Journal of Chromatography, 310 (1984) 361—371
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2215

REVERSED-PHASE PAIRED-ION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SEPARATION AND QUANTIFICATION OF MULTIPLE BLEOMYCIN CONGENERS

ROBERT P. KLETT, JAMES P. CHOVAN* and ILENE H.R. DANSE*

Department of Pharmacological Sciences, State University of New York at Stony Brook, Health Sciences Center, BHS T-8, Stony Brook, NY 11794 (U.S.A.)

(First received December 14th, 1983; revised manuscript received June 4th, 1984)

SUMMARY

A rapid, linear gradient chromatographic technique for separating and quantifying copper(II)-chelated bleomycin congeners is described. This method is also capable of (1) separating divalent from trivalent metal chelates; (2) determining the purity of many chemically modified bleomycins; and (3) assaying bleomycin hydrolase activity on complex mixtures. Quantification at 280 nm is sensitive to 50 pmol and is linear at least up to 10 nmol per injection.

INTRODUCTION

The phleomycin (PHLM) and bleomycin (BLM) groups of antitumor antibiotics were originally isolated by Maeda et al. [1] and Umezawa et al. [2]. These atypical glycopeptides (Fig. 1) are known to chelate both divalent and trivalent transition metal ions [3]. Although isolated from their respective *Streptomyces* cultures as the Cu(II) complexes, the most active form in vitro is Fe(II)—BLM, which rapidly degrades DNA producing both single and double strand breaks and releasing free bases and the recently described base propenals [4]. Several other radioactive metal chelates have been formulated for use as tumor imaging agents, the ^{57}Co complex having been studied most [5].

Both BLM and PHLM are isolated as mixtures of congeners, differing only by the structure of a C-terminal group (see Fig. 1 and Table I). In fact, most of the experimentation on these antibiotics has utilized mixtures, since pure

*Present address: Environmental Health Associates, Inc., 520 Third Street, Suite 208, Oakland, CA 94607, U.S.A.

0378-4347/84/$03.00

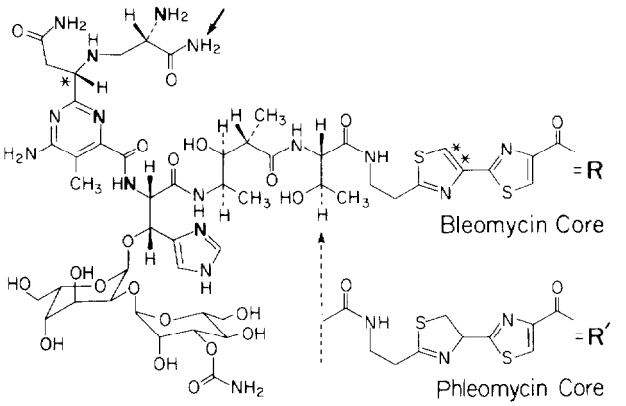


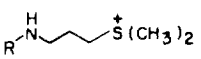
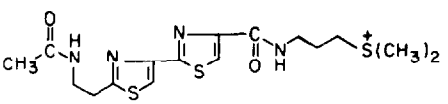
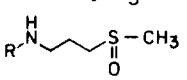
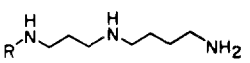
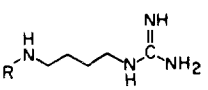
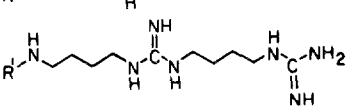
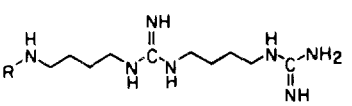
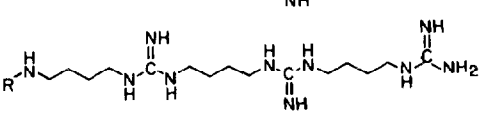
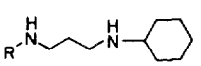
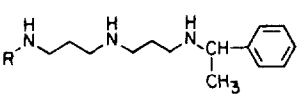
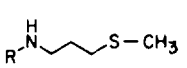
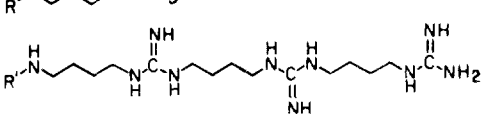
Fig. 1. The structures of the bleomycin (R) and phleomycin (R') cores. BLMs differ by the specific group attached to the thiazole carboxylate (see Table I), usually with a peptide bond, although esters have also been synthesized. The PHLM core differs from BLM only by reduction of the double bond at the paired asterisks [6]. The single asterisk indicates an *R*-configuration; epi-BLMs have an *S*-configuration at this site [7]. The darkened nitrogens are the proposed [3] sites of metal coordination. The arrow points to the amide group removed by BLM hydrolase [8], resulting in a desamido-BLM congener. Iso-BLMs have 2-O-carbamoyl-mannose sugars, rather than the 3-O-derivative shown [9].

