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PREPARATION, BIODISTRIBUTION AND STABILITY OF [^{65}ZN]BLEOMYCIN COMPLEX

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ABSTRACT

Bleomycin (BLM) has been labeled with various radioisotopes and widely used in therapy and diagnosis. In this study BLM was labeled with [^{65}Zn] zinc chloride and its distribution and stability in normal and tumor bearing mice was determined. The complex was obtained at the pH=2 in normal saline at 90 °C in 60 minutes. Radio-TLC showed an overall radiochemical yield of 95-97% (radiochemical purity >97%). The *in vitro* stability of the complex was determined in mice and human plasma. Preliminary studies were performed to determine distribution of [^{65}Zn]BLM in normal and tumor bearing mice on the basis of these results. [^{65}Zn]BLM may be used in therapeutic studies due to its suitable physico-chemical properties.

Keywords: Zinc-65, Bleomycins, Biodistribution, Euger emitters, Cyclotron

INTRODUCTION

Bleomycins are a group of peptidoglycoside antitumor antibiotoics that were initially isolated from *Sacharomyces* sp. cultures (figure 1.). Several radiolabeled bleomycin derivatives have been developed for imaging and/or therapy of neoplastic tissues. The most important imaging compounds contain indium-111(1), cobalt-57 (2), technetium-99m (3) and radioferric salts (4). Recently rhodium-105 labeled bleomycin has been studied for the therapeutic purposes (5). In the previous study, preparation of a radiolabeled bleomycin complex containing an euger-electron emitter isotope, gallium-67, as a therapuetic/ imaging agent (6) and [^{62}Zn]labeled bleomycin for diagnostic PET purposes (7) were reported.

Zn-65 (HL=244.3 d, Eγ:1115.5 keV; 50.8%) is a long-half life radioisotope that is mostly used in zinc metabolic studies in normal and malignant tissues (8,9). Other than a recent report about the use of zinc-65 as chloride in brain tumor imaging (10) there has not been any report so far about its uses in labeling and nuclear medicine studies. Preparation of [^{62}Zn] labeled bleomycin has been reported but without biological studies (11). Our recent studies on the preparation and tumor imaging properties of [^{62}Zn]bleomycin in normal and tumor-bearing mice showed a high tumor/blood and tumor/muscle ratio suggesting that it might be an appropriate candidate as a diagnostic agent (7).

The aim of this study was to investigate the possibility of incorporation of a therapeutic euger-electron emitter, *i.e.* zinc-65 with an antineoplastic compound, bleomycin, for the use in tumor therapy. Due to interesting properties and increasing importance of euger-electron emitting radiotracers (12, 13), conditions for formation of complex between zinc-65 and bleomycin was optimized, for further development of [^{65}Zn]BLM. The preparation, optimization, stability, formulation and biodistribution studies of [^{65}Zn]-bleomycin complex in normal and tumor-bearing mice were investigated in this study.

MATERIALS AND METHODS

Chemicals were purchased from Aldrich Chemical Company, Milwaukee, WI (USA). Bleomycin sulfate (BLEO-S) was a pharmaceutical sample purchased from Nippon Kayaku laboratories (Japan). Thin layer chromatography (TLC) was performed on polymer-backed silica gel (F 1500/LS 254, 20 × 20 cm, TLC Ready Foil, Schleicher & Schuell®-Germany). Methanol used for labeling was of "Sure-Seal" grade Aldrich. A mixture of ammonium acetate 10% and methanol (1:1) was used as eluent. Radio-chromatography was performed by counting different 5 mm slices of polymer-backed silica gel paper using a Canberra™ high purity germanium detector (model GC1020-7500SL). All calculations and TLC countings were based on 1115.5 keV peak. Murine fibroblastoma tumor cell lines were taken from the Department of Immunology, Shaheed Beheshti University of Medical Sciences.

