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Rapid High-Performance Liquid Chromatographic Determination of Bleomycin A_2 in Plasma

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Abstract □ A rapid and specific method for the determination of bleomycin A_2 is described. A 50-μl aliquot of 20% trichloroacetic acid was added to 200 μl of plasma. The sample was vortexed and centrifuged, and 50 μl of the clear supernate was injected into a liquid chromatograph equipped with a microparticulate reversed-phase column and a fixed wavelength detector. Elution was carried out using methanol-acetonitrile-0.0085 *M* heptanesulfonic acid-acetic acid. A linear calibration curve was found in the 0.05–5-μg/ml range with an estimated precision of ±6% (*CV*). Preliminary pharmacokinetic data in the rabbit also are reported.

Keyphrases □ Bleomycin A_2—high-performance liquid chromatographic analysis in plasma □ High-performance liquid chromatography—analysis, bleomycin A_2 in plasma □ Antineoplastic agents—bleomycin A_2, high-performance liquid chromatographic analysis in plasma

The antibiotic–antineoplastic agent bleomycin is actually a mixture of cationic polypeptides. The complex was isolated from fermentation products of *Streptomyces verticillus* (1). It is effective against human neoplasms, particularly squamous cell carcinoma, lymphoma, and testicular carcinoma (2–4). The most common toxic side effects are minor cutaneous reactions. However, about 10% of the patients develop pulmonary toxicity, occurring as pneumonitis and pulmonary fibrosis (5).

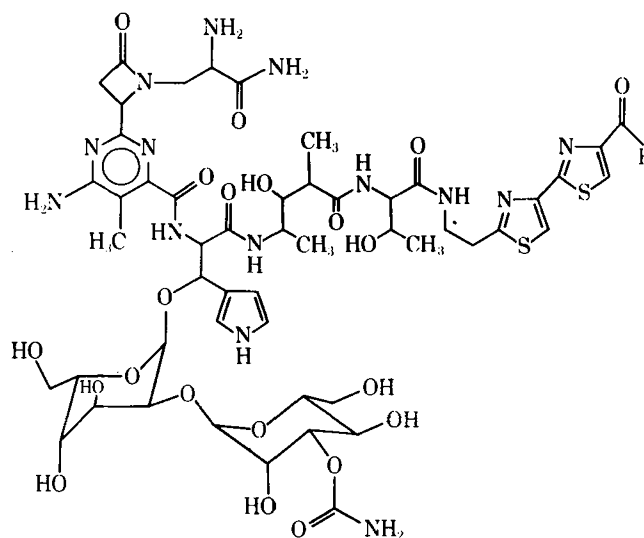
BACKGROUND

Crude bleomycin has been purified by chromatographic methods into two large fractions, A and B (6). Further fractionation provided six bleomycin A fractions and five bleomycin B fractions. Of the various highly hydrophilic polypeptides, 11 components have been completely purified. Each component consists of a carboxy terminal glycopeptide (~1300 daltons), designated as bleomycinic acid, which is substituted in an amide-type linkage with 3-(dimethylthionium)propylamine (bleomycin A_2, II), agmantine (bleomycin B_2, III), or 3-(methylsulfinyl)propylamine (bleomycin A_1, I).

The bleomycin administered clinically consists of, by weight, 55–70% bleomycin A_2, 25–32% bleomycin B_2, and the remaining percentage divided among the other subfractions (7). In all cases, at least 65% is from the bleomycin A group and less than 35% is from the bleomycin B group. The most commonly used regimen has been 15 mg/m² iv twice weekly (8).

Although almost all clinical work has been done using this formulation, there are indications that the individual bleomycin fractions should be studied. For example, bleomycin A_5 was more toxic to *Escherichia coli* than the more abundant bleomycin A_2 present in the formulation currently in use (9). Bleomycin acid was devoid of activity against *E. coli* (9). The possible effectiveness of bleomycin A_5 was reported by others (10, 11). Bleomycin B_4 caused kidney damage in dogs and had weak antitumor activity (12). However, few studies on individual components of the bleomycin mixture have been presented (12–14).