congeners are not readily available. The clinically used BLM is regulated [10] to be 60—70% BLM A_2, 25—32% BLM B_2, and less than 10% of other bleomycins. This mixture is said to have a higher therapeutic index than the individually pure substances, many of which appear to have unacceptable levels of pulmonary toxicity and/or antitumor activity [11]. Many new analogues have been prepared semisynthetically in an attempt to improve anticancer activity while reducing pulmonary side-effects [12]. These studies have shown that the BLMs vary widely in all of their biological activities, including DNA cleaving ability, antitumor activity, and systemic side-effects. We have found that the ability to induce pulmonary fibrosis is directly related to the structure of the terminal moiety [13—16]. Although it is presumed that, with regard to this effect, the distinction between congeners is expressed at the level of DNA [17], only one study on uptake and intracellular distribution has been reported [18]; this utilized only BLM A_2, and recoverable radioactivity was minimal. Thus, the search for new bleomycins has focused on the C-terminal moiety, without truly knowing the intracellular site of action.

In our studies of the cellular mechanism of cytotoxicity, we proposed to synthesize radiolabeled congeners of high specific activity. Thus we required a rapid and highly sensitive method of analysis. Unfortunately, published high-performance liquid chromatographic (HPLC) methods proved too cumbersome, not specific enough, or irreproducible [19—25]. Therefore, we have now developed a method for the separation and quantification of BLM congeners, which remedies these difficulties and which also is capable of separating the PHLMs. This is the subject of this communication.

TABLE I

CAPACITY FACTORS (k') OF SOME BLEOMYCIN AND PHLEOMYCIN CONGENERS

Bleomycin or phleomycin congener	Terminal structure	k'
Bleomycin or phleomycinic acid	R–OH, R'–OH	1.0
BLM B_1'	$R\text{-}NH_2$	2.9
BLM A_2		3.5
BLM A_2-BT		3.7
BLM A_1		4.2
BLM A_5		4.3
BLM B_2		6.1
PHLM E		6.9
BLM B_4		8.0
BLM B_6		9.2
BLM CHP		10.4
BLM PEPP		11.8
BLM DM-A_2		12.7
PHLM G		13.4

EXPERIMENTAL

Materials

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.). A single lot was used throughout these studies. Additional gifts from Bristol included BLMs A_2, B_2, A_1, and A_5. The BLM A_2-bithiazole C-terminus [BLM A_2-BT; 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-(3-dimethylsulfonio)-propylcarboxamide] was chemically synthesized by Mr. Mark Levin and Dr. Sidney Hecht of the Department of Chemistry, University of Virginia (Charlottesville, VA, U.S.A.). Dr. Hecht also kindly supplied bleomycinic acid. PHLMs E and G, as well as BLMs B_4, B_6, CHP, and PEPP, were gifts from Drs. Noel K. Hart and Alan Lane (CSIRO, Australia). BLM B'_1 was provided by Dr. Tomohisa Takita of the Institute of Microbial Chemistry (Tokyo, Japan). (See Fig. 1 and Table I for the structures of these compounds.) None of the above standards, except BLMs B'_1 and A_2-BT (and BLMs A_2 and B_2 prepared by ion-exchange chromatography [23] in our laboratory), were greater than 90% pure as judged by the HPLC procedures described below. The remaining congeners presented herein were 70—85% pure, and the major peak was taken to be the authentic BLM or PHLM.

HPLC-grade water and methanol were obtained through Fisher Scientific (Springfield, NJ, U.S.A.) or Milli-Q-purified (Millipore) water was used. Pentanesulfonic acid, sodium salt, was purchased from Aldrich (Milwaukee, WI, U.S.A.). Ultrapure indium chloride was obtained from Alfa Products (Danvers, MA, U.S.A.), and all other reagents were purchased from Fisher.

