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CHEMICAL AND BIOLOGIC PROPERTIES OF ISOLATED RADIOLABELED BLEOMYCIN PREPARATIONS

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Cobalt-57-bleomycin is a diagnostically useful radiopharmaceutical, but little is known about the nature of its individual fractions in regard to their metal-binding capacity and their in vivo distribution. Bleomycin was separated by high performance liquid chromatography (HPLC) into four major components. These were labeled and the distribution studied in tumor-bearing rats at 2 and 24 hr. In vivo radiochemical purity was also determined. Of the nine HPLC systems studied, Porasil A eluted with a mobile phase of 0.3% ammonium formate in methanol gave the best separation of the fractions. These fractions were copper free and retained their biologic activity and purity. An in vitro competitive binding study of ^{57}Co-bleomycin with either ^{57}Co-human serum albumin (HSA) or ^{57}Co-ethylenediaminetetraacetic acid (EDTA) showed the labeled bleomycin to be a strong chelate. The biologic distribution in tumor-bearing rats showed significantly higher concentration in tumors at 2 hr for fractions A_2 and B_2 as compared to the bleomycin mixture. The other fractions, A_1 and demethyl A_2, gave lower tumor concentrations than the bleomycin mixture. The tumor-to-blood ratios for A_2 and B_2 were not significantly different from the bleomycin mixture, suggesting that the concentration of the bleomycin in the tumor was related to blood concentration. Tumor-to-blood ratios of greater than 10:1 at 2 hr were achieved for A_2, B_2, and the mixture; ratios of greater than 31:1 were achieved at 24 hr for all three. From these data it appears that the major components A_2 and B_2 are the most useful for diagnostic tumor imaging.

TABLE 1. STRUCTURE OF BLEOMYCIN TERMINAL AMINES

Bleomycin component	Terminal amine group
A_1	$H_2N-(CH_2)_3-S-\overset{\overset{O}{\|}}{C}-CH_3$
Demethyl-A_2	$H_2N-(CH_2)_3-S-\overset{\overset{O}{\|}}{C}-CH_3$
A_2	$H_2N-(CH_2)_3-S^{+}(CH_3)-CH_3$
B_1	H_2N
A'_2-a	$H_2N-(CH_2)_4-NH_2$
A'_2-b	$H_2N-(CH_2)_5-NH_2$
A'_2-c	$H_2N-CH_2-CH_2-C(=N)-HC=CH-NH$
B_2	$H_2N-(CH_2)_4-NH-\overset{\overset{NH}{\|}}{C}-NH_2$
B_4	$H_2N-(CH_2)_4-NH-\overset{\overset{NH}{\|}}{C}-NH-(CH_2)_4-NH-\overset{\overset{NH}{\|}}{C}-NH_2$
A_5	$H_2N-(CH_2)_5-NH-(CH_2)_4-NH_2$
A_6	$H_2N-(CH_2)_5-NH-(CH_2)_4-NH-(CH_2)_5-NH_2$

Bleomycin is a mixture of closely related antibiotics that has been used extensively in the treatment of various malignant tumors. Chemically this antibiotic acts as a chelating agent and has been shown to bind a number of divalent and trivalent cations (*1*). This property has permitted the development of several radiolabeled bleomycins that have been

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used as radiopharmaceuticals for tumor scintigraphy. However, chelates of indium, technetium, gallium, and copper have not proven as stable in vitro and in vivo as the cobalt-bleomycin compound. Cobalt-57-bleomycin has proven to be a diagnostically sensitive radiopharmaceutical (*2–4*), but little is known about the nature of its individual fractions in regard to their metal-binding capacity, their antibiotic activity, and their in vivo distribution. Umezawa and coworkers (*5*) have isolated some 13 naturally occurring components that contain a common nucleus but differ in the terminal amine. The structure of eleven terminal amines has been established (Table 1).

Several investigators have presented distribution data for various fractions using ^{3}H-bleomycin (*6,7*) or microbiologic assay (*8,9*) but no report of the effect of chelation on distribution has appeared. We have investigated the chemical and biologic properties of several ^{57}Co-labeled fractions of bleomycin.

METHODS AND MATERIALS

Chromatography. Separations were carried out on a Waters Model ALC 202–6000 psi high performance liquid chromatography system (HPLC) equipped with stainless steel columns. Table 2 lists a summary of the chromatographic systems and conditions used. The identities of the separated components were confirmed by chromatography on silica gel plates eluted with 10% ammonium acetate: methanol (1:1) as suggested by Umezawa, et al (*10*). Retention values (R_f) were compared with the bleomycin mixture run on the same chromatographic plate. The R_f values were similar to those reported by Umezawa, et al (*10*), namely 0.40 for A_2, 0.68 for B_2, 0.74 for A_1, and 0.80 for demethyl A_2(DMA_2). Bleomycin labeled with ^{57}Co was prepared as previously described (*3*).

