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Note

Separation of bleomycins and their deamido metabolites by high-performance cation-exchange chromatography

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Bleomycin (BLM), a mixture of basic glycopeptides isolated from *Streptomyces verticillus*, is widely used as an antitumor antibiotic in the treatment of testicular carcinomas and lymphomas[1]. The clinical BLM mixture (Blenoxane) contains at least eleven different BLMs, although the two principal components are BLM A_2 (55–70%) and BLM B_2 (25–30%) (Fig. 1). It has been suggested that *in vivo* antitumor activity of these two compounds as well as their toxicity to non-malignant tissues is limited by the enzyme bleomycin hydrolase, which we have recently purified to homogeneity and characterized by fast protein liquid chromatography (FPLC)[2,3]. This enzyme generates the inactive metabolites, deamido BLM A_2 (BLM dA_2) and deamido BLM B_2 (BLM dB_2) from BLM A_2 and BLM B_2, respectively[4–7]. Characterization of these BLM metabolites has been greatly restricted due to the lack of a general method for their isolation in pure forms. Furthermore, the conventional method for isolation of components of BLM mixture is CM-Sephadex column chromatography, which is both laborious and time consuming[8–10]. In this paper, we describe the use of FPLC for the rapid separation of BLM dA_2 from BLM A_2 and BLM dB_2 from BLM B_2 as well as for the separation of BLM A_2 and BLM B_2 from the clinical BLM mixture.

EXPERIMENTAL

Reagents and materials

Clinical BLM mixture (Blenoxane) was a gift from Bristol-Myers Squibb (Wallingford, CT, U.S.A.). BLM A_2 and BLM B_2 were purified as previously described[6,11]. Enzymatically-generated, authentic BLM dA_2 and BLM dB_2 standards were gifts from Nippon Kayaku (Tokyo, Japan). Additional BLM dA_2 and BLM dB_2 were enzymatically prepared as described previously[6]. Ammonium formate was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Chromatographic equipment and conditions

The FPLC system (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) used in the present studies consisted of an LCC-500 gradient programmer controlling

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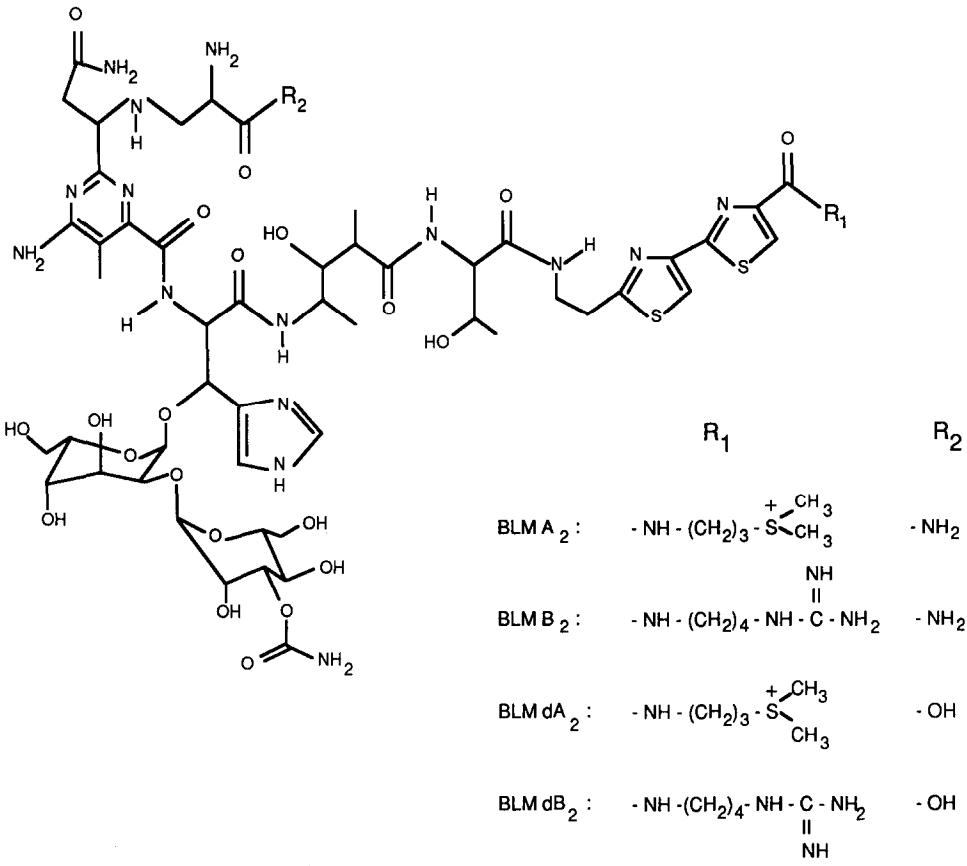