To elucidate the pharmacokinetics of individual bleomycins, a specific and sensitive assay must be available. Analytical techniques to estimate plasma levels of the bleomycin mixture include microbiological assays (11, 12, 15, 16), radioactive labeling assays (2, 17–19), and radioimmunoassays (20, 21). Unfortunately, all of these methods do not distinguish between the various components of the bleomycin mixture or their metabolites. Thus, specificity is a problem with all presently available techniques. In this report, a sensitive and specific paired-ion high-performance liquid chromatographic (HPLC) assay for one component of the bleomycin mixture, bleomycin A_2, is presented.



I: R = $NH(CH_2)_3SOCH_3$
II: R = $NH(CH_2)_3\overset{+}{S}{=}(CH_3)_2$
III: R = $NH(CH_2)_4NHC({=}NH)NH_2$

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EXPERIMENTAL

Apparatus—A modular high-pressure liquid chromatograph consisting of a constant-flow pump[1], a valve-type injector[2], a fixed wavelength (254-nm) UV detector[3], and a strip-chart recorder[4] was used. A stainless steel column (3.9 mm × 30 cm) packed with fully porous 10-μm silica particles with a chemically bonded monomolecular layer of octadecylsilane[5] was obtained commercially. A guard column (3.9 mm × 6 cm) filled with pellicular reversed-phase packing[6] was used to prolong column life.

Chromatographic Conditions—The mobile phase consisted of methanol-acetonitrile-0.0085 *M* heptanesulfonic acid–acetic acid (30:10:59:1). A flow rate of 2.0 ml/min was established (2500 psi).

Reagents and Materials—Bleomycin[7], bleomycin A_2[8], and sodium 1-heptanesulfonate[9] were used as received. Solvents were of HPLC grade. All other materials were reagent grade. Stock solutions of bleomycin and bleomycin A_2 were prepared by dissolving 10 mg of bleomycin and bleomycin A_2 in 100 ml of deionized water. Plasma standards were prepared by taking appropriate small volumes of stock solutions and adding them to control plasma.

HPLC Assay and Calculations—To 0.2 ml of plasma in a 12-ml glass-stoppered conical centrifuge tube was added 50 μl of a 20% trichloroacetic acid solution. The samples were mixed[10] and then centrifuged[11] at 3000 rpm for 10 min. A 50-μl aliquot of the clear supernate was then injected onto the column, and peak heights were determined. Calibration curves were constructed daily from results obtained from spiked control plasma samples containing 0.5–5 μg/ml by plotting peak heights *versus* bleomycin A_2 concentration.

Pharmacokinetics in Rabbits—Four female New Zealand White rabbits, 1.5–3 kg, received 4 units of bleomycin/kg iv in the marginal ear vein. Fifteen blood samples were withdrawn over 3 hr from the opposite marginal ear vein.

RESULTS AND DISCUSSION

In preliminary studies with methanol-0.01 *M* heptanesulfonic acid–acetic acid (48:51:1), reasonable separations of the major components were obtained, yielding three major fractions. By using TLC methods in which relative R_f values of the HPLC-separated components were compared with literature values, these fractions were determined to be bleomycins A_2, B_2, and A_1. Only bleomycin A_2 was available for direct comparison by TLC and HPLC.

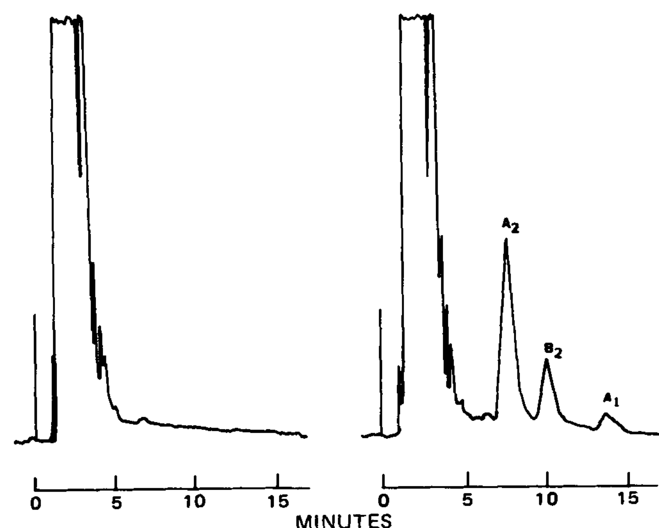

Figure 1—*Chromatogram obtained from HPLC assay of control rabbit plasma (left) and plasma from a rabbit administered a single intravenous dose of bleomycin (right). Peak for bleomycin A_2 corresponded to a concentration of 2.6 µg/ml.*