Chromatographic equipment and technique

A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatography system was utilized. This consisted of U6K injector, 440 dual-channel UV detector, 660 solvent programmer, 730 data module, M6000A and M-45 pumps, and a 300 × 3.9 mm μBondapak C_{18} column (10-μm particle size) preceded by a 23 × 3.9 mm guard column packed with C_{18} Corasil (particle size, 30—38 μm).

Solvent A (water) and solvent B (methanol) each contained 5.0 m*M* pentanesulfonic acid, sodium salt, and 0.5% (v/v) glacial acetic acid. The pH of solvent A was adjusted to 4.3 with concentrated ammonium hydroxide, while solvent B was used without further additions. Each was passed through a 0.2-μm filter and sonicated for 15 min at the beginning of every day.

The standard procedure was a linear gradient of 28—48% solvent B in solvent A run over 45 min at a flow-rate of 1.5 ml/min with a resulting pressure development of about 1.6 MPa (2200 p.s.i.). Detection was at 280 nm, and all chromatograms shown herein were obtained under these conditions. Except for BLM A_2-BT and as indicated, copper sulfate was added in equimolar amounts or in slight excess.

Bleomycin quantification

Stock solutions of known weights of Cu(II)-chelated BLM A_2 or B'_1 (greater than 98% pure as judged by the HPLC procedure outlined above) were prepared in HPLC-grade water, and the absorbance at 292 nm of a diluted sample was determined on a Zeiss MQ3 spectrophotometer. The extinction coefficient of $1.74 \cdot 10^4\ M^{-1}\ cm^{-1}$, defined for Cu(II)—BLM A_2 by Dabrowiak et al. [26], was utilized to determine the concentration of the specific BLM.

Dilutions of these stocks were made, as appropriate, so that a convenient volume (10—20 μl) could be injected. Triplicate samples were injected in the range of 50 pmol to 10 nmol, and the average peak area was determined for each set.

Separation of divalent and trivalent chelates

Several metals were found to form stable chelates under these chromatographic conditions, and the trivalent chelates could be separated from the divalent chelates. As an example, solutions of Blenoxane and the chlorides of Cu(II) and In(III) were prepared separately in unbuffered HPLC-grade water, and combined just prior to injection.

Bleomycin hydrolase analysis

Mouse liver was extracted by the method of Yoshioka et al. [28], except that dialysis of the crude extract was performed over 3 h with two changes of buffer at 4°C. Immediately after dialysis, 200 μl of the extract (about 50 μg protein) were incubated with 50 μl of either metal-free BLM B_2 or Blenoxane, lot FOX04, in 0.1 *M* sodium phosphate (pH 7.2), both at 0.3 mg/ml, for various times at 37°C in a shaking water bath. The reaction was stopped by addition of 0.25 ml of ice-cold HPLC solvent B, and after 15 min on ice the mixture was centrifuged at 15,000 *g*. The supernatant was collected, a slight excess of copper sulfate added, and the solutions were either chromatographed immediately or stored at 4°C until analyzed.

Chemical modifications of bleomycin A_2

BLM A_2 as the Cu(II) chelate was purified to greater than 98% homogeneity by CM-Sephadex C-25 ion-exchange chromatography [23]. BLM demethyl-A_2 (BLM DM-A_2) was prepared by pyrolysis at 100°C for 18 h as originally outlined by Fujii et al. [29] and more recently by Roy et al. [30]. BLM DM-A_2 was remethylated to BLM A_2 using methyl iodide [29,30]. BLM A_1 was synthesized from BLM DM-A_2 by treatment with hydrogen peroxide [29]. We also attempted to form BLM epi-A_2 [7] and BLM iso-A_2 [9], but yields were too poor for definitive analysis. This chemistry is discussed further below.

RESULTS AND DISCUSSION

Chromatography of metal(II)— and metal(III)—bleomycin congeners

The chromatographic procedure described in Experimental allows the separation and quantitation of BLM congeners in the form of their metal ion chelates. Fig. 2 shows the elution profile of the clinically used mixture in the metal-free form (Fig. 2A) and after conversion to the corresponding Cu(II) chelates (Fig. 2B). For analytical purposes, the requirement for metal complexation does not constitute a serious drawback, since their formation is rapid, complete (K_d of about 10^{-11} *M* [3]), and can even proceed while BLM is retained on the column. Excess metal ions do not interfere with the chromatographic separation since they elute in the void volume.

