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CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF BLEOMYCIN COMPLEX

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SUMMARY

Three thin-layer chromatographic methods were developed for the separation of the components of bleomycin which allowed direct bioautographic detection. The change in the composition of heat degraded samples of bleomycin was monitored by these methods and by high-performance liquid chromatography. It was found that the microbiological potency estimates of the heated material did not accurately reflect the changes which had occurred during degradation.

INTRODUCTION

The antitumour antibiotic bleomycin is a mixture of closely related water-soluble basic glycopeptides, which differ only in their terminal amine. The material available for clinical use in Great Britain consists mainly of bleomycins A_2 and B_2. Minor components A_1, A_5, B_4, demethyl A_2 and bleomycinic acid, may be present. The potency of bleomycin complex is determined by microbiological assay[1], even though the antibacterial activity of the different bleomycins does not necessarily parallel their antitumour activity[2]. Stability studies showed that the potency estimation of degraded samples was dependent upon the micro-organism used for the assay and that changes in the composition of the samples were not reflected by corresponding changes in the potency estimates[3,4]. The W.H.O. Expert Committee on Biological Standardization has emphasised that the potencies of bleomycin preparations determined by microbiological assays in terms of the international unit of activity are likely to provide a relative measure of antitumour activity only if the component composition of each preparation has been shown by chemical analysis to be within acceptable limits.[5] Specifications for the component composition of bleomycin determined by high-performance liquid chromatography (HPLC)[6] are being formulated[7].

Several chromatographic methods for the separation and quantitation of bleomycin have been described. The original time-consuming ion-exchange method[2] has been used for the certification of bleomycin[1], both the time and the amount of analyte required have been reduced[8–10]. Straight-phase[11,12] and reversed-phase[13,14] HPLC

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have been proposed; most workers currently prefer reversed-phase ion-pair systems[6,15,16]. The only quantitative method capable of resolving most of the components likely to be found in commercial preparations of bleomycin took at least 70 min[6]. The components of bleomycin have been separated as copper chelates on both silica and cellulose thin-layers[17,18] and bioautography has been used to differentiate some of the antibiotics of the pheomycin–bleomycin group after chromatographic separation on paper and cellulose plates[19].

Thin-layer chromatographic (TLC) separations of bleomycin with direct bioautographic detection were developed in order to demonstrate the antibacterial activities of the components present in the complex. The components of bleomycin were also separated by HPLC. Different estimates of potency have been obtained for degraded material with two organisms commonly used for the microbiological assay of bleomycin[3,4]. To investigate these differences, the changes in composition that occurred during the degradation of bleomycin were monitored by both TLC and HPLC.

EXPERIMENTAL

Materials

Bleomycin components A_1, A_2, A_5, B_2, demethyl A_2 and bleomycinic acid were very kindly provided by Dr. Yasuhiko Muraoka of Nippon Kayaku, Tokyo, Japan. Samples of bleomycin from China, Japan and U.S.S.R. were compared with the International Reference Preparation of Bleomycin[3]. Degraded material was obtained by heating aliquots stored under nitrogen at 80°C for up to 72 h and at 140°C for up to 16 h.

The following pre-coated chromatoplates were used; silica gel 20 × 20 cm (Type K5, Whatman Chemical Separation, NJ, U.S.A.) and silica gel 60 silanised (Art 5746, E. Merck, Darmstadt, F.R.G.). Acetonitrile (HPLC-grade, Rathburn Chemicals, Walkerburn, U.K.), hexanesulphonic acid sodium salt (HPLC-grade, Fisons Scientific, Loughborough, U.K.) and analytical grade solvents and reagents (BDH, Poole, U.K.) were used.

TLC

The following three chromatographic systems were employed: (A) silica gel K5, with mobile phase, 0.19 *M* diammonium hydrogen phosphate–methanol (1:1), (B) silica gel 60 silanised, with mobile phase, 0.357 *M* ammonium nitrate–methanol (7:3), (C) silica gel 60 silanised, with mobile phase, 0.1 *M* hexane sulphonic acid–methanol (1:1).

Plates were activated at 100°C for 60 min (system B) or sprayed with mobile phase then dried at room temperature (system C) before use. Samples were dissolved in 50% aqueous methanol (20 mg ml^{-1}) diluted as necessary with the same solvent and 5-μl aliquots applied to the surface of the plate with a micro-syringe. The plates were developed over a distance of 15 cm in a filter-paper-lined chromatography tank, which had been saturated for at least 2 h. The plates were dried at 100°C for 30 min, cooled, sprayed with ninhydrin reagent (1 g ninhydrin, 10 ml glacial acetic acid, 50 ml ethanol) and heated at 100°C for 2 min. The bleomycin components appear as purple spots on a white background.