Table I—Stability of Bleomycin A_2 in Rabbit Plasma

Days	Concentration[a], µg/ml	
	Sample A	Sample B
0	4.3 ± 0.1	3.0 ± 0.1
1	4.2 ± 0.2	3.1 ± 0.2
4	4.3 ± 0.2	3.0 ± 0.2
8	4.4 ± 0.1	3.1 ± 0.2

[a] Mean ± *SD*, *n* = 3.

Since the bleomycins are very water soluble, separation from normal plasma constituents was considered to be a major obstacle prior to the quantitation of bleomycin A_2. However, a simplistic approach was attempted. The proteins were removed by trichloroacetic acid precipitation, and then an aliquot of the supernate was injected onto a reversed-phase column. This approach proved to be very satisfactory, with no interfering peaks being found in the chromatograms. Comparison between spiked plasma samples and direct aqueous injections gave identical results, indicating complete recovery of the bleomycins.

Resolution between bleomycins A_2 and B_2 was good (R = 1.2) on the original HPLC column with the three-component system. However, resolution began to decrease after a relatively short time. After this same experience was noted on two other identical columns, attempts were made to prolong the useful lifetime of columns for this assay. Changing to the four-component system solved the problem. Since this system has been used, column life has been very satisfactory and resolution has been improved (R = 1.7).

Previously reported work on the HPLC separation of the bleomycins required long elution times (22, 23). With this ion-pairing technique, elution times were considerably reduced. This method is based on the ability to force drugs into their ionic forms by pH adjustment and then the addition of counterions to form ion-pair complexes. This lipophilicity allows for retention on the reversed-phase column.

Bleomycins reportedly are unstable in biological fluids and tissues. Since all assays could not be performed on the same day that the samples were obtained, a stability study in plasma was begun. The bleomycin mixture (3 units/kg) was injected into a rabbit, and two large blood samples (A and B) were withdrawn 10 and 20 min, respectively, after the intravenous infusion. Aliquots of these samples were assayed immediately, and then other aliquots were assayed after 1, 4, and 8 days. Samples were kept frozen prior to assay. No significant decrease in plasma concentration was noted over the period of study (Table I). In a previously reported study (16) that had shown instability of the drug in plasma, the microbiological assay was used. Since that method exhibited a very large standard deviation and reported decreases in concentration were relatively small, the conclusion is open to question.

Because of the convenience of using plasma as opposed to serum, it was necessary to consider the effect of anticoagulants on the assay. Heparin was used as the anticoagulant in most studies, although citrate also was used. Neither anticoagulant interfered with the method.

Chromatograms obtained on the analysis of control rabbit plasma and plasma containing 2.6 µg of bleomycin A_2/ml are shown in Fig. 1. The chromatogram of control plasma showed no interfering peaks. In the other chromatogram, peaks corresponding to bleomycin A_2 (7.8 min), B_2 (10.2 min), and A_1 (13.8 min) were observed. No internal standard was used because of the simple nature of the assay and the large sample volume injected.

To verify that the peak designated as bleomycin A_2 was indeed that

Table II—Precision and Accuracy for Assay of Bleomycin A_2 in Plasma

Actual Concentration, µg/ml	Experimental Concentration, µg/ml, Mean[a] (Range)	*CV*	Mean Relative Percent Error
Control	<MDL[b]	—	—
0.32	0.30 (0.28–0.34)	9.4	9.4
0.63	0.64 (0.59–0.70)	7.3	5.2
1.2	1.2 (1.1–1.3)	6.8	4.2
2.3	2.3 (2.1–2.4)	6.2	3.8
4.1	4.2 (4.1–4.3)	2.8	2.4
	Overall	(6.0)	(4.9)

[a] *n* = 4. [b] MDL = minimum detectable level (0.08 µg/ml); twice blank value.

