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High-Performance Liquid Chromatographic Determination of Components of Bleomycin Preparations

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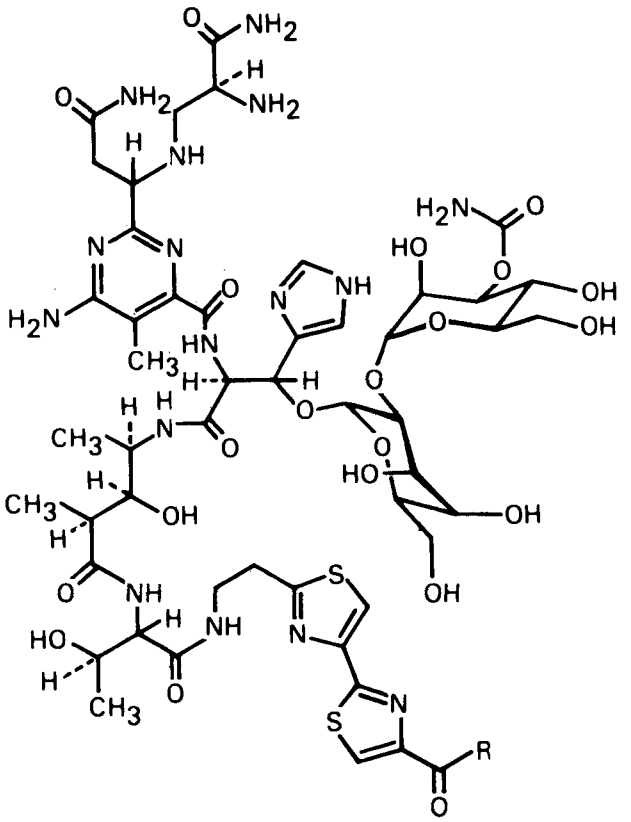
Abstract □ A fast and sensitive method was developed for the quantitative determination of at least 10 components of pharmaceutical bleomycin sulfate preparations. The method is based on the reversed-phase high-performance liquid chromatographic (HPLC) separation of the components on a μBondapak C_{18} column with a mobile phase having a linear gradient of 10–40% methanol in aqueous 0.005 *M* 1-pentanesulfonic acid at pH 4.3. With this assay, the average standard deviations for components A_2 and B_2 are 0.92 and 0.87, respectively, for a 7.5–22.5 × 10^{-3}-mg sample. Regulatory agencies presently use the official Code of Federal Regulations (CFR) method, which is based on CM-Sephadex column chromatography. It was demonstrated that this CFR method does not separate the bleomycin A_2 component from some other minor bleomycin components. After elution from the CM-Sephadex column, the "A_2 component" was separated into five components by the HPLC method. Bleomycin A_2 is stable under these HPLC conditions.

Keyphrases □ High-performance liquid chromatography—reversed phase, bleomycin assay, separation of bleomycin components □ Bleomycin—high-performance liquid chromatographic determination, separation of components □ Antineoplastics—bleomycin, high-performance liquid chromatographic analysis

Bleomycins (I) are a family of glycopeptide antibiotics obtained from the fermentation broth of *Streptomyces verticillus* (1). Bleomycins are effective against various human neoplasms, particularly against squamous cell carcinoma, sarcoma, and malignant lymphoma (2–4). Preparations used in the treatment of these neoplasms contain several bleomycins. The main components are bleomycins A_2 and B_2. Some minor components such as bleomycin acid, demethylbleomycin-A_2, and bleomycin B_4 also are present. Among these minor components, bleomycin B_4 is the most toxic (5).

The Code of Federal Regulations (CFR) (6) requires that bleomycin preparations for therapeutic use meet the following composition specifications: bleomycin A_2, not less than 60% and not more than 70%; bleomycin B_2, not less than 25% and not more than 32%; bleomycin B_4, not more than 1%; and bleomycins A_2 and B_2 combined, not less than 90% of the total bleomycins. The present established method is based on a lengthy CM-Sephadex column chromatographic procedure, essentially that described by Fujii *et al.* (7).

Reported high-performance liquid chromatographic (HPLC) methods were unsuitable for routine quantitative analysis. With one method (8), baseline resolution was incomplete; another method (9) was not designed for quantitative purposes; and a third method (10), which could be used to determine bleomycins A_2 and B_2, did not



bleomycin	R
demethyl-A_2	NH—CH_2—CH_2—CH_2—S—CH_3
A_2	NH—CH_2—CH_2—CH_2—S=$(CH_3)_2$
acid	OH
B_2	NH—$(CH_2)_4$—NH—C(=NH)—NH_2
B_4	NH—$(CH_2)_4$—NH—C(=NH)—NH—$(CH_2)_4$—NH—C(=NH)—NH_2
A_5	NH—$(CH_2)_3$—NH—$(CH_2)_4$—NH_2

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detect the minor components. The developed HPLC assay is suitable for the fast, quantitative determination of at least 10 components found in commercial bleomycin preparations.

