HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ACLACINOMYCIN A AND ITS RELATED COMPOUNDS

I. NORMAL PHASE HPLC FOR MONITORING FERMENTATION AND PURIFICATION PROCESSES

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A normal phase high performance liquid chromatographic method is presented for the separation and quantitative determination of aclacinomycin A (ACM) and its related compounds classified as anthracycline antibiotics. The sensitive, reliable and reproducible chromatography was achieved on a μ-Porasil column with a mobile phase consisting of chloroform - methanol - acetic acid - water - triethylamine (68: 20: 10: 2: 0.01, v/v). By use of MA144 M1, one of the ACM analogues, as an internal standard, selectivity of the HPLC method could be retained by virtue of the adjustment of the detecting wavelength to 436 nm. The HPLC method was applicable to monitoring the fermentation and purification processes.

Aclacinomycin A (ACM)[1,2] is an anthracycline antibiotic with potent antitumor activity[3,4] now under clinical study. The structure[5] of ACM and related compounds in the cultured broth of *Streptomyces galilaeus* MA144-M1 (ATCC 31133) is shown in Table 1. To improve the fermentation and purification processes more effectively, it was necessary to develop a more rapid and precise analytical method than thin-layer chromatography (TLC) which had been employed for years.

Recently, high performance liquid chromatography (HPLC) began to be used in the fermentation industry as well as in the pharmaceutical industry[6,7]. HPLC on adriamycin (ADM) and daunomycin (DM) which are also classified as anthracycline antibiotics has been recently reported[8~11]. However, the structure and physicochemical properties as well as biological activity of ACM is different from those of ADM and DM[1,2,5].

This paper describes a rapid, reliable and reproducible normal phase HPLC method for the quantitative determination of ACM in monitoring the fermentation and purification processes.

Experimental

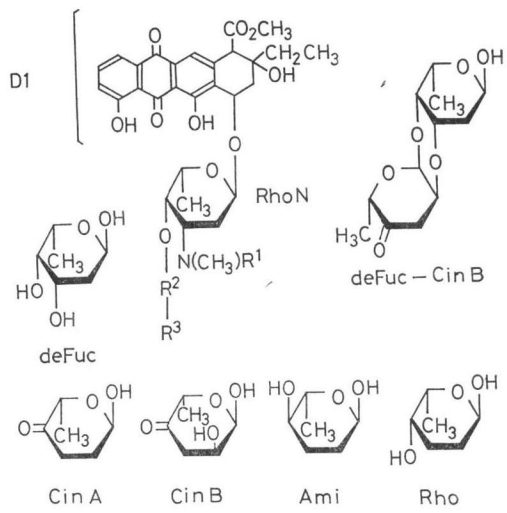
Apparatus

The liquid chromatography apparatus consisted of a Waters Assoc. Model M6000-A pump, U6K injector and 440 absorbance detector with fixed wavelength of 436 nm and 254 nm, and a Shimadzu Model C-R1A Chromatopak recorder and integrator. This system was equipped with a Waters Assoc. 30 cm $\times$ 3.9 mm i.d. stainless-steel column packed with normal phase μ-Porasil. The operating conditions were as follows: mobile phase, chloroform - methanol - acetic acid - water - triethylamine (68: 20: 10: 2: 0.01, v/v); pressure, about 1,000 psi; room temperature controlled at 23$\pm$1°C; detector sensitivity, 1.0 AUFS; recorder chart speed, 5.0 mm/min. At the end of each working day, the column should be rinsed with methanol for about 30 minutes.

Table 1. Structure of aclacinomycin A and its analogues.

Components	R^1	R^2	R^3
Aclacinomycin A	CH_3	deFuc	Cin A
Aclacinomycin B	CH_3	deFuc-Cin B	—
MA144 L1 (L1)	H	deFuc	Cin A
MA144 M1 (M1)	CH_3	deFuc	Ami
MA144 N1 (N1)	CH_3	deFuc	Rho
MA144 S1 (S1)	CH_3	deFuc	—
MA144 T1 (T1)	CH_3	—	—

RhoN=L-rhodosamine Ami=L-amicetose
deFuc=2-deoxy-L-fucose Rho=L-rhodinose
Cin A=L-cinerulose A Dl=aklavinone
Cin B=L-cinerulose B