Fig. 1. Structures of major BLM components and their deamido metabolites.

two P-500 reciprocating pumps, a mixing chamber, an MV-7 injector valve for introduction of sample, a Mono S HR 5/5 cation-exchange column (5 cm × 5 mm I.D., 10 μm particle size, 25 000 theoretical plates/m) and a 280-nm fixed-wavelength single-path UV monitor coupled to a single-channel Kipp & Zonen (The Netherlands) pen recorder. Fractions were collected with a FRAC-100 fraction collector (Pharmacia). The eluting buffers were: buffer A, 0.05 *M* ammonium formate (pH 6.5); buffer B, 1.0 *M* ammonium formate (pH 6.5). Appropriate gradients and flow-rates were chosen for each separation. For each separation method used, reproducible elution profiles were obtained. All chromatographic separations were carried out at 4°C. Buffer solutions were filtered through a 0.2-μm filter and degassed prior to use. All samples were also filtered before injecting onto the column.

RESULTS AND DISCUSSION

The use of ion-exchange chromatography as a modern biochemical separation technique has made it possible to isolate and characterize many peptides and proteins

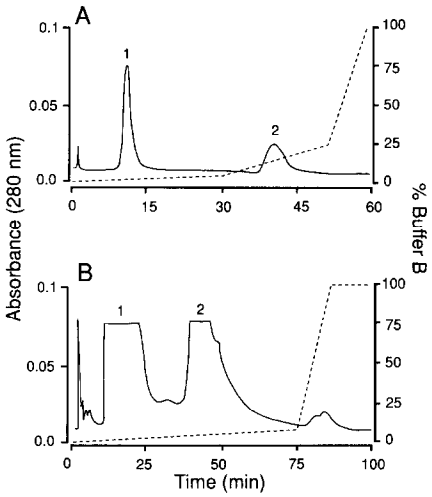

Fig. 2. Mono S (HR 5/5) cation-exchange chromatographic separation of (A) BLM A_2 and BLM B_2 from 10 μg of a clinical BLM mixture; elution was carried out with a gradient of 2–5% buffer B over 30 min, 5–25% buffer B from 30–50 min and 25–100% buffer B from 50–60 min; flow-rate = 1.0 ml/min; (B) BLM A_2, BLM B_2 and other minor BLM components from 1 mg of a clinical BLM mixture using gradients of 0–10% buffer B over 75 min and 10–100% buffer B from 75–85 min and a flow-rate of 1.0 ml/min. 1 = BLM A_2; 2 = BLM B_2. Solid lines, absorbance; dashed lines, percentage buffer B.

of biological importance. The antineoplastic agent BLM can be separated into its glycopeptide components, using CM-Sephadex chromatography[8–10]. Unfortunately, when conventional cellulosic ion-exchangers are used, the separation process is very lengthy. Polymer-based resins such as Mono S, Mono Q and Mono P have been used in place of conventional ion exchangers or chromatofocussing materials, because they possess high mechanical strength, are homogeneous in size and are stable over a wide pH range. Thus, we have examined the utility of Mono S cation-exchange resin to separate BLM components because of its superior resolving power and speed. In addition, we have used this methodology to address the more difficult problem of separating the known BLM metabolites.

Fig. 2A shows a gradient elution profile after cation-exchange chromatographic separation of BLM A_2 and BLM B_2 from clinical BLM mixture (Blenoxane). The clinical BLM (10 μg) was adsorbed to the cation-exchange column in the starting buffer (buffer A) and elution was carried out at a flow-rate of 1.0 ml/min, with a gradient of 2–5% buffer B over 30 min, 5–25% buffer B from 30–50 min and 25–100% buffer B from 50–60 min. As illustrated in Fig. 2A, BLM A_2 eluted first (retention time *ca.* 11 min). Due to strong electrostatic interactions between the basic guanidinium moiety of BLM B_2 and the strongly acidic SO_3^- groups on the Mono S beads, BLM B_2 eluted very slowly (retention time *ca* 40 min).

We have successfully extended the use of the analytical (HR 5/5) Mono S column to separate individual BLM components from quantities as large as 17.6 mg of the clinical BLM mixture. Fig. 2B illustrates the appearance and separation of other minor BLM components apart from BLM A_2 and BLM B_2, when 1 mg of BLM mixture