The Cu(II)—BLMs and —PHLMs are especially suitable for this analysis since they are stable under our assay conditions (pH 4.3), whereas the Zn(II)—

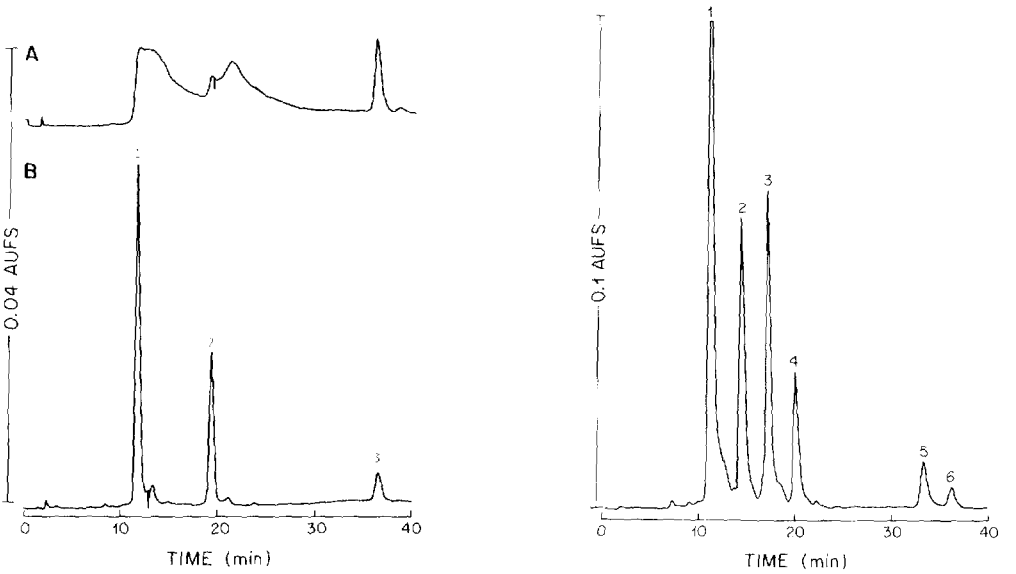


Fig. 2. Chromatographic profiles of the same amount of a Blenoxane mixture (lot FOX04) as (A) the metal-free drug and (B) the Cu(II) chelate. Conditions in this and all other figures are as given in Experimental. The terminal moieties of the indicated BLMs are given in Table I. Peaks: 1 = Cu(II)—BLM A_2; 2 = Cu(II)—BLM B_2; 3 = Cu(II)—BLM DM-A_2.

Fig. 3. Separation of Cu(II) and In(III) mixtures of Blenoxane (lot FOX04). Peaks: 1 = Cu(II)—BLM A_2; 2 = In(III)—BLM A_2; 3 = Cu(II)—BLM B_2; 4 = In(III)—BLM B_2; 5 = Cu(II)—BLM DM-A_2; 6 = In(III)—BLM DM-A_2.

BLMs are not [3]. Co(II) and Fe(II) complexes undergo oxidation to Co(III) and Fe(III) in the presence of oxygen [27], and long incubation periods are required before stable and uniform chelates are obtained. In addition, equimolar Cu(II) displaces Fe(III) [but not Co(III)] in the complex, so that its presence could interfere with results. In(III) chelates are also formed rapidly and are suitable for analysis in this system. The retention times of In(III) complexes are uniformly increased, due to the additional positive charge, and can be separated from the corresponding Cu(II) chelates, as seen in Fig. 3. The relative amount of Cu(II) to In(III) chelate shown in this chromatogram is 2:1, and quantitation of any of the congeners is not altered by the presence of either excess copper chloride or indium chloride.