For biological detection, air-dried plates were covered with a 4-mm thick layer of either nutrient agar [1.0% (w/v) peptone, 0.3% (w/v) beef extract, 0.2% (w/v) sodium chloride, 1.5% (w/v) agar nobel] inoculated with a spore suspension of *Bacillus subtilis* NCTC 10400 or medium 34 (ref. 1) inoculated with a suspension of *Mycobacterium smegmatis* NCTC 7017 and incubated at 35°C for 18 h. Antibacterial activity was revealed as a clear zone of inhibition of growth. The contrast between the zone and the surrounding growth was enhanced by spraying the inoculated medium with an aqueous solution of *p*-iodonitrotetrazolium violet (2 mg ml^{-1}) and incubating for a further 5 min.

HPLC

The apparatus consisted of two reciprocating pumps, a gradient controller (Constametric I, Constametric II G, and a gradient master, Model 1601, Laboratory Data Control) and a variable-wave length spectrophotometer (Model CE272, Cecil Instruments) fitted with a 75-μl flow-through cell. A pre-column (50 × 4.6 mm) packed with general purpose grade Spherisorb ODS was placed between the solvent mixing chamber and the injector; an Ultrasphere ion-pair column (150 × 4.6 mm) was used for the separation. The mobile phase was filtered through a glass microfibre filter and degassed prior to use. It consisted of acetonitrile, 0.01 *M* sodium citrate buffer pH 4, 0.02 *M* hexanesulphonic acid sodium salt and 0.005 *M* disodium ethylenediaminetetraacetate. The selected gradient elution profile gave a linear increase in acetonitrile concentration from 14 to 23% (v/v) in 50 min. The flow-rate was 1 ml min^{-1}. Aqueous solutions of bleomycin (0.75 mg ml^{-1}) were injected through a Rheodyne injector (Model 7125) with a 20-μl fixed loop. The eluent was monitored at 293 nm, sensitivity 0.2 a.u.f.s. The relative composition of the samples was determined by normalisation of peak areas measured using a computing integrator (Model 301, Laboratory Data Control). It was assumed that all the bleomycin components have the same absorbance at 293 nm.

Microbiological assay

Degraded samples were assayed against the International Reference Preparation of Bleomycin using both *Bacillus subtilis* NCTC 10400 and *Mycobacterium smegmatis* NCTC 7017. A large plate, 3 + 3 Latin Square diffusion assay was employed.

TABLE I

R_F VALUES OF THE COMPONENTS OF BLEOMYCIN IN THE THREE TLC SYSTEMS USED

System A = silica gel K5; 0.19 *M* diammonium hydrogen phosphate–methanol (1:1). System B = silica gel 60 silanised; 0.357 *M* ammonium nitrate–methanol (7:3). System C = silica gel 60 silanised; 0.1 *M* hexanesulphonic acid–methanol (1:1).

Component of bleomycin	*A*	*B*	*C*
Bleomycin A_1	0.63	0.51	0.77
Bleomycin A_2	0.23	0.53	0.45
Bleomycin A_5	0.06	0.20	0.04
Bleomycin B_2	0.35	0.39	0.36
Demethylbleomycin A_2	0.73	0.24	0.57
Bleomycinic acid	0.80	0.83	0.98

Solutions (50 μl) were applied to the surface of the medium with fish spine heads. After 18 h incubation at 35°C the zones of inhibition of growth were measured (Optomax, Micro Measurements, Saffron Walden). The precision of the assays were ± 15% using *B. subtilis* and ± 6% using *Myco. smegmatis*. The antibacterial activities of the individual components were measured relative to bleomycin B_2.

