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High-performance liquid chromatographic determination of bleomycins

H.-P. FIEDLER* and J. WACHTER
Universität Tübingen, Biologisches Institut, LB Mikrobiologie/Antibiotika, Auf der Morgenstelle 28, D-7400 Tübingen (F.R.G.)

ABSTRACT

A rapid and specific ion-pair reversed-phase high-performance liquid chromatographic method was developed for the determination of bleomycins. The use of 5-μm particles of less adsorptive reversed-phase packings and sodium perchlorate as ion-pairing reagent permitted a short analysis time and the transferability of the separations on different batches of the reversed-phase materials. The detection sensitivity and precision of the method demonstrated that the system is suitable for routine analysis.

INTRODUCTION

Commercially available bleomycin is a mixture of highly hydrophilic, cationic glycopeptide antibiotics. The complex is isolated from the fermentation broth of *Streptomyces verticillus* [1]. Bleomycins are effective against a variety of human neoplasms, sarcoma, malignant lymphoma and testicular carcinoma. Preparations used in cytostatic therapy consist of bleomycin A_2 (55–70%) and bleomycin B_2 (25–32%), the remainder being divided among the minor congeners. Bleomycin A_2 will be partially converted into demethylbleomycin A_2 during lyophilization. The structures of the main components of the bleomycin complex are shown in Fig. 1.

The most commonly used dosage has been 15 mg of bleomycin biological activity (15 units) twice weekly. The wide use of bleomycins for therapeutic purposes and pharmacokinetic studies of the individual bleomycins require a specific and sensitive assay. Several high-performance liquid chromatographic (HPLC) methods have been developed for the separation and determination of these compounds. Separations on silica gel lacked baseline resolution, showed peak tailing and required long analysis times [2,3]. Reversed-phase ion-pair HPLC using alkane sulphonic acids as lipophilic counter ions proved to be the only method to achieve baseline resolution of most bleomycins and their quantification in a sensitive detection range [4–9].

In the course of investigations on the separation behaviour of basic peptides depending on the stationary and mobile phases [10], we tested the results for the HPLC determination analysis of bleomycins. Basic compounds often show an unfavourable adsorption on the stationary phase caused by highly acidic, isolated silanol groups of

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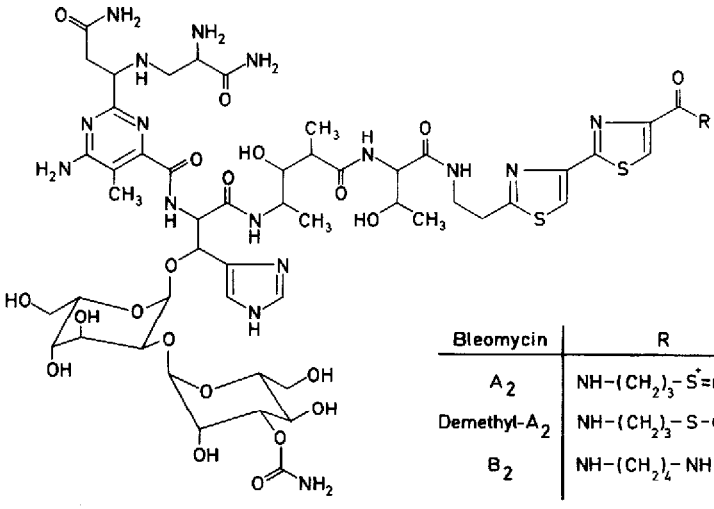

Bleomycin	R
A_2	$NH-(CH_2)_3-\overset{+}{S}=(CH_3)_2$
Demethyl-A_2	$NH-(CH_2)_3-S-CH_3$
B_2	$NH-(CH_2)_4-NH-C(=NH)-NH_2$

Fig. 1. Structure of bleomycins.

the reversed-phase packings [11–13]. We investigated the applicability of different reversed-phase materials and the addition of perchlorate as a strong ion-pairing reagent to the mobile phase to achieve selectivity in the separation of bleomycins.

EXPERIMENTAL

Chromatographic system

The chromatographic system consisted of an HP 1090M liquid chromatograph equipped with a diode-array detection system and workstation (Hewlett-Packard, Waldbronn, F.R.G.). A detection wavelength of 240 nm and a reference wavelength of 550 nm were used.