Competitive binding study. Equimolar solutions (0.18 n*M*) of ^{57}Co-bleomycin and ethylenediaminetetraacetic acid (EDTA); ^{57}Co-EDTA and bleomycin; ^{57}Co-bleomycin and human serum albumin (HSA); and ^{57}Co-HSA and bleomycin were mixed at pH 6.5. Mixtures were analyzed chromatographically on silica gel as noted above after reaction times varying from 0 to 72 hr (Table 3). Retention values were compared with those reported in the literature. The R_f value of ^{57}Co-EDTA is 0.84; ^{57}Co-HSA did not move from the origin.

Biologic distribution studies. The distribution studies of several radiolabeled bleomycin fractions were performed in female Fischer-344 rats implanted with 13762 mammary adenocarcinoma (Mason Research Institute, Worcester, Mass.) (Table 4). Rats weighed approximately 150 gm at the time of study 2 weeks after tumor implantation; average tumor weight was 4 gm. Under light halothane anesthesia, rats were intravenously injected with one of the radiopharmaceuticals. Either 2 or 24 hr later, a cardiac blood sample and samples of tumor, muscle, fat, and liver were obtained and weighed. Tumor samples were carefully dissected and only solid portions without

TABLE 2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF BLEOMYCIN MIXTURE

Column system	Mobile phase	Operating conditions
1. Bondapak phenyl or C_{18} pellicular 0.21 × 183 cm	Acetonitrile:water (1:1)	Room temperature flow = 1 ml/min
2. Vydac pellicular strong cation exchange 0.21 × 274 cm	NH_4OOCH in water; pH 6.4; linear gradient 0–1.0 *M*	Room temperature or 60°, flow = 1 ml/min and higher
3. Bio-Rex-70 0.95 × 122 cm	NH_4OOCH in water; pH 6.4; linear gradient 0–1.0 *M*	Room temperature or 60°, flow = 1 ml/min and higher
4. Chelex-100 copper form, 0.95 × 122 cm	NH_4OOCH in water; pH 6.4; 0.1, 0.2, or 0.5 *M*	Room temperature or 60°, flow = 1 ml/min
5. Biogel P-2 2.54 × 91 cm	0.1 *M* NH_4OOCH in 10% aqueous methanol; pH 6.4	Room temperature or 60°, flow = 0.3 ml/min
6. Hydrogel IV 0.95 × 122 cm	NH_4OOCH in 0.1% aqueous polyethylene glycol; pH 6.4; linear gradient 0–1 *M*	Room temperature flows up to 5 ml/min (2000 psi)
7. Pellidon pellicular polyamide 0.21 × 274 cm	Methanol:water (1:1); linear gradient of acetic acid from 0–2%	Room temperature flows up to 5 ml/min
8. Porasil A 0.95 × 122 cm	5% NH_4OOCH: methanol (1:1); pH 6.4	Room temperature flow = 1 ml/min
9. Porasil A 0.95 × 122 cm	0.3% NH_4OOCH: methanol (1:1); pH 6.4; flow gradient	Room temperature flow gradient 1 ml/min to 5 ml/min (400–2500 psi)

TABLE 3. COMPETITIVE BINDING STUDY

Reaction mixture	Percent unchanged ^{57}Co-chelate reaction time (hr) 0	24	72
^{57}Co-bleomycin/EDTA	100	100	100
^{57}Co-EDTA/bleomycin	100	26	6
^{57}Co-bleomycin/HSA	100	100	100
^{57}Co-HSA/bleomycin	100	0	0

TABLE 4. DISTRIBUTION OF ^{57}Co-LABELED BLEOMYCIN FRACTIONS IN TUMOR-BEARING RATS

Organ	Time (hr)	Bleomycin mixture	A_2	B_2	A_1	DMA_2
Tumor	2	0.240 ± 0.065(5)*	0.556 ± 0.296(15)†	0.642 ± 0.229(19)†	0.228 ± 0.103(14)	0.223 ± 0.092(11)
	24	0.228 ± 0.034(5)	0.142 ± 0.034(10)†	0.246 ± 0.093(18)	0.058 ± 0.043(11)†	0.066 ± 0.038(11)†
Blood	2	0.023 ± 0.013(5)	0.036 ± 0.018(15)	0.055 ± 0.033(19)†	0.027 ± 0.011(14)	0.041 ± 0.036(11)
	24	0.007 ± 0.001(5)	0.005 ± 0.002(10)	0.007 ± 0.004(18)	0.004 ± 0.002(11)†	0.004 ± 0.001(11)†
Liver	2	0.130 ± 0.047(5)	0.160 ± 0.112(15)	0.184 ± 0.043(19)	0.072 ± 0.023(14)	0.070 ± 0.019(11)
	24	0.227 ± 0.031(5)	0.088 ± 0.022(10)†	0.140 ± 0.042(18)†	0.046 ± 0.014(11)†	0.036 ± 0.005(11)†
Tumor-to-blood ratio‡	2:24	12:34	15:31	19:37	14:16	11:17