RESULTS AND DISCUSSION

TLC

The three systems developed were each capable of separating the bleomycin components which were examined. The R_F values for the components in each system are given in Table I, typical chromatograms of bleomycins are shown in Fig. 1. The

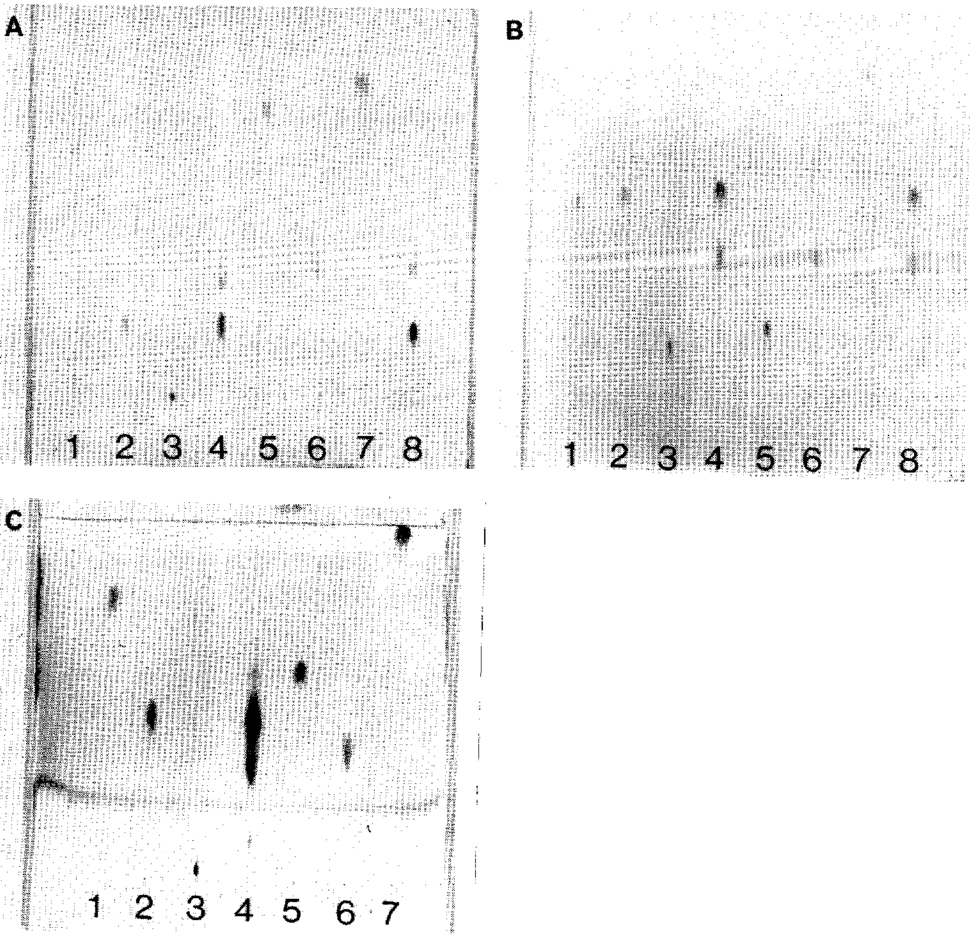

Fig. 1. Thin-layer chromatograms of the components of bleomycin detected with ninhydrin. (A) Silica gel K5; 0.19 *M* diammonium hydrogen phosphate–methanol (1:1). (B) Silica gel 60 silanised; 0.357 *M* ammonium nitrate–methanol (7:3). (C) Silica gel 60 silanised; 0.1 *M* hexanesulphonic acid–methanol (1:1). 1 = Bleomycin A_1, 10 μg; 2 = bleomycin A_2, 10 μg; 3 = bleomycin A_5, 10 μg; 4 = IRP bleomycin, 100 μg; 5 = demethylbleomycin A_2, 25 μg; 6 = bleomycin B_2, 10 μg; 7 = bleomycinic acid, 25 μg; 8 = IRP bleomycin, 100 μg. For system C the amounts were halved.

