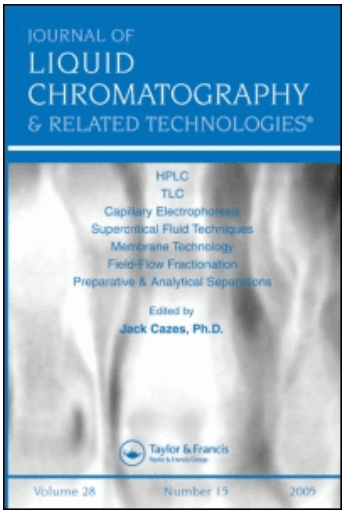


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Rapid and Inexpensive Thin Layer Chromatographic Method for Quantitative Analysis of Bleomycin Complex

K. Datta[a]; S. K. Das[a]; S. K. Roy[b]
[a] Department of Biochemistry, [b] Director Central Drugs Laboratory, Calcutta, India

To cite this Article Datta, K. , Das, S. K. and Roy, S. K.(1989) 'A Rapid and Inexpensive Thin Layer Chromatographic Method for Quantitative Analysis of Bleomycin Complex', Journal of Liquid Chromatography & Related Technologies, 12: 6, 949 — 956
To link to this Article: DOI: 10.1080/01483918908051772
URL: http://dx.doi.org/10.1080/01483918908051772

JOURNAL OF LIQUID CHROMATOGRAPHY, 12(6), 949-956 (1989)

A RAPID AND INEXPENSIVE THIN LAYER CHROMATOGRAPHIC METHOD FOR QUANTITATIVE ANALYSIS OF BLEOMYCIN COMPLEX

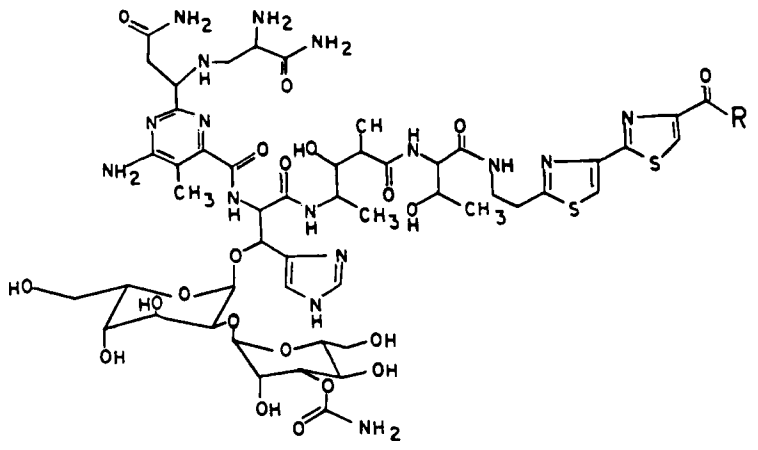
K. DATTA[1], S.K. ROY[2] AND S.K. DAS[1]
[1]*Department of Biochemistry*
[2]*Director*
Central Drugs Laboratory
3 Kyd Street
Calcutta, India

ABSTRACT

A densitometric and a spectrophotometric method for rapid but accurate determination of different components of bleomycin injections has been described. The bleomycin components were separated by reversed phase thin layer chromatography on silanized silicagel plates using a mixture of aqueous ammonium nitrate (2.5%) : methanol :: 7 : 3 (v/v) as mobile phase. Assay was done at the absorption maxima of the components (291 nm) by in situ densitometry or by spectroscopy after extracting the drugs from the adsorbent with the mobile phase. Results obtained by both the methods agreed well with each other and with those obtained by an official HPLC method. The densitometric method described was highly suitable for routine quality control of bleomycines as a large number of samples could be analysed within a short time (68 samples/analyst/day).

INTRODUCTION

Bleomycin, a potent anticancer antibiotic, is a mixture of closely related water soluble basic glycopeptides differing only in their terminal amino group (Fig.1). Potency of bleomycin preparations are determined by measuring their antimicrobial activity (1). However, the antitumer activity of the bleomycin complex may not parallel their antimicrobial activity (2) and the microbiological assay results are acceptable only when the relative proportions of the different bleomycin components are within certain limits (3). The usual method for the compositional analysis of bleomycin prepara-



Bleomycin A_2 : R = - NH - CH_2 - CH_2 - CH_2 - $\overset{+}{S}(CH_3)_2$ - CH_3

Bleomycin B_2 : R = - NH - CH_2 - CH_2 - CH_2 - CH_2 - NH - C(=NH) - NH_2

Bleomycin A_5 : R = - NH - CH_2 - CH_2 - CH_2 - NH - CH_2 - CH_2 - CH_2 - CH_2 - NH_2