Chromatography of Cu(II)—BLM and —PHLM congeners

The different BLM and PHLM congeners listed in Table I with their k' values were analyzed as the Cu(II) chelates. The void volume of our system is 3.77 ml, so that the k' is defined as $k'_i = V_i/3.77 - 1$, where V_i is the volume from time of injection to that of elution of the ith peak. Although several of these BLMs and PHLMs were only 70—85% pure by this method, we do not attribute this to instability of the compounds, since samples stored at room temperature in unbuffered HPLC-grade water for longer than one year gave the same chromatographic profiles as when freshly prepared. From the results shown in Table I, several conclusions can be drawn. (1) The negatively charged

bleomycinic acid has relatively little interaction with the octadecyl groups of the chromatographic column. However, fairly minor changes in the C-terminal group (e.g., BLM B'_1) drastically influence the retention time. This is confirmed by the k' determined for the terminal fragment BLM A_2-BT, which is eluted very near the corresponding intact congener. (2) As originally seen for ion-exchange chromatography [31], the A group BLMs, with the exception of BLM DM-A_2, generally are eluted before the B group, indicating in both cases that there is less formal charge density at the termini of the A group. However, these results are also consistent with either a greater interaction of the A group terminal moieties with the core portion of BLM (± metal) or lower lipophilicity. (3) Within each group, the greater the charge of the end-piece the greater the ion-pairing and thereby the longer the retention time. The notable exception is BLM DM—A_2, whose methylsulfide group might interact non-ionically with silane and/or silanol groups, as well as the C_{18} portion of the column. The retention of BLMs A_1, CHP, and PEPP is also consistent with the greater effect of lipophilicity over ion-pairing of the terminal group.

A typical separation of an artificial mixture of ten Cu(II)-complexed BLM congeners is shown in Fig. 4, obtained with the standard chromatographic conditions. This specific combination can be separated adequately on a μBondapak C_{18} within 25 min by increasing the gradient slope (data not shown), but some of the smaller, undefined peaks, contaminants in the reference compounds, seen in Fig. 4 are no longer resolved. However, for practical purposes, the Blenoxane (lot FOX04) mixture can be analyzed much more rapidly by increasing the gradient slope and/or by starting the gradient at a higher percentage of solvent B. In addition, preliminary results using a Novapak C_{18} (3.9 × 150 mm, 5-μm particle size) indicate about 50% solvent reduction with no significant loss of resolution.

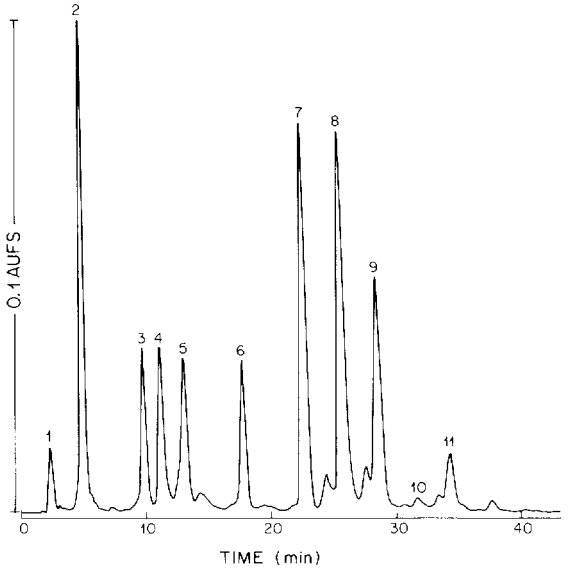


Fig. 4. Chromatogram of ten different Cu(II)—BLMs. The sturctures of the terminal groups are given in Table I. Peaks: 1 = free Cu(II); 2 = bleomycinic acid; 3 = BLM B'_1; 4 = BLM A_2; 5 = BLM A_5; 6 = BLM B_2; 7 = BLM B_4; 8 = BLM B_6; 9 = BLM CHP; 10 = BLM PEPP; 11 = BLM DM-A_2.

Quantitative aspects

The area under the absorbance curve (AUC) for BLM detected at 280 nm is linearly related to the amount injected over a range of 50 pmol to 10 nmol, or, in this system over a concentration range of 25 n*M* to 10 m*M*. For BLM A_2, automated integration gave AUC = $2.38 \cdot 10^{12} X - 129$ ($r = 0.9995$), while for BLM B'_1, AUC = $2.33 \cdot 10^{12} X - 41$ ($r = 0.9991$). The standard deviations of every triplicate sample injected were less than 0.5% by this method, and spot checks with a planimeter gave similar results. There is no detectable difference in estimation accuracy when different volumes containing the same amount of a single BLM are injected, but peak broadening with a large volume of a complex mixture might be expected to affect results. However, several combinations of different BLMs in 1—100 μl injected volumes gave the same AUC for each congener as they did when they were injected alone in similar volumes. Of course, the AUC is the same for all congeners with identical chromophores, but not, for instance, for BLM PEPP or the PHLMs (Table I and Fig. 1), whose terminal groups contribute to absorbance at 280 nm.