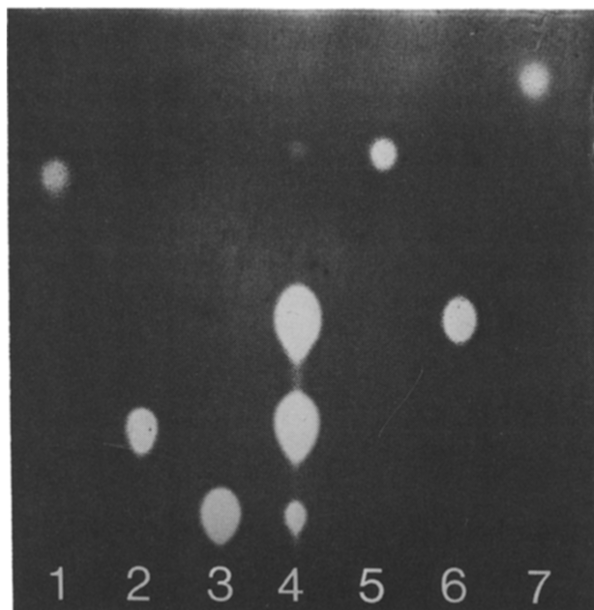

Fig. 2. Thin-layer chromatogram of the components of bleomycin detected with *Bacillus subtilis* NCTC 10400. Silica gel K5; 0.19 *M* diammonium hydrogen phosphate–methanol (1:1). 1 = Bleomycin A_1, 10 μg; 2 = bleomycin A_2, 2.5 μg; 3 = bleomycin A_5, 3.5 μg; 4 = IRP bleomycin, 37.5 μg; 5 = demethylbleomycin A_2, 3 μg; 6 = bleomycin B_2, 6 μg; 7 = bleomycinic acid, 60 μg.

presence of divalent metals which complex with bleomycin was found to affect the R_F value. A sample of bleomycin A_5 with the R_F value reduced from 0.06 to 0.04 in system A was shown to be saturated with zinc[20] and the addition of zinc acetate to bleomycin A_5 produced an identical reduction in R_F value. Though all three systems were suitable for bioautographic development, system A was the most sensitive. Detection of the components of bleomycin with *B. subtilis* is demonstrated (Fig. 2). The five-fold decrease in antibacterial activity in systems B and C was attributed to the stronger binding of the bleomycins to the silanised silica. An additional biologically active component (R_F = 0.12) was detected in the reference preparation with system C.

The samples heated at 80°C showed an increase in the content of demethylbleomycin A_2. An unidentified minor degradation product was detected (R_F = 0.65, system B, and R_F = 0.04, system C) which did not exhibit any antibacterial activity. Bleomycins A_2 and A_5 were not detectable either biologically or chemically in samples after 8 h at 140° and the amount of bleomycins B_2, demethylbleomycin A_2 and bleomycinic acid had fallen (Figs. 3 and 4). The decrease in the demethylbleomycin A_2 content was clearly demonstrated with the *B. subtilis* bioautograph, but was less apparent with the *Myco. smegmatis* bioautograph (Fig. 4), indicating the greater sensitivity of the latter organism to demethylbleomycin A_2.

HPLC

The method developed revealed up to 15 components (Table II, Figs. 5 and 6). Bleomycins A_5 and B_2 could only be resolved by extending the gradient time to