Column

The LiChrospher RP-Select B column (5 μm) (125 × 4 mm I.D.), fitted with a precolumn 4 × 4 mm I.D., was obtained from Merck (Darmstadt, F.R.G.). Columns of Nucleosil-100 C_{18} (7 μm) and Shandon Hypersil ODS (5 μm) (125 × 4.6 mm I.D.), fitted with precolumns 20 × 4.6 mm I.D., were purchased from Grom (Herrenberg, F.R.G.).

Mobile phases

Solvent A was 10 m*M* sodium perchlorate in 0.1% aqueous phosphoric acid and solvent B was acetonitrile. A linear gradient was applied from 5% to 25% solvent B in 13 min, increasing in 1 min to 100% B with a 1-min hold and followed by a post-time of 5 min under the initial conditions. The flow-rate was 1.5 ml/min for the 4-mm I.D. column and 2 ml/min for the 4.6-mm I.D. columns.

Chemicals

Acetonitrile (HPLC grade), sodium perchlorate and phosphoric acid (analytical-

reagent grade) were obtained from Merck. Water was purified by means of a Milli-Q system (Millipore, Eschborn, F.R.G.). Bleomycin sulphate (Bleomycinum) was purchased from Mack (Illertissen, F.R.G.); the composition specification was 68.6% bleomycin A_2, 0.7% demethyl-bleomycin A_2, 30.6% bleomycin B_2 and 0.1% bleomycinic acid.

Sample preparation

Bleomycin sulphate preparations in vials containing a biological activity of 15 mg of bleomycin (15 units) were dissolved in 15 ml of water and 20 μl of this bleomycin solution and of dilutions were injected onto the HPLC column.

RESULTS AND DISCUSSION

Fig. 2 shows the separation of a commercial bleomycin preparation by ion-pair reversed-phase HPLC using sodium perchlorate as ion-pairing reagent. The UV spectra of bleomycins which were recorded during the HPLC analysis by the diode-array detection system are congruent, as shown. Elution with a linear gradient of 10 m*M* sodium perchlorate dissolved in 0.1% aqueous phosphoric acid and acetonitrile resulted in a successful separation of the bleomycin mixture. The minor peaks which elute after bleomycin A_2 and B_2 are minor congeners of the bleomycin mixture because the UV spectra are identical with those of the major compounds. The retention behaviour is dependent on the concentration of the ion-pairing reagent, the gradient conditions being kept constant, as shown in Fig. 3. The same selectivity in the separation of bleomycins was reported using alkanesulphonic acids, such as pentanesulphonic acid, as the ion-pairing reagent [7–9]. However, these separations efforted elution times in the range of 45 and 75 min per analysis and problems with batch-to-batch reproducibility of the packings were observed. The use of 5-μm

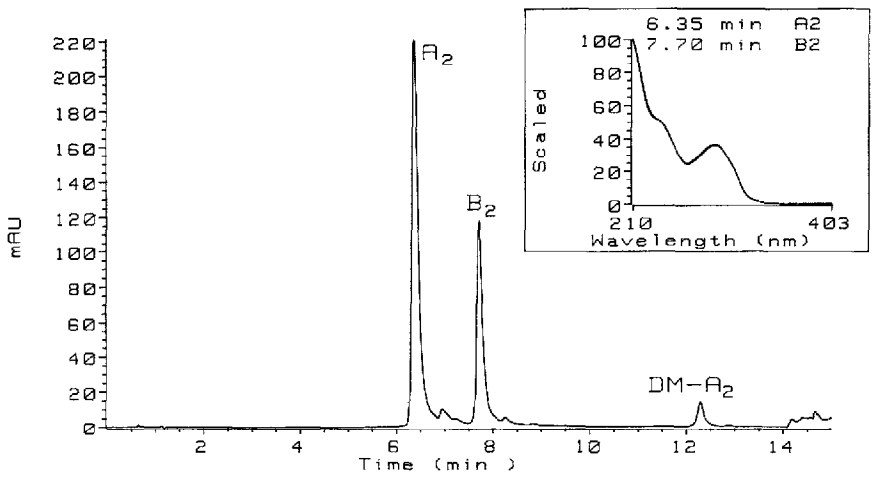

Fig. 2. HPLC separation profile of a bleomycin sulphate preparation plotted at 240 nm, and UV spectra of bleomycin A_2 and B_2 recorded during the HPLC analysis. Sample, 20 μl of Bleomycinum mixture (1 mg/ml bleomycin biological activity); column, LiChrospher RP-Select B.