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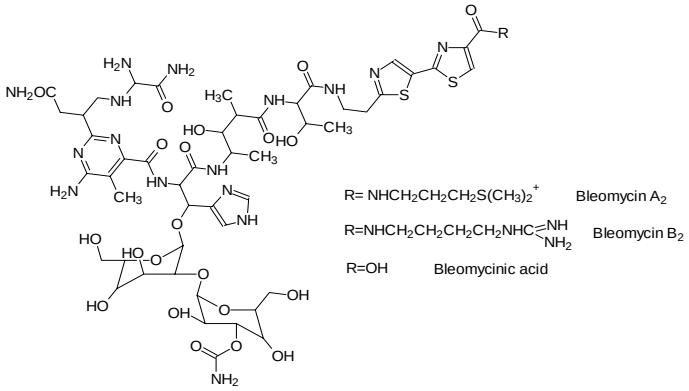

Figure 1. Structures of commercial bleomycin components

Animal experiments were carried out in compliance with the *United Kingdom Biological Council`s Guidelines on the Use of Living Animals in Scientific Investigations*, 2nd edn. Male Balb-C mice weighing 15-20 grams were purchased from Razi Institute of Iran.

Preparation of [^{65}Zn]zinc chloride from natural copper solid target

[^{65}Zn]Zinc chloride was prepared by 29.5 MeV proton bombardment of a natural copper-target in a 30 MeV cyclotron (Cyclone-30, Nuclear Research Center for Agriculture & Medicine, Karaj, Iran) based on a non-carrier-added method described previously (14) with slight modifications. The copper target was bombarded with a current intensity of 150 μA for 20 minutes. The resultant activity of ^{65}Zn was 4.6 mCi and the production yield was 92 μCi/μAh. After dissolution of irradiated target in 7N HNO_3, the solution was heated under a flow of nitrogen gas until a precipitate was formed. The residue was rinsed 2 times with distilled water (10 ml) and heated to drynerss. A portion of 6N HCl (75 ml) was added and mixed gently. The solution was passed through a cation exchange resin (Dowex 1×8, Cl^- form) which had been pre-conditioined by passing 5 ml of 6 N HCl. The column was then washed by 50 ml of 2N HCl to remove all copper ion contents. Finally ^{65}Zn cations were washed out by 150 ml of 0.05 N HCl. The resulting high-purity Zinc- 65 chloride solution was used directly in the labeling step. Radionuclide purity was checked by a Canberra™ high purity germanium detector (model GC1020-7500SL), comparison of area under the curve of 1115.5 peak to the rival reaction products showed a purity of at least 99%. All calculations and TLC counting were based on 1115.5 keV peak (figure 2.).

Labeling of bleomycin with [^{65}Zn]zinc chloride

Labeling procedure was performed using some reported guidelines for the production of In-111 bleomycin (15) with slight modifications. [^{65}Zn]Zinc chloride (0.25-2.5 mCi) was dissolved in acidic media which was obtained as above (0.5-2 ml) and was transferred to a 2 ml-vial and pH of the solution was adjusted to 1-7 using 1M HCl and/or 1M NaOH. The mixture was evaporated by slight warming under a nitrogen flow. A mixture of BLM (0.25-2.5 mg) in normal saline (0.1 ml) was then added and heated at different temperatures (25, 50, 80 and 100°C). The mixture was cooled in an ice bath and was rapidly sent for using it. The active solution was checked for radiochemical purity by polymer-backed silica gel layer chromatography using a 1:1 mixture of 10% ammonium acetate and methanol as mobile phase. Radio thin layer chromatography showed two major and distinct radio peaks at R_f s of 0.40 and 0.70. The radiochemical yields (>95% in each case) were also determined by RTLC method. These analyses were carried out every 30 minutes after the labeling step. The final solution was then passed through a 0.22 μ filter and pH was adjusted to 5-7 by addition of sodium acetate (1M) buffer. The gamma spectroscopy of the final sample was carried out by a HPGe detector and showed a radio-nuclide purity higher than 99% by comparison of 1115.5 keV to other intruder peaks. Pyrogen test was performed using a commercial LAL kit (Sensitivity 0.125 EU/ml, Charles River Endosafe Co., U.S.). Microbial-fungal tests showed suitable pharmaceutical sterility.

Stability of [^{65}Zn]BLM complex in final product

Stability studies were based on the previous studies performed for other radiolabeled bleomycins (16). A sample of [^{65}Zn]BLM (0.5 mCi) was kept at room temperature for 48 hours while checked by RTLC at various time intervals

(2, 12, 24, 72 and 96 hours). A micropipet sample (50 µl) was taken from the shaking mixture and the ratio of free radiozinc to [^{65}Zn]BLM was checked by radio thin layer chromatography (eluent: 10% NH_4OAc buffer and methanol 1:1). The patterns for [^{65}Zn]$ZnCl_3$ and [^{65}Zn]BLM did not change during 24 hrs.