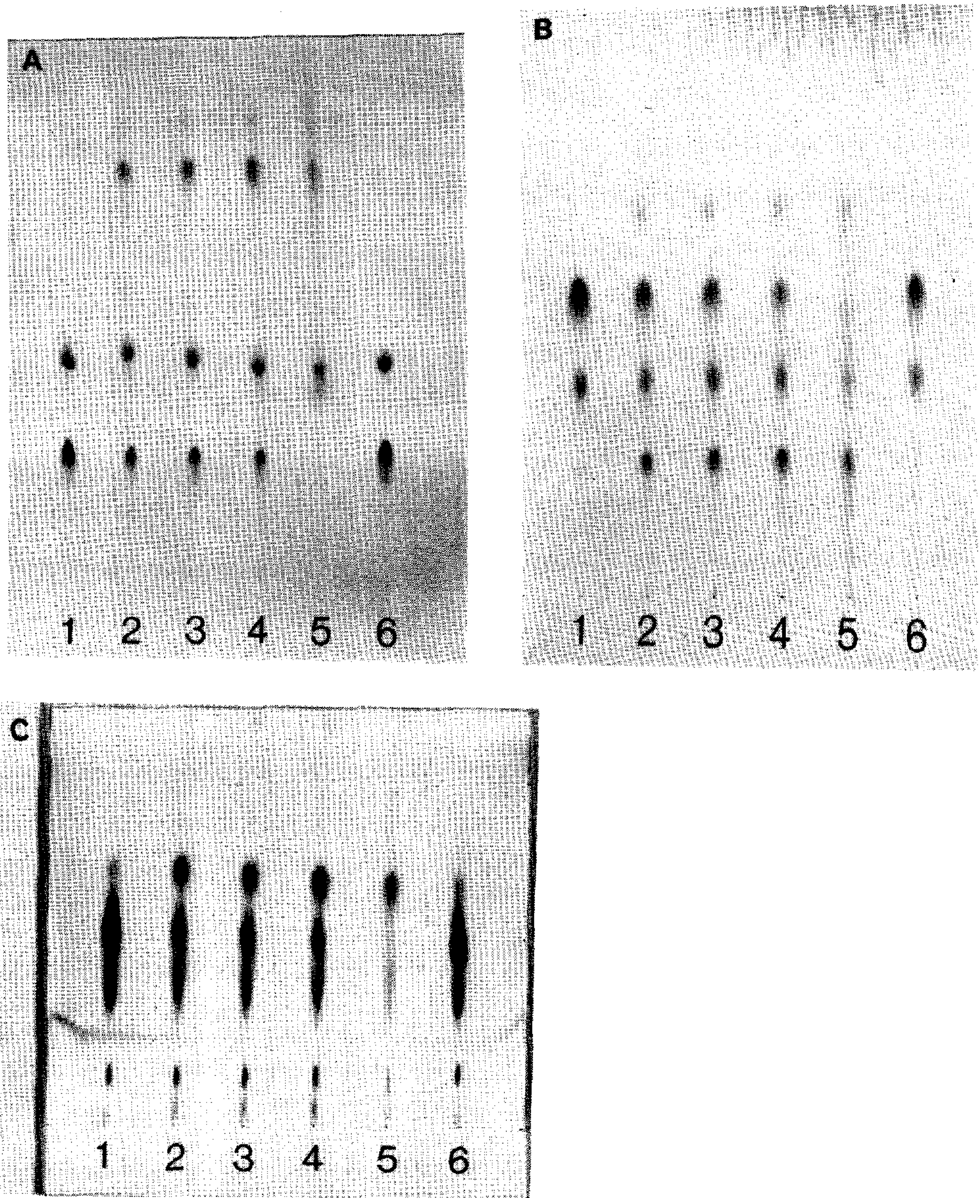

Fig. 3. Thin-layer chromatograms of bleomycin and samples heated at 80°C for 1, 3, 24 h and 140°C for 8 h detected with ninhydrin. (A) Silica gel K5; 0.19 *M* diammonium hydrogen phosphate–methanol (1:1). (B) Silica gel 60 silanised; 0.357 *M* ammonium nitrate–methanol (7:3). (C) Silica gel 60 silanised; 0.1 *M* hexanesulphonic acid–methanol (1:1). 1 and 6 = unheated control; 2 = 80°C/h; 3 = 80°C/3 h; 4 = 80°C/24 h; 5 = 140°C/8 h. Amounts applied in A and B, 125 μg and in C, 62.5 μg.

75 min. As bleomycin A_5 is usually considered a minor component in the samples of bleomycin currently in use, the gradient time was held at 50 min. Two samples of Chinese and Russian origin, G and H respectively, consisted mainly of bleomycin A_5; this was confirmed by TLC. The composition of the other samples of bleomycin

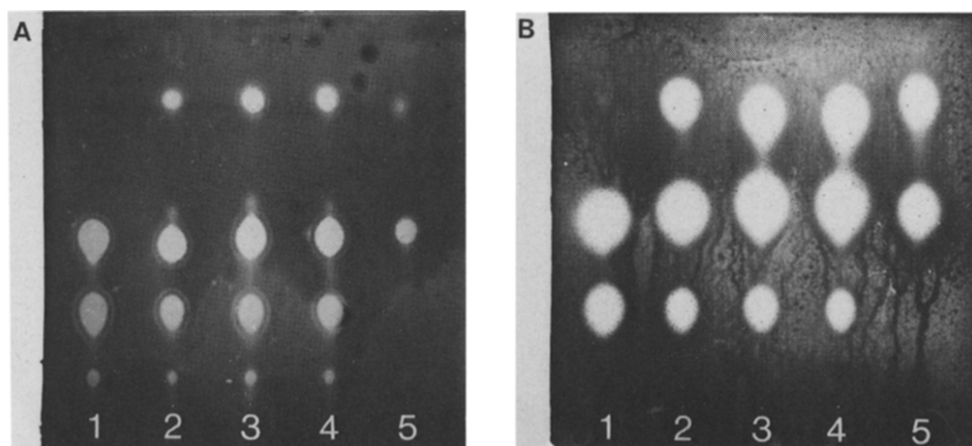

Fig. 4. Thin-layer chromatograms of bleomycin and samples heated at 80°C for 1, 3, 24 h and 140° for 8 h detected biologically. (A) *Bacillus subtilis* NCTC 10400. (B) *Mycobacterium smegmatis* NCTC 7017. Silica gel K5; 0.19 *M* diammonium hydrogen phosphate–methanol (1:1). 1 = Unheated control; 2 = 80°C/1 h; 3 = 80°C/3 h; 4 = 80°C/24 h; 5 = 140°C/8 h. Amounts applied in A, 37.5 μg; in B, 18.75 μg.