Demethyl Bleomycin A_2 : R = - NH - CH_2 - CH_2 - CH_2 - $\overset{+}{S}H$ - H

Fig.1. Structure of some bleomycin components

tions is high performance liquid chromatography (HPLC) using ion pair reversed phase technique (4, 5, 6) which has also been recommended by some compendia (7, 8). HPLC systems are costly. The operational cost is also very high due to high cost of HPLC grade solvents and other consumables. But the most serious draw back of the HPLC method used for analysis of bleomycins (7, 8) is that a single analysis takes about 2 hours running time which makes the method unsuitable for analysis of a large number of samples at a time.

Bleomycin complex may be fractionated to individual components by thin layer chromatography (TLC) on silicagel, cellulose or silanized silicagel layers (9, 10, 11). TLC coupled with bioautography has successfully

been used for qualitative analysis of bleomycin complex (11, 12). Since TLC is an inexpensive and simple analytical tool capable of handling a large number of samples at a time, we used this method for quantitative analysis of bleomycin components and the results are reported in this paper.

MATERIALS

Apparatus

Spectrophotometer - Shimadzu UV-VIS dual beam recording spectrophotometer, model Graphicord UV-240.

TLC Scanner - Shimadzu dual beam Zigzag scanner, model CS-930.

HPLC System - Waters' gradient HPLC system equipped with model 510 HPLC pump, model 680 gradient controller, model U6K universal injector and model 440 UV/VIS detector with Omniscribe single pen recorder and 30 cm X 0.4 cm μ Bondapak C 18 column.

Ultraviolet Viewer - Desaga UVIS system.

Sample applicator - Camag Nanomat II with 1 μl micropipette and holder and 20 μl micropipette.

Table centrifuge (Swing head, 5000 rpm) - Model Remi 8R.

TLC plates - 20 X 20 cm, coated with 0.4 mm thick layer of

a) silanized silicagel, E. Merck (60H :60HF 254 :: 2 : 1 w/w)

b) silicagel, E. Merck (60G : 60HF 254 :: 10 : 3, w/w) containing a 5 cm wide sample concentrating zone of Kieselgurh G.

Developing tank - Camag twin trough developing chamber.

Reagent

Bleomycin test samples (bleomycin hydrochloride injection 15 mg/ ampoule) were bought from local market. Reference standards of bleomycins A_2, B_2 and A_5 and demethyl bleomycin A_2 were kind gift from M/s Nippon Kayaku Co. Ltd., Tokyo, Japan.

Pentane sulfonic acid (PIC reagent) was bought from M/s Waters Associates. Solvents for TLC were of GR, E. Merck grade and used as such. Solvents for HPLC were of Lichrosolv, E. Merck grade.

Mobile phase

1) Partition TLC - Aqueous ammonium nitrate (2.5%) : methanol :: 7 : 3 (v/v).

2) Adsorption TLC - Aqueous diammonium hydrogen phosphate (2.5%) : methanol :: 1 : 1 (v/v).

3) HPLC - solvent system described in USP XXI (7) was used. A linear gradient of 10% to 40% methanol in 0.08N acetic acid, pH 4.3 and containing 0.096%

of sodium-1-pentane sulfonate was used. The gradient mixing time was 60 minutes and the final mixture was allowed to flow for further 20 minutes.

Sample preparation

Content of one ampoule of sample was dissolved in a mixture of water : ethanol (1:1 v/v) and concentration was adjusted to a nominal 5 mg/ml.

Standard preparation

The following amount of different bleomycin standards were dissolved in 5 ml of water : ethanol (1:1 v/v).

a) Bleomycin A_2 5 mg, b) Bleomycin B_2 5 mg, c) Bleomycin A_5 5 mg, d) Demethyl bleomycin A_2 5 mg, e) Bleomycin A_2 (11.7 mg), Bleomycin B_2 (11.4 mg), Bleomycin A_5 (0.65 mg) and demethyl bleomycin A_2 (1.25 mg)

PROCEDURE

For assay by scanning densitometry, 1 µl of the test solutions and 1 µl of the standard solution 'e' were applied as separate compact spots 10 mm apart on an imaginary line 15 mm from the bottom of the TLC plate 'a'. The plate was then developed upto 50% of the total length of the plate in usual way in a filter paper lined tank previously saturated with mobile phase '1' for 1 h and containing 15 ml of the mobile phase in each trough. For adsorption TLC, plate 'b' and mobile phase '2' were used. After development, the plate was dried in a current of air for 10 minutes. The different bleomycin spots were visualised under short wave (254 nm) ultraviolet (UV) lamp and identified by comparison of Rf values of the different components of the standard mixture 'e'. The starting and end point for the first lane were then marked and the plate was scanned in the densitometer with liniarizer setting at X=3, background scanning wave length at 350 nm and sample scanning wave length at 291 nm. Other parameters were set as per the instruction mannual of the instrument.