[1] Model M 6000A, Waters Associates, Milford, Mass.
[2] Model SVOV-6-1, Glenco Scientific, Houston, Tex.
[3] Model 440, Waters Associates, Milford, Mass.
[4] Model 255, Linear Instruments Corp., Irvine, Calif.
[5] Prepacked µBondapak C_{18}, Waters Associates, Milford, Mass.
[6] Co:Pell ODS, Whatman, Inc., Clifton, N.J.
[7] Blenoxane, lot E4095, supplied by Bristol Laboratories, Syracuse, N.Y.
[8] Supplied by Bristol Laboratories, Syracuse, N.Y.
[9] Eastman Kodak Co.
[10] Vortex-Genie, Fisher Scientific Industries, Springfield, Mass.
[11] IEC EXD centrifuge 460G, Damon/IEC Division, Needham Heights, Mass.

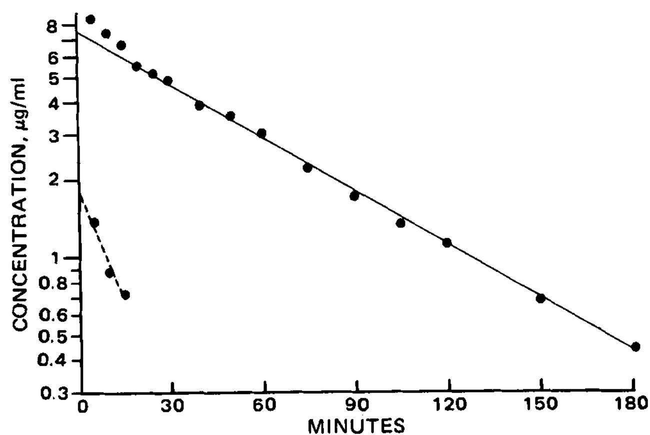

Figure 2—*Plot of plasma concentrations of bleomycin A_2* versus *time obtained after a single intravenous dose of the commercially available bleomycin mixture.*

compound, the retention times and peak widths at half-height were compared to the standard. In addition, the standard addition technique was used. These methods confirmed that bleomycin A_2 was present in the rabbit plasma samples and that no interfering peaks were hidden under that bleomycin A_2 peak.

The standard curve for the assay was linear over the 0.5–5-μg/ml range. All samples were diluted to bring them within this range. To study the precision and accuracy of the method, known amounts of the drug were added to control plasma. These samples were assayed subsequently in quadruplicate as described. The coefficient of variation and relative error for the assay of bleomycin A_2 are reported in Table II.

The utility of this method was shown by studying the pharmacokinetics of bleomycin A_2 in rabbits. Since the activity of the commercially available bleomycin mixture varied from batch to batch, all reported data were obtained from a single batch. A dose of 4 units of bleomycin/kg iv was given to each rabbit in the marginal ear vein. The activity of this batch was reported to be approximately 2 units/mg. Blood samples were drawn from the opposite marginal ear vein over 3 hr; plasma was then separated by centrifugation and assayed as described.

The data, plotted on semilog coordinates, are shown in Fig. 2. A biphasic curve was noted, suggesting the data should be fit to a two-compartment model: a central plasma compartment and a peripheral tissue compartment. The elimination half-life was calculated to be about 40 min for the β-phase. The volume of distribution for the central compartment was about 0.2 liter/kg.

In mice, the decrease in plasma levels of the bleomycin mixture did not follow first-order kinetics (16). Samples in that study, however, were assayed by a nonspecific microbiological method. In the present study, preliminary findings indicate that first-order kinetics are followed. This result suggests that each component of the bleomycin mixture may have a different clearance.

Further studies on bleomycin A_2 pharmacokinetics are in progress. With this simple HPLC method for bleomycin A_2 as a model, it is hoped that assays for the other major components may be developed and their pharmacokinetics studied.

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