In vitro studies of the stability of [^{65}Zn]BLM complex in human and mice sera

A previously reported method (17) was used for the serum stability studies. A mixture of 5 parts of serum and one part of radiopharmaceutical (0.2 mCi) was shaked in a 37-degree incubator under nitrogen atmosphere. A micropipet sample (50 µl) was taken from the shaking mixture every 30 minutes. The ratio of free radiozinc (R_f=0) to [^{65}Zn]BLM (R_f= 0.4 & 0.7) was checked by radio thin layer chromatography (eluent: pH 5.6 NH_4OAc buffer and methanol 1:1).

Cell line formation

Cell line of murine fibroblastoma was employed for this investigation. Culture of 1-2 × 10^4 cells of murine fibroblastoma were seeded into a 75 cm^3 flask containing 20 ml of medium supplemeted with 10% fetal bovine serum and 1% glutamine. Cells were incubated at 37 °C in 5% CO_2 and were maintained in exponential growth phase and passaged twice per week.

Animal studies

Tumoral animal studies and procedures were performed by the previous methods for evaluation of bleomycin labeled compounds (18). Fibrosarcoma cells (about 10^4) were injected subcutanously to the dorsal area of Balb C mice weighing 20-25 g. After 14 days the animals were sacrificed and tumor tissues which were not grossly necrotic weighed to a suitable amount (0.7±0.05 g). The distribution of [^{65}Zn]$ZnCl_2$ and [^{65}Zn]BLM in tissues were determined for untreated mice and for mice with fibrosarcoma. A volume (0.1 ml) of final [^{65}Zn]BLM solution containing 20-40 µCi radioactivity (≤ 6 µg bleomycin in 50 µL) was injected into the dorsal tail vein. The total amount of radioactivity injected into each mouse was measured by counting the 1-ml syringe before and after injection in a curiemeter with a fixed geometry. The animals were sacrificed by ether asyxphycation at selected times after injection (1,2 and 3 days), stool and the tissues (blood, heart, lung, spleen, intestine, skin, bladder, kidneys, liver, muscle and bone) weighed and their specific activities determined with a γ-ray scintillation detector and the results are expressed as percentage of injected dose per gram of tissues.

RESULTS AND DISCUSSION

Bleomycin is an antineoplastic agent, widely used in therapy (16). This compound produces suitable and stable complexes with cations like Mg^{2+}, Ca^{2+}, Fe^{2+}, In^{3+} (figure 1.) (19).

It is believed that this antibiotic interferes with DNA as false nucleotides and it is assumed that dithiazole moiety acts like a purine base (20). On the other hand, these compounds are activated by a cation insertion as anti-neoplastic agents. The whole complex can then act like a peroxidase system producing hydrogen peroxide, which causes DNA decomposition (2).Thus, labeling of bleomycin with bi/trivalent radioisotopes produces pharmacologically active compounds carrying a diagnostic and/or therapeutic radioisotope (21). In-111 labeled bleomycin ([^{111}In-BLM) has been widely used as a therapy/diagnostic agent (22,23). Zinc cation coordinates with at least five nitrogen atoms of bleomycin, based on NMR studies (24,25). This coordination forms a rather stable complex. Cell toxicity of Zn-Bleomycin has been studied and tested in human and different animals (26). The antitumor activity of Zn-Bleomycin complex has been elucidated in some human tumor models (27), suggesting the possibilty of application of radiozinc-bleomycin complexes in human tumor imaging.

Due to euger-emitting property of zinc-65 and the selective therapeutic effects of this radioisotope, the strategy of incorporation of such an isotope and a famous antineoplastic compound, *i.e.* bleomycin appeared of great interest.

Labeling

Because of the presence of several polar functional groups (NH, OH, ..) in its structure, labeling of bleomycin with a cation, does not affect its chromatographic properties extensively (28), and thus the labeled and unlabeled bleomycin almost migrate with the same R_f in RTLC method. The more polar bleomycin fractions, i.e., bleomycin A_2 & B_2 , correlates to the lower R_f, while other less polar fraction come at the higher R_fs (bleomycinic acid). In all radiolabeling procedures (n=5), the area under the curve ratio of the two peaks were constant (0.7:0.3), showing the isomeric ratio of two bleomycin chromatogram peaks (Figure 3.).