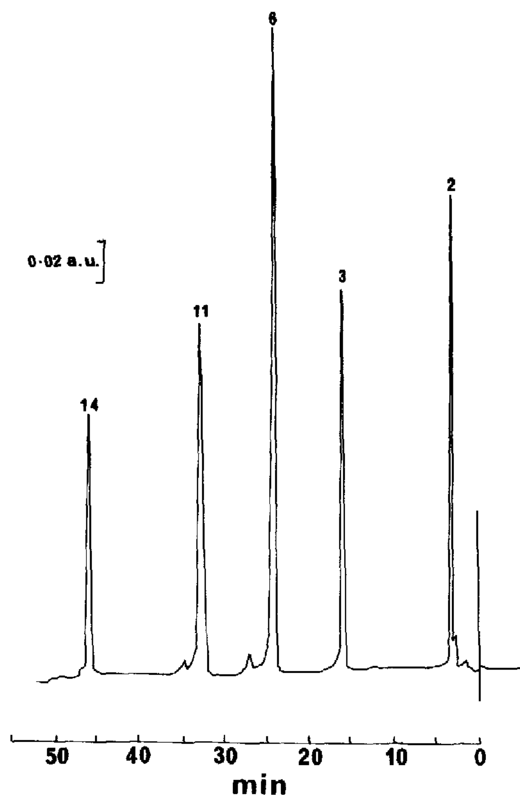

Fig. 5. Chromatogram of a mixture of bleomycins. Ultrasphere ion pair column, 150 × 4.6 mm. Eluent 0.02 *M* hexanesulphonic acid sodium salt, 0.005 *M* disodium ethylenediaminetetraacetate, 0.01 *M* sodium citrate buffer pH 4, acetonitrile (14%, v/v) increasing linearly to (23%, v/v) in 50 min, flow-rate 1 ml min^{-1}. Peak numbers refer to identification as in Table II.

TABLE II

COMPOSITION (%) OF BLEOMYCIN COMPLEX DETERMINED BY HPLC

Composition (%) of The International Reference Preparation of bleomycin, samples degraded by heating at 80°C for 1, 3, 24 and 72 h, at 140°C for 2, 8 and 16 h, and commercial samples based on the measurement of peak areas of the components separated by HPLC. Ultrasphere ion pair column, 150 × 4.6 mm, eluent 0.02 *M* hexanesulphonic acid sodium salt, 0.005 *M* disodium ethylenediaminetetraacetate, 0.01 *M* sodium citrate buffer pH 4, acetonitrile (14%, v/v) increasing linearly to (23%, v/v) in 50 min, flow-rate 1 ml min^{-1}. Identification: 2 = bleomycinic acid, 3 = bleomycin A_1, 6 = bleomycin A_2, 11 = bleomycin A_5 or B_2, 14 = demethylbleomycin A_2.

Sample	*Peak number*														
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
	k'														
	1.94	*8.90*	*31.58*	*43.76*	*48.10*	*50.44*	*51.82*	*56.30*	*64.00*	*66.70*	*68.10*	*69.80*	*73.20*	*93.68*	*95.10*
IRP bleomycin	–	–	–	–	–	61.87	–	3.32	–	–	31.22	–	1.24	2.35	–
R.S.D. $p = 0.95$, $n = 7$	–	–	–	–	–	0.72	–	4.96	–	–	1.27	–	7.75	5.86	–
Heated 80°C/1 h	–	3.75	–	–	1.05	39.19	1.20	1.88	–	–	26.55	3.44	1.09	13.86	1.25
80°C/3 h	–	3.85	–	–	0.80	38.20	1.24	1.44	–	–	26.61	2.95	0.81	14.51	0.92
80°C/24 h	0.23	3.84	0.42	–	0.47	35.56	0.77	1.46	–	–	26.66	0.80	0.80	14.46	–
80°C/72 h	0.17	4.86	0.72	–	0.96	32.93	1.00	1.34	–	0.29	24.17	0.94	0.69	15.47	–
140°C/2 h	0.73	9.91	–	–	–	5.13	–	–	–	1.63	14.39	6.47	0.96	16.24	5.84
140°C/8 h	3.08	8.73	0.66	1.23	1.81	–	–	–	2.72	–	4.32	7.64	0.82	6.13	7.96
140°C/16 h	2.83	3.97	0.28	1.13	0.84	–	–	–	1.48	–	3.52	4.68	–	4.10	3.67
Commercial sample															
A	–	1.77	0.46	–	–	54.99	–	2.82	–	–	28.92	–	1.16	9.13	–
B	–	0.48	–	–	–	62.91	–	4.12	–	–	27.54	–	1.02	3.37	–
C	0.49	–	–	–	0.48	63.04	–	2.49	–	–	28.76	–	0.99	3.46	–
D	–	0.46	–	–	–	64.23	–	1.86	–	–	28.87	–	0.99	3.59	–
E	–	0.35	–	–	0.68	63.88	–	1.74	–	–	29.33	–	–	3.17	–
F	0.17	0.30	–	–	–	64.19	–	2.18	–	–	28.93	–	0.86	3.26	–
G	0.26	–	–	–	–	–	2.38	1.22	0.72	–	95.40*	–	–	–	–
H	–	–	–	–	–	–	–	–	8.11	–	86.77*	–	3.66	1.19	–