We have chosen measurement at this wavelength since the baseline absorbance at 254 nm (about 15% more sensitive) is less stable during the gradient and affects accuracy. A variable-wavelength detector set at 292 nm also would be expected to increase sensitivity by about 10%. The most exact detection method (down to about 1.0 pmol) should be by utilizing the fluorescence properties of BLM, as reported for the analysis of BLM hydrolase [32,33], but we have not explored these possibilities. However, since bithiazole fluorescence is being measured [34], this is not applicable for the PHLM. Furthermore, this method is comparable in sensitivity to published radioimmunoassay (RIA) [35,36] and enzymeimmunoassay (EIA) [37,38] techniques with much greater specificity and simplicity. In addition, recovery from tissue extracts (as in the BLM hydrolase assay) or sera from several species (data not shown) is essentially 100%, with no UV interference observed.

Bleomycin hydrolase analysis

Fig. 5 shows the usefulness of this system for the analysis of the action of BLM hydrolase on the Blenoxane mixture. If only one congener is to be used as a substrate (e.g., BLM A_2 or B_2), simply increasing the slope of the solvent gradient can reduce the analysis time to less than 15 min. There is no difference in chromatographic results due to storage in 50% methanol at 4°C or room temperature over twenty days, the longest time tested. Total recovery of original BLM and/or the corresponding desamide analogue(s), regardless of reaction time in the range 0—4 h, is always 100 ± 5%. In addition, the 3-h dialysis effectively removed low-molecular-weight material, so that concentration with a CF-25 Centriflo cone (Amicon, Lexington, MA, U.S.A.) was not found to be necessary to prevent loss of activity during overnight dialysis, as indicated by Yoshioka et al. [28].

This enzyme is known to interact with the various BLMs at different rates [39], BLM B_2 being affected most, as can be seen in Fig. 5. Since BLM is presently used clinically as a mixture, and since there is no guarantee that different tumors inactivate each BLM to the same relative extent, we believe that the mixture should be used to analyze the level of activity of this enzyme.

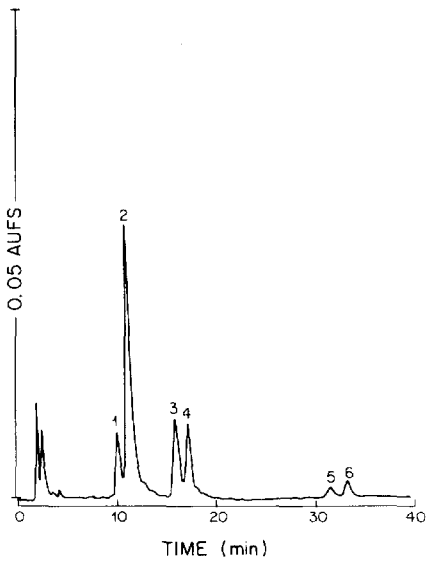


Fig. 5. Assay of BLM hydrolase activity in mouse liver extracts using Blenoxane (FOX04), as indicated in Experimental. Structures of the following identifiable peaks can be defined by referring to Fig. 1 and Table I. Peaks: 1 = BLM desamido-A_2; 2 = BLM A_2; 3 = BLM desamido-B_2; 4 = BLM B_2; 5 = BLM desamido-DM-A_2; 6 = BLM DM-A_2.

This would be especially important when comparing relative rates in cells which respond differently to drug treatment. This technique, therefore, gives an additional dimension to published methods of determining BLM hydrolase activity [28,32,33,40] and provides more useful inactivation rates. Our calculations for mouse liver (1 h incubation) indicate about 25% less activity when the complex is used as compared to BLM B_2 alone [5.6 versus 7.7 μg BLM (mg protein)$^{-1}$ min^{-1}].

Chemical modifications of bleomycin A_2

Fig. 6 shows the analysis of some of the published chemical modifications of BLM A_2. If Cu(II)—BLM A_2 (greater than 98% pure as shown in Fig. 6A) is subjected to pyrolysis at 100°C for 18 h, about 95% is converted to BLM DM-A_2 (Fig. 6B). This peak has a shoulder, just prior to that of authentic BLM DM-A_2, which we attribute to the formation of BLM epi- or iso-DM-A_2. However, preliminary attempts to form these analogues in sufficient yields for analysis were not successful, so that we cannot at this time adequately define these alternate peaks.