EXPERIMENTAL

Materials—Spectrograde methanol was used throughout the experiments. Acetic acid[1], concentrated ammonium hydroxide[2], and 1-pentanesulfonic acid[3] were used without further purification. Solvents were deaerated under vacuum with stirring for 1 hr before use. Sterile bleomycin sulfate preparations[4] in vials containing 15 units of bleomycin activity were dissolved in 3 ml of deaerated water and stored at 5°.

Apparatus—A modular high-pressure liquid chromatograph was equipped with a constant-flow pump[5], a valve-type injector[5], and a fixed-wavelength (254-nm) UV detector[6]. A stainless-steel column (4.6 × 250 mm) was packed with fully porous 10-μm silica particles to which a monomolecular layer of octadecylsilane was chemically bonded. In this work, generically identical column packing materials from two different suppliers were used (referred to as Columns 1[7] and 2[8]). Columns were washed with methanol–water (1:1) after use each day and with methanol before each weekend. Calculations for the percentage of total UV absorbance at 254 nm were performed for each component by a microprocessor[9] with the peak area calculation program (Figs. 1 and 2). The microprocessor was set as follows: sensitivity, 10%; attenuator, 16; and initial peak width, 0.5.

Analytical Procedure—The sample size for injection was 3-9 μl of bleomycin solution. The solvent system consisted of 0.005 *M* 1-pentanesulfonic acid in 0.5% acetic acid deaerated aqueous solution, adjusted to pH 4.3 (concentrated ammonium hydroxide), and methanol with a linear gradient of 10–40% methanol. Gradient mixing time was 60 min, but chromatography was continued with the final gradient mixture (40% methanol) up to 75 min to allow for the appearance of demethylbleomycin-A_2, the last peak. The column pressure at a flow rate of 1.2–1.8 ml/min, depending on column conditions, was equivalent to the initial column pressure of 1600 psi.

The relative percent of each bleomycin component was calculated on the basis of the area under the peak by the microprocessor with the given parameters.

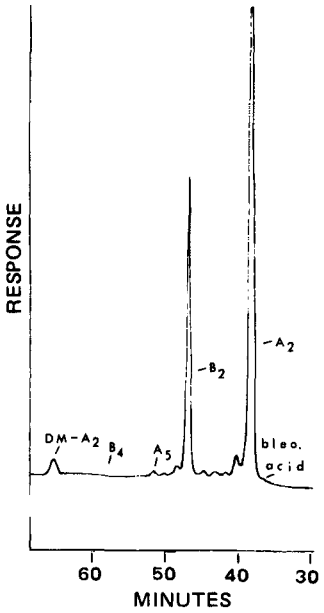

Figure 1—*HPLC separation profile of a bleomycin sulfate preparation obtained with Column 1 (6 μl of bleomycin solution injected). Key: bleo. acid, bleomycin acid; A_2, bleomycin A_2; B_2, bleomycin B_2; A_5, bleomycin A_5; B_4, bleomycin B_4; and DM-A_2, demethylbleomycin-A_2. Microprocessor quantitation indicates 58.29, 28.18, and 4.32 "relative %" composition for A_2, B_2, and demethyl-A_2, respectively.*

[1] Mallinckrodt Chemical Co., St. Louis, Mo.
[2] J. T. Baker Chemical Co., Phillipsburg, N.J.
[3] Eastman Organic Chemicals, Rochester, N.Y.
[4] Nippon-Kayaku Co. Ltd., Chugai Boyeki (America) Corp. Importers and Exporters, New York, N.Y.
[5] Model 6000 solvent delivery system and model 660 solvent programmer, Waters Associates, Milford, Mass.
[6] Model 440, Waters Associates, Milford, Mass.
[7] μBondapak C_{18}, Waters Associates, Milford, Mass.
[8] Chromegabond MC-18, ES Industries, Marlton, N.J.
[9] Columbia Instrument Supergrator I.

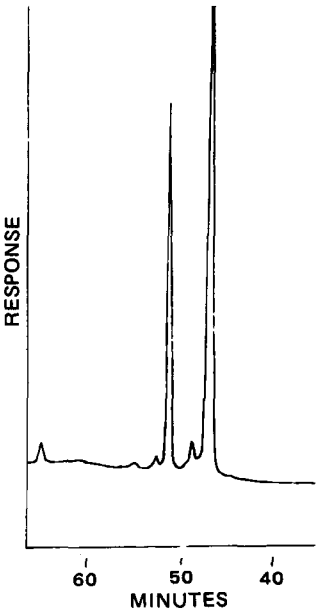

Figure 2—*HPLC separation profile of a bleomycin sulfate preparation obtained with Column 2 (6 μl of bleomycin solution injected). Relative peaks for separated components are the same as in Fig. 1. Microprocessor quantitation indicates 59.14, 29.62, and 4.09 "relative %" composition for A_2, B_2, and demethyl-A_2, respectively.*

RESULTS AND DISCUSSION

In developing this HPLC method, different parameters such as solvent, column, pH, operating time, gradient composition, and flow rate were investigated for optimum separation. Mobile phases of acetonitrile, acetone, or methanol with aqueous solutions of perchloric acid or 1-pentanesulfonic acid in dilute acetic acid were studied. With aqueous 0.005 *M* 1-pentanesulfonic acid solution, adjusted to pH 4.3, and mixed with 10–40% methanol in a linear gradient, 10 or more components were