For spectrophotometric assay after TLC, 1 µl of the standard solution 'e' was spotted as a compact spot and 20 µl of the sample solutions were applied as 5 mm wide bands 15 mm apart on TLC plate 'a' or 'b' as the case may be. The plates were then developed with respective solvent systems and visualized as described above. The different bleomycin bands, identified by comparing with the reference standard spots, were marked and adsorbent containing the different components were scrapped into separate 15 ml stoppered glass centrifuge tubes. Bleomycin components were extracted from the adsorbent by shaking with 2 x 2 ml of the mobile phase used on a vortex mixer for 30 sec. followed by centrifugation each time at 1000 g for 5 minu-

tes and optical density of the combined extract of each component was measured at 291 nm. A blank obtained by extraction of adsorbent from an area equivalent to respective sample bands but not containing any sample was used in the reference beam for each sample. For HPLC, method described by the USP XXI (7) was followed using the solvent system 'c'.

RESULTS AND DISCUSSION

Resolution of the bleomycin components after reversed phase partition TLC (RPTLC) and adsorption TLC (ATLC) are shown in Fig.2. The test sample could be resolved into only 3 components by our method or by HPLC (7) and the components were identified as bleomycin A_2, bleomycin B_2 and demethyl bleomycin A_2 by comparing their Rf values (ATLC and RPTLC) or retention time (HPLC) with those of authentic reference standards. In RPTLC, all the components in the sample (reference standard mixture and sample) were clearly separated and allowed precise determinations. ATLC on ordinary plate showed different Rf values for different components (Rf difference between A_2 and A_5 being marginal) but excessive tailing made assay of the individual components difficult. Thus we used a zone concentrating layer of Kieselgur G which prevented tailing and made assay possible but A_2 and A_5 did not separate clearly in the system (Fig.2D). However, since our sample did not contain any bleomycin A_5, we assayed the components after ATLC and included these results in this report for comparison. The Rf values of bleomycin A_5 obtained by us in RPTLC or ATLC differed from those reported elsewhere (11) but we used laboratory made plates and for ATLC, we used a sample concentrating zone and the adsorbent was from a different manufacturer.

The United States Pharmacopoeia (7) and the International Pharmacopoeia (8) have indicated the elution order of different bleomycin components but are silent about the retention time. This makes identification difficult if one or more components are missing (in the sample) and makes use of reference standards mandatory resulting in increase in analysis time. Identification is more authentic, easy and do not take extra time by the proposed method as the reference standard is chromatographed simultaneously with the sample in same TLC plate.

The assay results of bleomycin components of a bleomycin injection procured from local market is shown in table 1. Results obtained by the proposed method agreed excellently with those by HPLC (pharmacopoeial method, 7,8). The low standard deviation values obtained by UV spectroscopy after