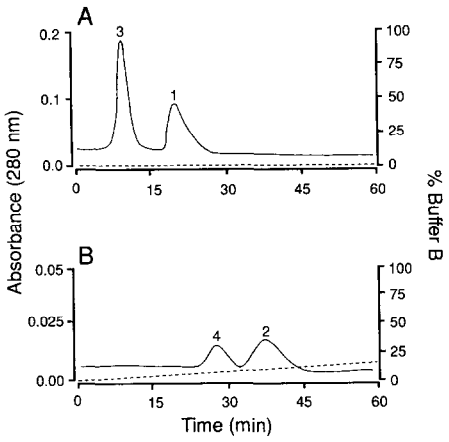

Fig. 3. Mono S (HR 5/5) cation-exchange chromatographic separation of the deamido BLM metabolites. (A) Separation of BLM dA_2 and BLM A_2, using buffer A and a flow-rate of 0.9 ml/min; (B) separation of BLM dB_2 and BLM B_2, using a gradient of 0–15% buffer B over 60 min and a flow-rate of 1.0 ml/min. 1 = BLM A_2; 2 = BLM B_2; 3 = BLM dA_2; 4 = BLM dB_2. Solid lines, absorbance; dashed lines, percentage buffer B.

(Blenoxane) was loaded onto the column. Elution was carried out at a flow-rate of 1.0 ml/min, with a linear gradient of 0–10% buffer B over 75 min followed by a gradient of 10–100% buffer B at the end of 85 min. Various fractions containing BLM A_2 and BLM B_2 were collected and lyophilized. The amount of BLM A_2 and BLM B_2 obtained was 0.55 mg and 0.235 mg, respectively, (based on absorbance at λ_{max} = 292 nm); this is in good agreement with previously calculated ratios of BLM A_2 and BLM B_2 found in the clinical mixture of BLM[1,10]. Assuming that *ca.* 90% of the mixture consisted of BLM A_2 and BLM B_2, the combined recovery of the two components was 87%. BLM A_2 and BLM B_2 from the clinical mixture eluted at the same relative positions as authentic BLM A_2 and BLM B_2 standards. The identity of BLM A_2 and BLM B_2 that eluted from the Mono S column was further confirmed by analysing the corresponding fractions by reversed-phase HPLC as described previously[12].

We then used Mono S column chromatography to separate the BLMs from their deamido metabolites, which were generated by the enzyme bleomycin hydrolase. A mixture of BLM A_2 and BLM dA_2 (total 25 μg) was injected onto a Mono S column and eluted at a flow-rate of 0.9 ml/min with 0.05 *M* ammonium formate. As shown in Fig. 3A, BLM dA_2 eluted first (retention time *ca.* 9 min) followed by BLM A_2 (retention time *ca.* 20 min). The metabolic cleavage of the amide bond in the β-aminoalanine moiety of BLM A_2 by the enzyme bleomycin hydrolase, produces BLM dA_2, a molecule with a lesser positive charge than the parent BLM A_2. Therefore, BLM dA_2 binds less to the Mono S column and elutes first. The identity of BLM dA_2 was also confirmed by reversed-phase HPLC as described previously[12]. Recovery of the two compounds was 90%.

Similar results were obtained with a mixture of BLM B_2 and its inactive metabolite BLM dB_2. Fig. 3B demonstrates a Mono S cation-exchange chromatographic separation of a mixture of BLM dB_2 and BLM B_2 (total 7 μg). A flow-rate of 1.0 ml/min and a linear gradient of 0–15% buffer B over 60 min were found to be

optimum for the separation since a steeper gradient did not resolve the compounds and a slower gradient resulted in very wide peaks on the chromatogram. Retention times of BLM dB_2 and BLM B_2 were found to be *ca.* 28 min and *ca.* 38 min, respectively. Confirmation of the identity for the separated components was provided by comparison with the migration of the authentic standards.

The chromatographic method described in this report can have broader biological applications beyond the separation of the BLMs. For example, a number of endogeneous bioactive peptides such as enkephalins, substance P and thyrotropin-releasing hormone contain terminal carboxamide moieties, which are essential for their biological activity[13]. Separation of the deamidated peptides from the carboxamide-containing peptides can be problematic. Therefore, it is possible that the Mono S/FPLC system could be generally useful for separating inactive deamido metabolites from other bioactive peptides that contain the protective carboxamide moieties[13].

In conclusion, the Mono S/FPLC provides a rapid method to separate individual components of BLM. The Mono S column chromatography also allows for the rapid separation of the deamido metabolite of BLM from the parent compound. Although we have separated relatively small amounts of BLM dA_2 and BLM dB_2 due to their limited availability, the existence of preparative columns should permit even greater amounts to be isolated. In contrast to ion-pair reversed-phase HPLC methods, the FPLC approach provides for a BLM that is free of contaminating organic acids or ion-pair agent, which are difficult to remove[14–16]. The use of ammonium formate as a buffer system in the FPLC method is advantageous because the salt can be easily removed from the separated compounds upon lyophilization. When BLM is complexed to Cu(II) the resolution is improved further. This versatile method should now allow for a more detailed characterization of the metabolites of an important class of biologically active glycopeptides.

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