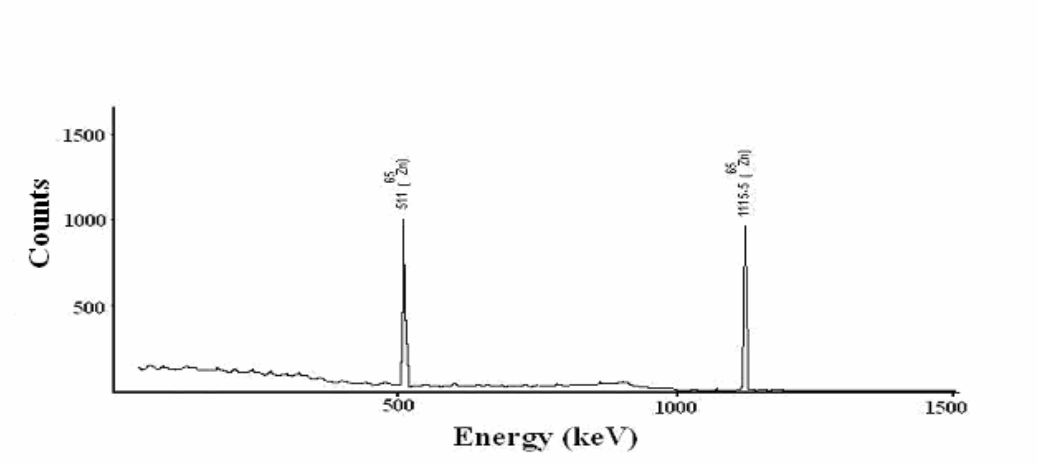

Figure 2. Gamma spectrum of the final purified [^{65}Zn]$ZnCl_2$ solution obtained by high purity germanium detector. A purity of at least 99% can be attributed to the product.

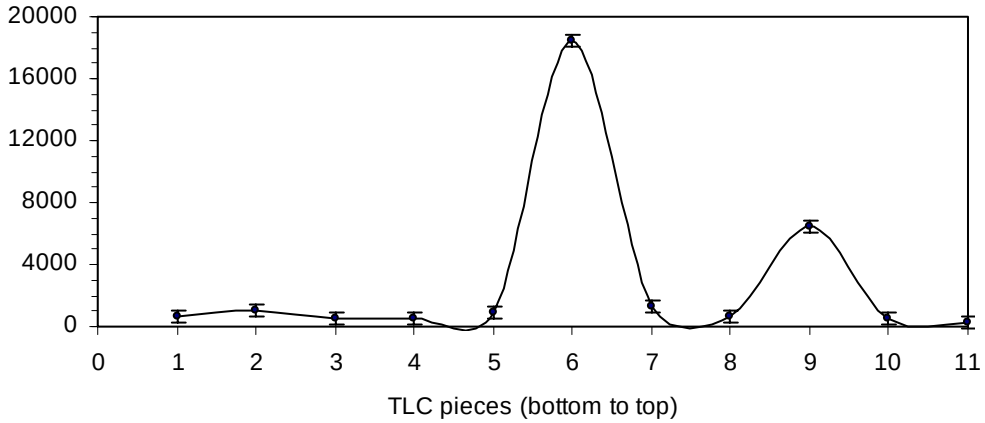

Figure 3. Radiochromatogram of a [^{65}Zn] bleomycin sample at optimized conditions, Left peak: Bleomycins A_2 & B_2 (lower R_f) ; Right peak: Bleomycinic acid (higher R_f)