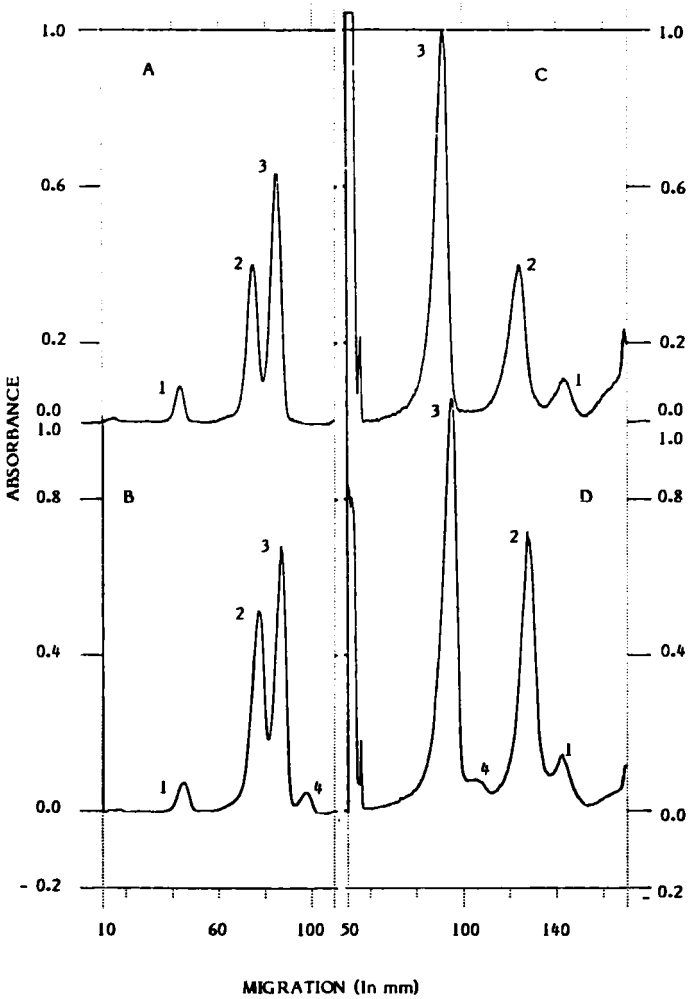


Fig.2. Resolution of bleomycin components by reversed phase partition TLC (A and B) and adsorption TLC (C and D) of a bleomycin injection (A and C) and a simulated mixture of bleomycin components (B and D). Chromatogramme obtained by scanning densitometry of the TLC plates as described in text. 1) Demethyl bleomycin A_2, 2) Bleomycin B_2, 3) Bleomycin A_2 and 4) Bleomycin A_5

TABLE 1

Percent composition of a bleomycin injection and a simulated bleomycin mixture determined by the proposed method and by HPLC

	Bleomycin injection					Simulated mixture	
	Proposed method				HPLC		
	Partition TLC		Adsorption TLC				
Bleomycin components	Spectroscopy	Densitometry	Spectroscopy	Densitometry		Calculated	Partition TLC and Densitometry
Bleomycin A_2	61.9±0.6	59.3±2.5	60.6±0.4	63.7±3.3	61.7±3.6	46.8	46.7±0.7
Bleomycin B_2	32.2±0.5	36.3±1.2	33.0±0.4	31.2±2.5	32.7±2.0	45.6	45.5±0.8
Demethyl Bleomycin A_2	5.9±0.1	4.4±0.4	6.3±0.1	5.0±0.4	5.4±0.3	5.0	5.0±0.2
Bleomycin A_5	-	-	-	-	-	2.6	2.6±0.1

Results expressed as average of 6 independent determination ± standard deviation

extraction from RPTLC plates indicated high precision of the method but densitometry after TLC was much more rapid, easy to operate and showed a standard deviation comparable to the official method (7,8). A mixture of different bleomycin components prepared in the laboratory and assayed by densitometry after RPTLC also showed excellent agreement with the theoretical values (Table 1). The TLC methods described here are suitable for analysing at least 68 samples (two 20 x 20 cm plates) by densitometry or 18 samples by spectroscopy by an analyst on a working day. The cost of operation is also very low which makes these methods ideal for quality control laboratories.

REFERENCE

1. Code of Federal Regulations Food and Drugs. Title 21, Part 450.10a, U.S. Government Printing Office, Washingtom DC, p615. (1980)

2. H. Umezawa. Pure Appl.Chem. 28, 665 (1971)

3. W.H.O. Expert Committee on Biological Standardization, W.H.O. Technical Report Series No.658, p7 and p243 (1981)

4. A. Aszalos, J. Crawford, P. Vollmer, N. Kantor and T. Alexander, J. Pharm.Sci. 70, 878 (1981)

5. T.T. Saki, J. Chromatogr. 161, 389 (1978)

6. G.K. Shin and T.J. Goehl, J. Chromatogr. 181, 127 (1980)

7. The United State Pharmacopoeia XXI Rev. United States Pharmacopoeial Convention, Inc. Rockville, MD. p.93 (1985)

8. The International Pharmacopoeia, Vol. 3. World Health Organisation, Geneva (1987)

9. H. Umezawa, Y. Suhara, T. Takita and K. Maeda. J. Antibiotic. 19, 210 (1966)

10. A. Fuji, T. Takita, K. Maeda and H. Umezawa, J. Antibiotic. 26, 396 (1973)

11. A.H. Thomas, P. Newland and N. Sharma. J. Chromatogr. 291, 219, (1984)

12. Y. Ito, Y. Ohashi, Y. Egawa, T. Yamagnehi, T. Furumai, K. Enomoto and T. Okuda. J. Antibiotic. 24, 727 (1971)