* Shown to be bleomycin A_5 by TLC.

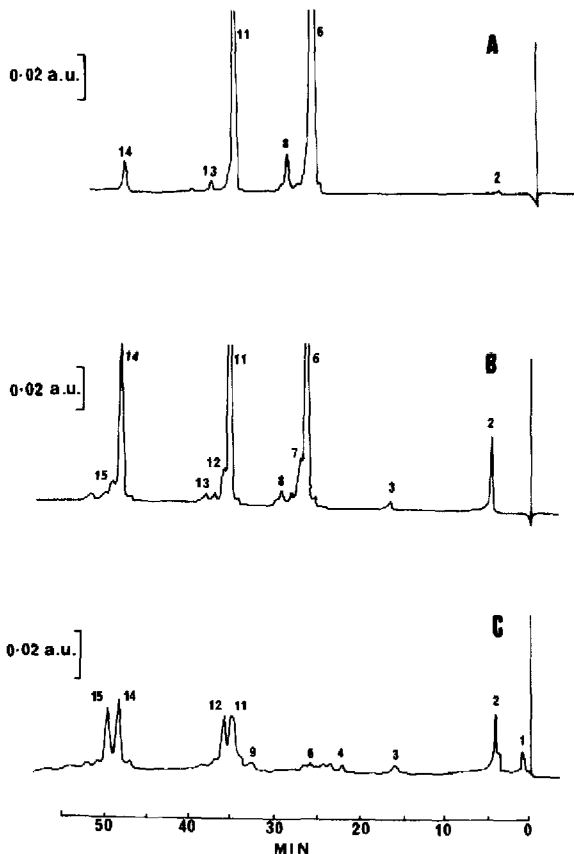

Fig. 6. Chromatograms of bleomycin and samples heated at 80°C for 24 h and 140° for 8 h. Conditions as in Fig. 5. (A) Unheated control. (B) Sample heated at 80°C for 24 h. (C) Sample heated at 140°C for 8 h. Peak numbers refer to identification as in Table II.

was found to be in good agreement with the results obtained in a recent collaborative study (unpublished results). Sample A, with the high demethylbleomycin A_2 content, had been stored in a sealed ampoule for 9 years. The composition of the bleomycin reference preparation was determined on eight separate aliquots: the relative standard deviation of the estimations varied from 0.72% for the main component, bleomycin A_2, to 7.75% for an unidentified minor component (see Table II). The composition of the bleomycin samples and the degraded samples is shown (Table II).

Bleomycin A_2 was susceptible to degradation upon heating at 80°C, half the amount of bleomycin A_2 was lost in 72 h, with a concomitant increase in the content of demethylbleomycin A_2 and bleomycinic acid. There was a slight fall in the content of bleomycin B_2. After 72 h, about 23% of bleomycin complex had been lost. Prolonged heating at 140°C completely destroyed bleomycin A_2 and caused the loss of the resulting demethylbleomycin A_2 and bleomycinic acid; the content of bleomycin B_2 decreased. After 16 h at 140° only 26.5% of the bleomycin complex could be detected.