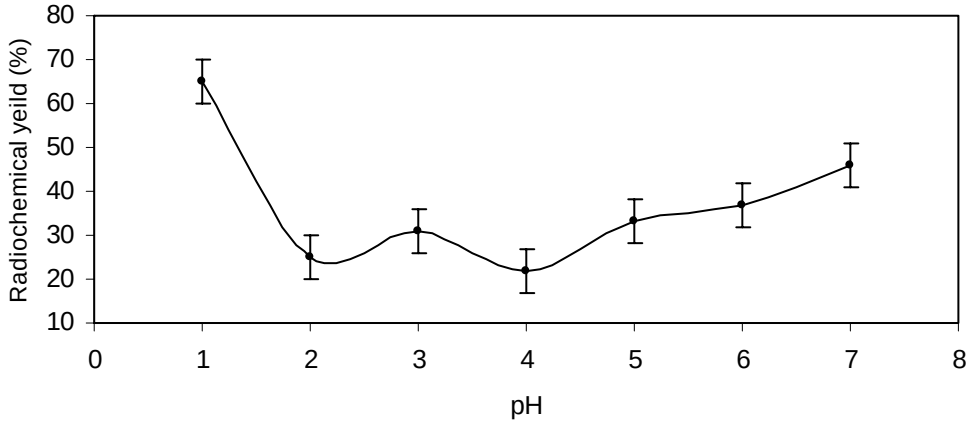

Figure 4. Variation of radiochemical yield of [^{65}Zn]bleomycin with pH at 80ºC

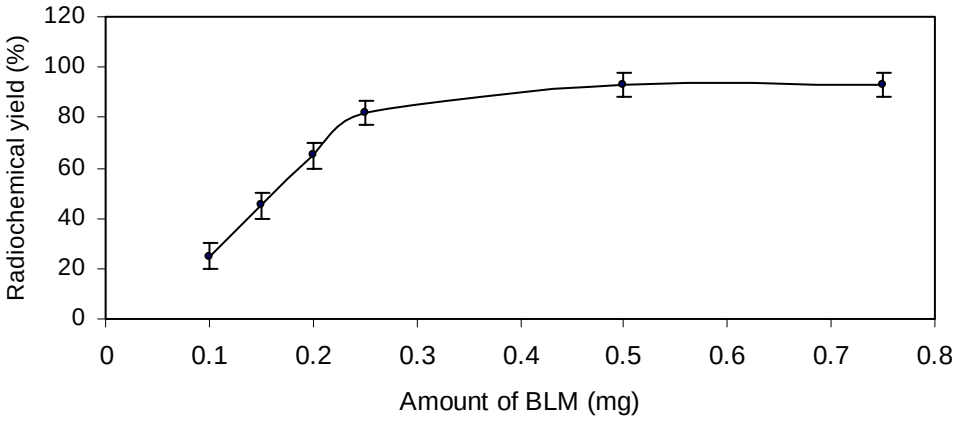

Figure 5. Variation of [^{65}Zn] bleomycin radiochemical yield with bleomycin amount (mg) at an experimental temperature (80ºC) and pH=2

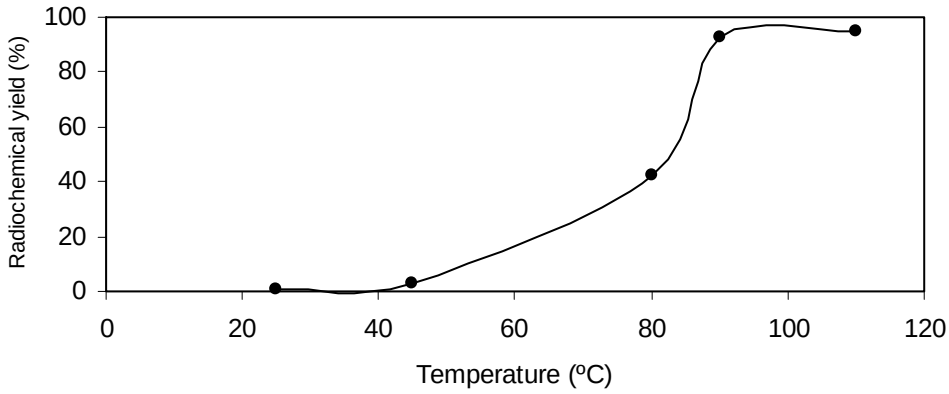

Figure 6. Variation of radiochemical yield of [^{65}Zn] bleomycin with temperature at optimized conditions