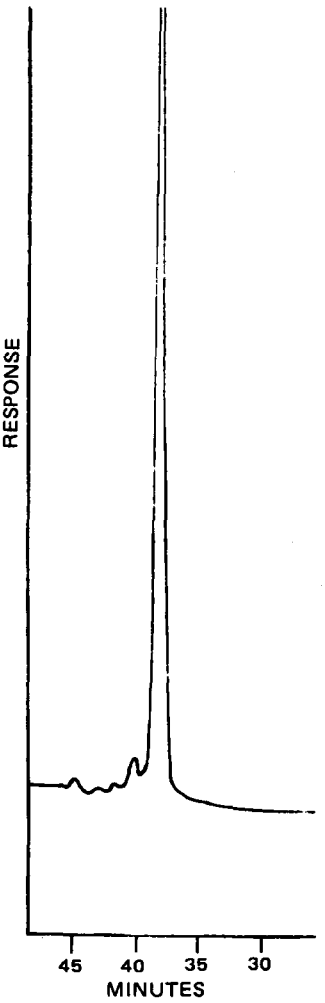

Figure 3—*HPLC separation profile obtained with Column 1 of the bleomycin "A_2 fraction" after elution from the CM-Sephadex column.*

Table I—Comparison of the CFR and HPLC Methods for the Determination of Bleomycins A_2 and B_2 in Bleomycin Sulfate Preparations

Sample	Bleomycin A, %			Bleomycin B, %		
	CFR Method	HPLC Method	*SD*	CFR Method	HPLC Method	*SD*
1	68.43 (64.15)[a]	62.30[b]	0.46	28.33	28.10[b]	0.80
2	67.60 (63.82)[a]	63.16[c]	1.12	28.54	29.49[c]	1.25
3	66.52	59.50[d]	1.26	30.85	28.48[d]	0.77
4	65.78	60.33[b]	0.85	31.39	29.89[b]	0.66
5	69.27	65.12[e]	—	26.63	29.30[e]	—
Average			0.92			0.87

[a] Calculation after HPLC separation. [b] Average of 10 determinations. [c] Average of nine determinations. [d] Average of eight determinations. [e] Single determination.

detected. The last peak was observed at ~70 min, with a gradient mixing time of 60 min. Other gradient compositions and contours did not give satisfactory chromatograms. The same resolution of the 10 components was obtained between pH 4.0 and 6.0.

Another system that showed considerable promise was water, adjusted to pH 2.0 with perchloric acid, and mixed with 10–40% acetonitrile in a linear gradient. A separation profile like that in Fig. 2 was obtained, but the low pH of this solvent system made it impractical for routine use.

An initial column pressure of 1600 psi must be applied to obtain a flat baseline, suitable for area calculations with the microprocessor. Lower pressures result in baseline drift and poor area calculations. Typical separation profiles, obtained with Columns 1 and 2, are shown in Figs. 1 and 2, respectively. As used in this laboratory, Column 1 gave a better separation of the components. Specifically, the components between bleomycins A_2 and B_2 appeared as one peak with Column 2 but four peaks with Column 1. The quantitative results were comparable, however, for the bleomycin A_2, B_2, and demethyl-A_2 fractions from both columns; quantitative information for the A_2, B_2, and demethyl-A_2 fractions from the same sample is given for Columns 1 and 2 in the legends of Figs. 1 and 2, respectively.

The presumed identities of some individual peaks confirmed by coinjection of reference materials are shown in Fig. 1. No reference materials were available for the unidentified separated components.

Four bleomycin preparations were analyzed eight to 10 times each with Column 1 under the described HPLC conditions. These results (Table I), expressed as average percentages of the A_2 and B_2 components in bleomycin samples, were compared with those obtained by the official CFR method (6). The respective A_2 percentages obtained from the HPLC analyses were lower than those found by the CFR method. To explain this discrepancy, A_2 components isolated by the CFR method were collected from several column separations and combined, lyophilized, redissolved, and injected into the chromatograph. As shown in Fig. 3, the HPLC method separated this fraction into five components, with the major component having the retention time of bleomycin A_2. The amount of A_2 in this fraction was ~92% of the total bleomycin content of the fraction. After the A_2 percentages found by the CFR method were recalculated on the basis of the HPLC fraction separation, the official CFR column and HPLC results were in close agreement (Table I). This confirmation is significant since the accurate quantitation of bleomycins A_2 and B_2 in clinically used preparations is essential.

Bleomycin A_2 material collected from HPLC separations showed a single peak when rechromatographed by HPLC. This finding is evidence of the stability of bleomycin A_2 (and probably the other components) under these HPLC conditions.

Separate standard deviations for bleomycin A_2 and B_2 were calculated from multiple determinations of each component in four bleomycin samples (Table I). Calculations with these values give average standard deviations for components A_2 and B_2 of 0.92 and 0.87, respectively, and demonstrate good reproducibility for the HPLC assay.

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