* Results expressed as percent dose per gram ±1 s.d. (number of animals).
† $p < 0.01$ compared to bleomycin mixture.
‡ The average tumor-to-blood ratios were calculated from individual tumor-to-blood ratios and not from the average values.

gross necrosis or cyst formation were analyzed. The tissue samples were counted in a well scintillation counter [NaI(Tl) crystal] and compared to an appropriately prepared standard.

The results for tissue concentration were expressed as the percentage of the injected dose per gram of tissue wet weight. Each group contained five or more animals. The ^{57}Co-labeled fractions obtained from the HPLC were compared with the ^{57}Co-labeled radiopharmaceutical using bleomycin currently available for therapeutic purposes (Blenoxane, Bristol Laboratories, Syracuse, N.Y.). The commercial mixture is stated to contain approximately 55% A_2, 27% B_2, 9% A_1, and 3% DMA_2. This material is labeled directly with $^{57}CoCl_2$ and is referred to as ^{57}Co-labeled bleomycin mixture.

Pharmacology. Samples of serum, urine, and saline extracts of homogenized liver, tumor, and kidney from tumor-bearing rats euthanatized at 2 hr were chromatographed on silica gel as noted above to determine the radiochemical purity of the labeled bleomycin in tissue. Blood clearance in rabbits was determined and these blood samples were also analyzed chromatographically (Table 5).

TABLE 5. IN VIVO RADIOCHEMICAL PURITY OF ^{57}Co-LABELED BLEOMYCIN MIXTURE IN RABBITS

Percent	Time (min) 10	30	60	120	240	1440
Dose in blood*	18.3	12.3	8.5	4.9	1.5	0.4
^{57}Co-bleomycin	95.7	95.4	95.0	93.9	77.3	46.9
Unbound ^{57}Co	4.3	4.6	5.0	6.1	22.7	53.1

* Disappearance from blood of ^{57}Co-bleomycin mixture in a rabbit. Chromatographic results are expressed as percent of activity in blood at the indicated times.

RESULTS

Chromatography. The bleomycin mixture supplied commercially is copper free. All chromatographic techniques reported by Umezawa, however, have required preliminary chelation with copper in order to effect acceptable separations (*11*). This presents a problem if one attempts to label individual fractions since the added copper necessary for isolation of the fraction must first be removed before adding the radioactive species of interest. Furthermore, it has been found that removal of copper leads to partial damage of bleomycin as shown by altered chromatographic properties. This is most easily detected in the A_2 fraction where the R_f changes from 0.40 to approximately 0.80 after removal of copper by hydrogen sulfide precipitation. The aim, therefore, was to develop a system for separating the bleomycin complexes in copper-free form. Separation of bleomycin by reverse-phase chromatography (System 1) was not successful. This method has been used to separate amines but only at higher pH values that deactivate bleomycin. No separation was obtained on Systems 4, 6, and 7 because of strong adsorption of bleomycin to the solid support. Systems 2 and 3, which are cation-exchange columns similar to the CM Sephadex C25 used by Umezawa, et al (*10*) for separation of Cu^{2+} chelates of bleomycin, required concentrated ammonium formate solution to elute the components. This was also true of System 5. The necessity of performing separation on these systems at high temperatures, of removing relatively large amounts of ammonium formate, and of lyophilizing large volumes of solution all contributed to a difficult recovery process. Under these circumstances, the fraction A_2 is demethylated to DMA_2 by the heat required in the lyophilization process and may then oxidize to A_1. Moreover, separation of ^{57}Co-bleomycin in these systems has resulted in substantial removal of cobalt by the resin.

To procure pure fractions of bleomycin, great care is required to avoid excessive heat and oxygen.

A silica gel column (Porasil A) eluted with methanol:0.3% ammonium formate (1:1) separated the fractions of copper-free bleomycin in a small elution volume and did not require the high salt concentrations that interfere with the lyophilization process. The capacity of this column is such that milligram quantities of A_2, A_1, DMA_2, and B_2 have been separated. The identity of these peaks has been confirmed by radiolabeling with ^{57}Co either before or after HPLC, followed by thin-layer chromatography (TLC). The chromatographic behavior on TLC was identical for the ^{57}Co fractions labeled before or after HPLC.