Oxidation of BLM DM-A_2 with hydrogen peroxide [29] produces the predicted BLM A_1 (Fig. 6C), and the alternative form(s) is seen to carry over in relatively the same amount. If, however, the BLM DM-A_2 formed in Fig. 6B is remethylated with methyl iodide [29,30], the chemistry does not appear to be as specific. Although the amounts of BLM A_2 and the additional form produced are considerable, these account for no more than 60% of the products. It also appears that BLM A_1 is formed along with other undetermined products. This result is consistent with our analyses of two lots of commercial [^{3}H]S-CH_3-BLM A_2 prepared by this method [29,30], in which 70% and 60%, respectively, of the label was separated from the authentic BLM A_2 peak (data not shown).

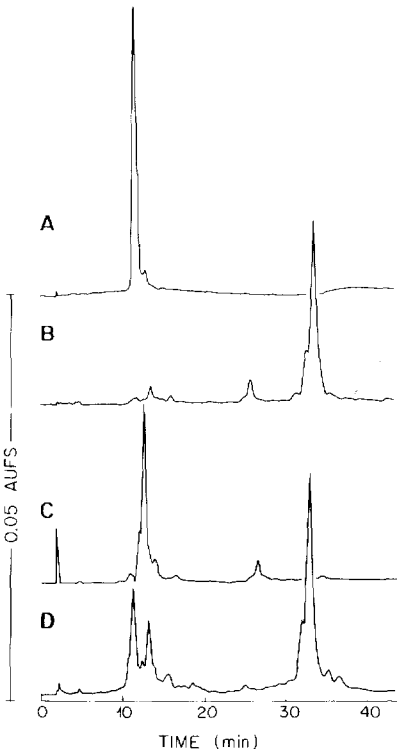


Fig. 6. Chemical modifications of BLM A_2. The chemistry is described in Experimental. A, BLM A_2; B, BLM DM-A_2; C, BLM A_1; D, BLM remethylated-DM-A_2.

CONCLUSIONS

We have developed a procedure which precisely separates many of the common BLMs and/or PHLMs and is also readily adaptable to the analysis of the chemical purity of newly synthesized derivatives of this class of antibiotics. The sensitivity of 50 pmol and the ease of sample preparation make this technique suitable for studies of tissue distribution, metabolism, and pharmacokinetics of specific BLMs, as well as mixtures of different congeners. Moreover, the method may be easily modified to provide increased separation capacity for complex mixtures, or shortened analysis time for samples of simpler composition.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Heart, Lung, and Blood Institute Division of Lung Diseases (HL23874-05 TOX) and from the American Cancer Society's Robert A. Sullivan Memorial Grant for Cancer Research (CH-252).

We are grateful to all who have provided specific BLM/PHLM congeners for our use, and to Mrs. Karen Grodsky for her help in the preparation of this manuscript.