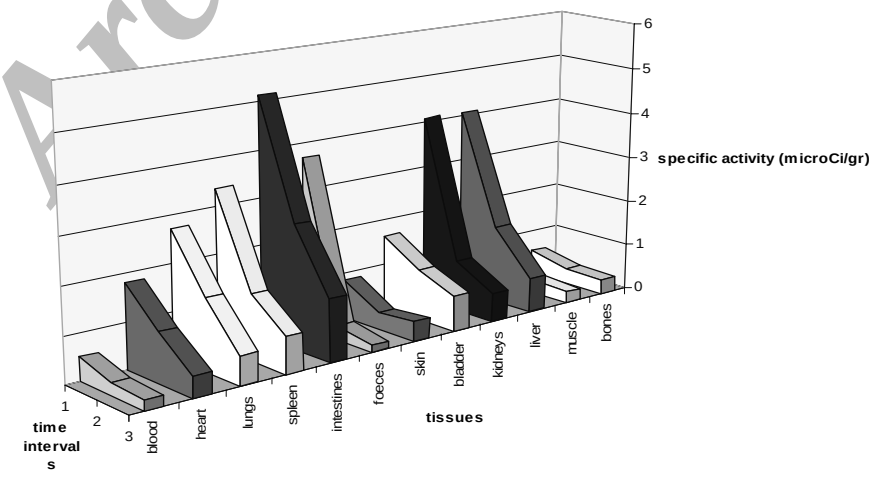

Figure 7. Biodistribution of [^{65}Zn]$ZnCl_2$ in organs of normal mice (n=5) (%ID/g tissue), : Average ± Standard Deviation;(standared deviation: less than 5%); time intervals (1: 24h, 2: 48h, 3: 72h)

Table 1. Bio-distribution of [^{65}Zn]-$ZnCl_2$ in organs of tumor-bearing mice (n=5) (%ID/g tissue), (Avg : average, SD: standard deviation: less than 5%)

Organ	Time (h)							
	24		48		72		96	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Blood	1.33	0.04	1.02	0.14	0.35	0.06	0.19	0.07
Liver	0.12	0.03	0.19	0.06	0.34	0.02	0.56	0.18
Kidney	1.0	0.06	1.16	0.18	1.30	0.12	0.86	0.15
Stomach	0.63	0.08	2.7	0.54	1.7	0.44	1.12	0.01
Colon	0.64	0.05	1.25	0.05	0.84	0.15	0.34	0.09
Stool	0.62	0.07	1.92	0.1	0.58	0.13	0.52	0.09
Bladder	3.12	0.56	2.79	0.15	2.48	0.21	1.84	0.15
Sternum	0.80	0.02	0.73	0.02	0.79	0.05	0.72	0.02
Lung	1.02	0.03	0.94	0.25	0.80	0.04	0.64	0.14
Skin	0.63	0.09	0.75	0.12	0.75	0.15	0.81	0.16
Muscle	0.26	0.11	0.32	0.11	0.29	0.02	0.17	0.05

Table 2. Biodistribution of [^{65}Zn]bleomycin in organs of tumor-bearing mice (n=5) (%ID/g tissue), Avg: average, SD: standard deviation

Organ	Time (h)							
	24		48		72		96	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Blood	2.49	0.44	3.92	0.54	4.46	0.26	4.99	0.27
Liver	3.1	0.23	2.99	0.6	3.44	0.71	3.96	0.78
Kidney	1.0	0.06	3.66	0.88	7.30	1.12	7.86	1.15
Stomach	1.3	0.08	2.7	0.54	4.7	0.84	5.12	1.01
Colon	0.44	0.05	0.85	0.05	1.21	0.05	1.34	0.09
Stool	1.62	0.07	1.92	0.1	2.18	0.13	3.52	0.19
Bladder	1.12	0.06	2.39	0.15	3.48	0.21	4.84	0.35
Sternum	0.81	0.02	0.78	0.02	0.73	0.05	0.67	0.02
Lung	1.1	0.03	1.74	0.55	2.20	1.94	2.64	0.54
Skin	1.65	0.09	1.75	0.12	1.76	0.15	1.81	0.12
Muscle	7.26	1.11	5.12	0.91	3.32	0.42	1.87	0.25
Tumor	4.33	0.85	5.32	0.99	5.34	0.92	5.77	1.01

In order to obtain the best labeling reaction conditions, the complex formation was optimized for pH, temperature, time, and the amount of bleomycin. At a random temperature (80°C for instance), the best pH for the labeling step was 2, while at higher pH (5-6) the radiochemical yield increased again due to the formation of different labeled species (figure 4.). At the alkaline conditions the radiochemical yield decreased drastically due to the degradation of bleomycin to the less soluble compounds (29).