Competitive binding studies and pharmacology. Results in Table 3 show that bleomycin is a strong chelating agent for cobalt. The equilibrium is established slowly, however, as is seen in the case of the transfer of ^{57}Co from EDTA to bleomycin. The radiochromatographic pattern of ^{57}Co-bleomycin in urine, plasma, and liver extracts from tumor-bearing rats was identical to that of the original material. Also, chromatography of urine collected from patients showed that ^{57}Co-bleomycin was excreted unchanged. However, chromatography in rat tumor extracts revealed a ^{57}Co distribution that differed from that of ^{57}Co-bleomycin in that the majority of the activity remained at the origin on the TLC system. The clearance of bleomycin from the blood in rabbits was rapid and the ^{57}Co activity remaining in the blood migrated as bleomycin (Table 5).

Biologic distribution studies. Tissue distribution studies were carried out on the separated fractions from column Systems 8 and 9 in which the Porasil A support had been eluted with aqueous ammonium formate:methanol mixtures. In System 8, 5% ammonium formate was used and, although a clearly defined separation on HPLC was obtained, the subsequent chromatography on the TLC system showed damage to the A_2 fraction. The DMA_2, A_1, and B_2 fractions exhibited the expected R_f values, but the A_2 fraction appeared to be less polar and migrated with R_f of 0.80 instead of the expected 0.40. The distribution obtained in the tumor-bearing rats indicated that the biologic behavior of all fractions had changed; the blood concentrations were significantly higher ($p < 0.01$) than those obtained with the ^{57}Co-labeled mixture, and the tumor values were slightly lower. Consequently, the tumor-to-blood ratios achieved with the fractions from System 8 were much lower than the corresponding value for the mixture.

When the study was repeated using 0.3% ammonium formate (System 9), the fractions migrated identically to those in the radiolabeled bleomycin mixture on TLC. Distribution studies in tumor-bearing rats (Table 4) showed that A_2 and B_2 had significantly higher tumor concentrations at 2 hr than did the mixture ($p < 0.01$). Nevertheless, the tumor-to-blood ratios for A_2, B_2, and the mixture were similar, and all three radiopharmaceuticals exceeded 10:1 at 2 hr and 31:1 at 24 hr. The remaining fractions, DMA_2 and A_1, showed lower tumor-to-blood ratios at both times. The mean muscle concentration for all fractions was 0.027% dose/gm at 2 hr and 0.010% dose/gm at 24 hr. The mean fat concentration for all fractions was 0.092% dose/gm at 2 hr and 0.07% dose/gm at 24 hr.

DISCUSSION

The chromatographic separation and isolation of bleomycin fractions must be carried out under mild conditions in order to prevent the transformation of the A_2 fraction to dimethyl A_2 and A_1. Bleomycin labeled with carrier-free amounts of ^{57}Co, either before or after chromatography on System 9, does not show a change in biologic distribution as measured by tumor uptake and blood clearance. The labeling procedure with ^{57}Co does not seem to inhibit the ability of bleomycin to bind to DNA. A recent report indicates that ^{57}Co-bleomycin is associated with the DNA fraction in tumor homogenates obtained from mice implanted with Ehrlich solid tumor (*12*). This finding may explain the altered chromatographic behavior of bleomycin in the tumor homogenates in our study. The chromatographic technique reported here allowed us to prepare labeled individual fractions that appear to remain physically, chemically, and biologically intact. Results from the in vivo distribution studies of these compounds suggest that the major components, A_2 and B_2, are the most useful fractions for diagnostic tumor imaging because they show high tumor-to-nontumor ratios and have high absolute tumor concentrations. The tumor concentrations of these compounds seem to be related to blood concentration, since the tumor-to-blood ratios for the mixture, A_2, and B_2 were all similar whereas the absolute concentrations varied. No significance can be attached to the two- to fourfold decrease in A_2, B_2, A_1, and DMA_2 concentration in tumor at 24 hr. It appears that those fractions with charged side chains, namely A_2 and B_2, achieve high tumor-to-nontumor ratios whereas the neutral side chains in DMA_2 and A_1 do not give such differential uptake (Table 1). Since A_1 and DMA_2 are degradation products of A_2, the use of a purified A_2 and B_2 mixture might give better diagnostic results. The implication of these findings for possible modification of the use of the bleomycin mixture for chemotherapy is unknown but worthy of further study.

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