The absorbance measurements showed no marked decrease, suggesting that not all the bleomycin was recovered from the chromatography column. But neither

TABLE III

ANTIBACTERIAL ACTIVITY OF INDIVIDUAL BLEOMYCINS

Comparison of antibacterial activities of some of the components of bleomycin complex against the assay organisms *Bacillus subtilis* NCTC 10400 and *Mycobacterium smegmatis* NCTC 7017; the results are expressed as the potency ratio relative to bleomycin B_2.

Component	*Potency ratio B_2*	
	B. subtilis	*Myco. smegmatis*
Bleomycin A_1	0.07	0.16
Bleomycin A_2	0.69	0.29
Bleomycin A_5	10.27	0.97
Demethylbleomycin A_2	0.16	1.06
Bleomycinic acid	0.02	0.04

extensive elution nor inspection of top of the column for polymeric material revealed any retained substance. Bleomycin A_2 was readily demethylated upon heating, though the absence of oxygen prevented the formation of bleomycin A_1.

Microbiological assay

The relative antibacterial activity of the individual components of bleomycin against the two test organisms commonly used for the assay of bleomycin is shown (Table III). Compared with *Myco. smegmatis*, *B. subtilis* exhibited a threefold greater susceptibility to bleomycin A_2 and a tenfold greater susceptibility to bleomycin A_5. Demethylbleomycin A_2 was five times more active against *Myco. smegmatis* than against *B. subtilis*. These findings agree closely with the original work[21].

The low precision of *ca.* 15% of the *B. subtilis* assay was due to the poor dose–response relationship, even though the zones of inhibition of growth were well defined. By using a base and a seed layer for *Myco. smegmatis*, distinct zones of

TABLE IV

DEGRADATION OF BLEOMYCIN

Comparison of the absorbance $E^{1\%}_{1cm}$ 293 nm, the content of bleomycin complex determined by HPLC as the sum of the peak areas relative to the unheated control and the potency determined by microbiological assay with *B. subtilis* NCTC 10400 and *Myco. smegmatis* NCTC 7017, of samples of bleomycin degraded by heating.

Bleomycin samples	*$E^{1\%}_{1cm}$ 293 nm*	*Bleomycin content (%)*	*Potency ratio*	
			B. subtilis	*Myco. smegmatis*
Unheated control	101.67	100.00	1.06	1.03
Heated 80°C/1 h	95.83	93.26	0.51	1.03
80°C/3 h	92.17	91.33	0.42	1.05
80°C/24 h	97.50	85.63	0.56	1.01
80°C/72 h	95.00	83.60	0.54	0.99
140°C/2 h	91.66	61.30	nd	nd
140°C/8 h	95.34	45.10	0.52	0.52
140°C/16 h	100.00	26.50	nd	nd

inhibition were obtained, the precision of the assay was *ca.* 6%.

The degraded material was assayed against an unheated control using both test organisms and the results compared with absorbance values and bleomycin contents, the latter estimated as the sum of peak areas relative to the unheated control (Table IV). Neither microbiological assay indicated the extent of the damage to bleomycin, in fact no loss of potency was shown by the *Myco. smegmatis* assay until more than 50% of the bleomycin complex had been destroyed. The activity of demethylbleomycin A_2 against *Myco. smegmatis* assay alone is insufficient to explain the lack of correlation between potency and degree of bleomycin degradation. *Myco. smegmatis* also appears to be susceptible to the minor unidentified degradation products. The *B. subtilis* assay recorded a 50% reduction in the potency of the heated samples irrespective of the amount of degradation.

CONCLUSION

The improved separations obtained by TLC allowed direct bioautographic detection without the risk of the zones of inhibition of the minor components being obscured by those of the major components. Three TLC systems were used to minimize the chance of failing to detect any biologically active degradation products. HPLC used was developed from the method introduced for the quantitative determination of bleomycin[6], however, the analysis time has been reduced by 20 min without any loss of precision.

The marked changes in the composition of the bleomycin complex detected during degradation by TLC and HPLC were not reflected by corresponding changes in potency. These results reinforce the reservations expressed regarding the use of an antibacterial assay to quantify the potency of an antitumour antibiotic[5]. And as demethylbleomycin A_2 has been shown to cause significantly less damage to DNA in the presence of iron(II) ions than bleomycin A_2[22] the value of the microbiological potency estimation for assessing antitumour activity may be questioned. Work is in progress to determine the DNA reactivity of degraded samples of bleomycin complex. The chromatographic methods developed here proved invaluable for monitoring the composition of bleomycin during degradation and should lead to the choice of a more rational assay for bleomycin.

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