At the optimum reaction temperature and pH, the yield reached a maximum within 25 minutes, and stayed constant for longer reaction times. Increasing the ratio of bleomycin to radioactivity increased the labeling yield, presumably due to the more availability of chelate in solution (figure 5.). Heating the reaction mixture to 90°C increased the yield, which remained constant for temperatures up to 100°C. Further heating reduced the radiochemical yield due to decomposition of bleomycin and/or product (figure 6.).

Twenty five to 40% of the activity remained on 0.22 Millipore filters when filtration was used to sterilize the product. The thermal stability of [^{65}Zn]BLM was excellent in a way that autoclaving a [^{65}Zn]BLM preparation showed no changes in the amount of free zinc present. The presence of 3-5% free zinc on the RTLC before and after autoclaving indicates that the final product may be sterilized by this technique. Due to decay of zinc-65 to copper-65, [^{65}Zn]BLM complex produces the stable complex, copper-65 BLM, which maintains tumor toxicity. The biological stability of [^{65}Zn]BLM was high enough to perform scanning due to high stability of the final product in the presence of murine/human blood serum so that RTLC showed no changes in the amount of free zinc up to 6 hours. The presence of 3-5 % free zinc on the RTLC was unchanged even after 6 hours.

Biodistribution in animal tissues

Final [^{65}Zn]BLM and [^{65}Zn]$ZnCl_2$ solutions were injected into the dorsal tail vein of test animals. The animals were sacrificed by ether asyxphycation at selected times after injection, the tissues were weighed and washed with saline and their specific activities (percentage of injected dose per gram) were determined by γ-ray scintillation method. Figure 7 demonstrates the biodistribution of [^{65}Zn]$ZnCl_2$ in organs of normal mice and table 1. shows the biodistribution of [^{65}Zn]$ZnCl_2$ in tumor-bearing mice.

Liver and spleen uptake increased 24-48 hours after administration of [^{65}Zn]BLM. Lung uptake increased after 48 hours. Twenty four hours after administration, the radioactivity of the bladder and kidney increased and remained constant like unlabeled bleomycin for the next few hours (4), suggesting the stable incorporation of Zn-65 into bleomycin core. These observations were quite different from the biodistribution of [^{65}Zn]$ZnCl_2$ which shows rapid wash-out from kidneys in the first 2-4 hours. A late increase in liver uptake was observed that might be due to the accumulation of metalloproteins in this tissue (Tables 1,2). Table 2 demonstrates the biodistribution of [^{65}Zn]BLM in organs of tumor-bearing mice.

Our results were similar in some aspects with *in vivo* biodistribution experiments previously performed for [^{111}In]bleomycin. [^{65}Zn]Bleomycin is rapidly tagged in tumor and scanning may be achieved in rather short times after I.V. injection. Higher half-life of Zn-65 in contrast to In-111 is another important advantage leading to higher irradiation to tumor cells for therapeutic purposes. Due to our current studies on the positron emitter zinc-62 (HL: 9.6 h) as an interesting PET radioisotope having labeling potentials with appropriate ligands like bleomycins, results of this paper may be of great importance. Higher half life of zinc-65 affords longer experiments in optimization studies as well as less cyclotron working hours. On the other hand long half life of zinc-65 (244 d) as a auger emitter may afford a possibly suitable therapeutic agents for ovary, testicle and some other bleomycin-responsing tumors having synergistic radiation/cell toxicity effects.

Total labeling and formulation of [^{65}Zn]BLM took about 60 min, with a yield of 95-97% at 90°C and pH. 2. A suitable specific activity product was formed *via* insertion of [^{65}Zn]zinc cation. The radio-labeled complexes were stable in aqueous solutions at least for 6 days, and no significant amount of other radioactive species were detected by TLC 6 days after labeling. Trace amounts of [^{65}Zn]zinc chloride (≈3%) were detected by paper chromatography. TLC showed that radiochemical purity of the [^{65}Zn]labeled components was >95%. In contrast to other labeled bleomycins, [^{65}Zn]bleomycin, has a high half life causing long irradiation effects on the microorganules in tumor cell by a short range of its auger-electron emission. Relatively high chemical stability for this complex suggests its use as a possible therapeutic agent.

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