REFERENCES

1 K. Maeda, H. Kosaka, K. Yagishita and H. Umezawa, J. Antibiot. Ser. A, 9 (1956) 82.
2 H. Umezawa, K. Maeda, T. Takeuchi and Y. Okami, J. Antibiot. Ser. A, 19 (1966) 200.
3 J.C. Dabrowiak, J. Inorg. Biochem., 13 (1980) 317.
4 L. Giloni, M. Takeshita, F. Johnson, C. Iden and A.P. Grollman, J. Biol. Chem., 256 (1981) 8608.
5 P. Raban, J. Brousil and P. Svihovcova, Eur. J. Nucl. Med., 4 (1979) 191.
6 T. Takita, Y. Muraoka, T. Yoshioka, A. Fujii, K. Maeda and H. Umezawa, J. Antibiot., 25 (1972) 755.
7 Y. Muraoka, H. Kobayashi, A. Fujii, M. Kunishima, T. Fujii, Y. Nakayama, T. Takita and H. Umezawa, J. Antibiot., 29 (1976) 853.
8 H. Umezawa, S. Hori, T. Sawa, T. Yoshioka and T. Takeuchi, J. Antibiot., 27 (1974) 419.
9 Y. Nakayama, M. Kunishima, S. Omoto, T. Takita and H. Umezawa, J. Antibiot., 26 (1973) 400.
10 Code of Federal Regulations, Food and Drugs, Title 21, part 450.10a, U.S. Government Printing Office, Washington, DC, 1980.
11 H. Umezawa, GANN Monogr. Cancer Res., 19 (1976) 3.
12 A. Matsuda, O. Yoshioka, K. Ebihara, H. Ekimoto, T. Yamashita and H. Umezawa, in S.K. Carter, S.T. Crooke and H. Umezawa (Editors), Bleomycin: Current Status and New Developments, Academic Press, New York, 1978, p. 299.
13 I.H. Raisfeld, Toxicol. Appl. Pharmacol., 56 (1980) 326.
14 I.H. Raisfeld, Toxicol. Appl. Pharmacol., 57 (1981) 355.
15 I.H. Raisfeld, J.P. Chovan and S. Frost, Life Sci., 30 (1982) 1391.
16 I.H. Raisfeld, P. Chu, N.K. Hart and A. Lane, Toxicol. Appl. Pharmacol., 63 (1982) 351.
17 M. Takeshita, L.S. Kappen, A.P. Grollman, M. Eisenberg and I.H. Goldberg, Biochemistry, 20 (1981) 7599.
18 M. Miyaki, T. Ono, S. Hori and H. Umezawa, Cancer Res., 35 (1975) 2015.
19 W.C. Eckelman, W.J. Rzeszotarski, B.A. Siegel, H. Kubota, M. Chelliah, J. Stevenson and R.C. Reba, J. Nucl. Med., 16 (1975) 1033.
20 W.J. Rzeszotarski, W.C. Eckelman and R.C. Reba, J. Chromatogr., 124 (1976) 88.
21 T.T. Sakai, J. Chromatogr., 161 (1978) 389.
22 G.K. Shiu, T.J. Goehl and W.H. Pitlick, J. Pharm. Sci., 68 (1979) 232.
23 Y. Muraoka, in S..M. Hecht (Editor), Bleomycin: Chemical, Biochemical, and Biological Aspects, Springer, New York, 1979, p. 92.
24 G.K. Shiu and T.J. Goehl, J. Chromatogr., 181 (1980) 127.
25 A. Aszalos, J. Crawford, P. Vollmer, N. Kantor and T. Alexander, J. Pharm. Sci., 70 (1981) 878.
26 J.C. Dabrowiak, F.T. Greenway, W.E. Longo, M. van Husen and S.T. Crooke, Biochim. Biophys. Acta, 517 (1978) 517.
27 R.M. Burger, S.B. Horwitz, J. Peisach and J.B. Wittenberg, J. Biol. Chem., 254 (1979) 12299.
28 O. Yoshioka, N. Amano, K. Takahashi, A. Matsuda and H. Umezawa, in S.K. Carter, S.T. Crooke and H. Umezawa (Editors), Bleomycin: Current Status and New Developments, Academic Press, New York, 1978, p. 35.
29 A. Fujii, T. Takita, K. Maeda and H. Umezawa, J. Antibiot., 26 (1973) 398.
30 S.N. Roy, G.A. Orr, C.F. Brewer and S.B. Horwitz, Cancer Res., 41 (1981) 4471.
31 H. Umezawa, Y. Suhara, T. Takita and K. Maeda, J. Antibiot. Ser. A, 19 (1966) 210.
32 J.S. Lazo, C.J. Boland and P.E. Schwartz, Cancer Res., 42 (1982) 4026.
33 J.S. Lazo and C.J. Humphreys, Proc. Nat. Acad. Sci. U.S., 80 (1983) 3064.
34 M. Chien, A.P. Grollman and S.B. Horwitz, Biochemistry, 16 (1977) 3641.
35 A. Broughton and J.E. Strong, Cancer Res., 36 (1976) 1418.
36 M.K. Elson, M.M. Oken and R.B. Shafer, J. Nucl. Med., 18 (1977) 296.
37 K. Fujiwara, M. Yasuno and T. Kitagawa, Cancer Res., 41 (1981) 4121.
38 K. Fujiwara, M. Isobe, H. Saikusa, H. Nakamura, T. Kitagawa and S. Takahashi, Cancer Treat. Rep., 67 (1983) 363.
39 H. Umezawa, Prog. Biochem. Pharmacol., 11 (1976) 18.
40 W.E.G. Muller, A. Totsuka, R.K. Zahn and H. Umezawa, Z. Krebsforsch., 83 (1975) 151.