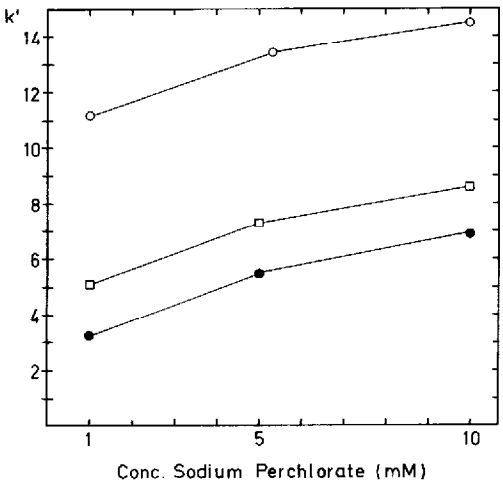

Fig. 3. Capacity factor (k') *versus* concentration of the ion-pairing reagent in the mobile phase. ● = Bleomycin A_2; □ = bleomycin B_2; ○ = demethyl-bleomycin A_2.

particles and perchlorate as the ion-pairing reagent permitted a reduction in the analysis time to 13 min and the transferability of the separations from one batch to another.

Not all reversed-phase packings proved to be suitable for the separation of such basic compounds, even by ion-pair HPLC. As shown in previous investigations [10], pore size, coverage, reaction type and end-capping of the packings have no effect on selectivity and resolution in the HPLC analysis of basic compounds. Undesirable

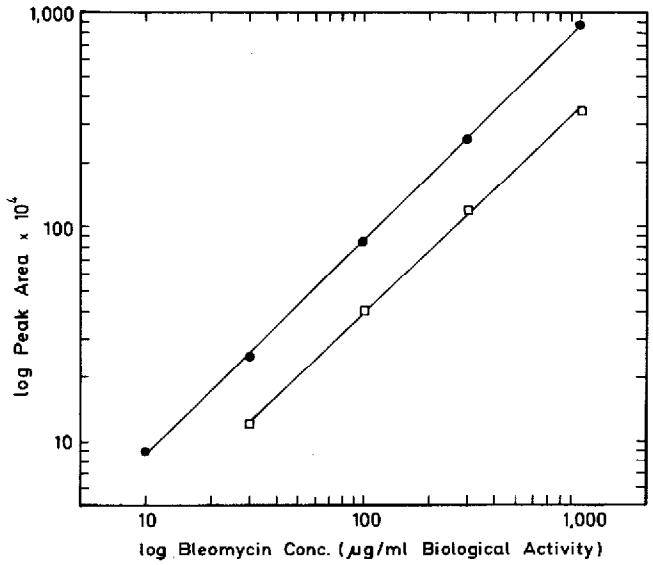

Fig. 4. Linear dynamic range of bleomycin determination. ● = Bleomycin A_2; □ = bleomycin B_2.

TABLE I

PRECISION OF RETENTION TIMES AND PEAK AREAS

Compound	Relative standard deviation (%)[a]	
	Retention time	Peak area
Bleomycin A_2	0.60	0.30
Bleomycin B_2	0.45	0.36
Demethyl-bleomycin A_2	0.20	1.37

[a] Based on 20 replicate analyses.

adsorption is caused by the presence of highly acidic, isolated silanol groups on the reversed-phase materials [11–13]. The separation of basic compounds, such as the strongly basic bleomycins, can be performed more favourably using reversed-phase materials of a less adsorptive type such as LiChrospher RP-Select B or Nucleosil C_{18}. The separation of the bleomycin mixture by ion-pair HPLC on reversed-phase packings of a strongly adsorptive type is characterized by peak tailing and incomplete baseline resolution (data not shown) and is therefore unsuitable for routine quantitative analysis.

The quantification of compounds is facilitated by a linear response in the range of analytical interest. With UV detection at 240 nm, linearity was achieved over the concentration range 10–1000 μg/ml of bleomycin biological activity (bleomycin units), as shown in Fig. 4, which corresponds to 5–500 μM bleomycin A_2 (biological activity). The detection limit for bleomycin A_2 was 100 nmol injected amount of biological activity. The detection sensitivity can be enhanced by using a fixed-wavelength UV detector instead of a relatively insensitive diode-array detector or by fluorescence detection.

The precision of the method was tested by assaying the Bleomycinum mixture 20 times (concentration 300 μg/ml biological activity), as shown in Table I. The reproducibility of both the retention times and the peak areas of bleomycin A_2 and B_2 and demethyl-bleomycin A_2 showed that the system is suitable for routine analysis.

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