antimicrobial activity [4, 5]. These data are consis-

tent with a reduced metal affinity in the iso-bleomycins as well as the involvement of the carbamoyl function in metal coordination [6].

Apparently, removing the amide from the β-amino-L-alanine amide N-terminus affects molecular conformation and metal binding similar to epimerization in this region, although to a lesser degree. This can be seen in Fig. 3D, as well as in our previous work [1]. The desamido modification reported greatly reduces the drug's activity with respect to both its DNA cleaving and cytotoxic effects [2].

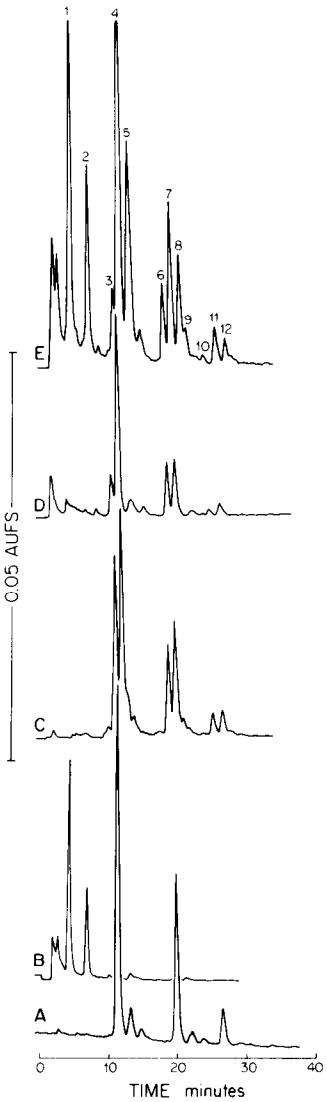

Fig. 3. Modified HPLC procedure as indicated in Fig. 2. (A) Blenoxane lot FOX04; (B) epi-FOX04; (C) 60% iso-FOX04 with unmodified conformers; (D) various proportions of the desamido-analogues eluting just prior to the parent bleomycin; and (E) a mixture of epi-, iso-, desamido-, and unmodified bleomycins identified as follows: (1) epi-A_2, (2) epi-B_2, (3) desamido-A_2, (4) A_2, (5) iso-A_2, (6) desamido-B_2, (7) B_2, (8) iso-B_2, (9) epi-DM-A_2, (10) desamido-DM-A_2, (11) DM-A_2, and (12) iso-DM-A_2. AUFS = absorbance units full scale.

A powerful example of the separation capacity of this technique is shown in Fig. 3E, in which a mixture of epi-, iso-, desamido-, and unmodified drug (Blenoxane lot FOX04) is dramatically separated in a single run. The authors know of no other published HPLC method which has this capability.

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