Chemicals and reagents

Chloroform and methanol were spectroscopic grade obtained from Junsei Chemical Co., Tokyo, and acetic acid and triethylamine analytical grade commercial materials also were obtained from the same company. Water was deionized and distilled. ACM and its related compounds were prepared by isolating and purifying from the cultured broth of *Streptomyces galilaeus* MA144-M1 according to the procedure described in the previous paper[12]. MA144 M1 (hereinafter abbreviated as M1), one of the ACM analogues, was prepared by reduction of ACM with sodium borohydride and confirmed to be identical with authentic M1[5].

Sample preparation

Reference mixture solution: About 1~2 mg each of ACM and its related compounds (B1, L1, M1, N1, S1, T1 and D1) were weighed into a test tube, and added with 5 ml of chloroform just before the analysis. Ten μl of the solution were injected onto the HPLC column.

Fermentation broth: An appropriate quantity (2~5 ml), accurately measured, of the fermentation broth was pipetted into a centrifuge tube. Two ml of 0.5 M tris(hydroxymethyl)-aminomethane-hydrochloric acid buffer solution (pH 7.5) and 2 ml of toluene containing an adequate amount, accurately weighed, of an internal standard were added. The mixture was shaken vigorously for three minutes on a shaker (Thermomixer, Thermonics Co., Ltd., Tokyo) to extract ACM and its related compounds, and centrifuged for 15 minutes at 3,000 rpm. Depending upon the sample, an emulsion may form at the solvent interface, and the emulsion can be broken with a glass rod by addition of salt. A suitable quantity of the toluene layer was taken out and filtered with a Millipore membrane filter (FH membrane, pore size: 0.5 μ, Millipore Co., Bedford, Mass., U.S.A.). Twenty μl of the filtrate were injected onto the column.

Purification sample: Either a powder or solvent sample taken from the purification process was subjected to HPLC analysis. A powdered sample (1~2 mg) was weighed and dissolved in 1 ml of chloroform, and 10 μl of the solution was injected.

Results and Discussion

Chromatographic Conditions

ACM and all of its related compounds having the same aglycone, aklavinone, showed the same molecular absorbancy index at 436 nm ($\varepsilon=1.3\times10^4$) in the mobile phase. To retain selectivity on HPLC especially in a sample of fermentation broth, which contained a large amount of UV absorbing materials, the detector wavelength was selected to be 436 nm.

For application of the chloroform - methanol elution system which had been employed for TLC analysis to HPLC use as a mobile phase, it was necessary to add acetic acid and water to the HPLC system to prevent "tailing" of the elution bands. Although relatively clean chromatograms were obtained, the elution bands still spread and the retention volume gradually increased and no reproducibility was observed. Further addition of a small amount of triethylamine was found to give reproducibility. Therefore, the optimum mobile phase for HPLC was determined to be as follows: chloroform - methanol - acetic acid - water - triethylamine (68: 20: 10: 2: 0.01, v/v). A HPLC chromatogram of the reference mixture with the optimum mobile phase is shown in Fig. 1. Each component was clearly separated from the others without any tailing.

Determination of Internal Standard for Quantitative Analysis

A typical chromatogram of the fermentation broth sample is shown in Fig. 1. As a mutant of the ACM-producing microorganism treated with UV radiation was used, the main product was ACM and fortunately one of the analogues, M1, was not produced. In order to hold the selectivity on the HPLC monitoring at the wavelength of 436 nm, M1 which was easily obtained as a purified analogue by reduction of ACM with sodium borohydride was selected as an internal standard for the HPLC quantitative analysis. Fig. 2 shows a calibration curve of ACM on the HPLC with the internal standard, concentration of which was adjusted at 0.5 mg/ml in samples. The curve was constructed from five replicate measurements of five concentrations over the range, and plots of peak area ratio (peak area of ACM/peak area of internal standard) against ACM concentration were straight-line passing through the point O: $y=a\cdot x$, where a was equal to 0.505 and the relative standard error

Fig. 1. Chromatograms of (a) the reference mixture containing aclacinomycin A (ACM) and its analogues and (b) the extract from the fermentation broth.
Column: 30 cm×3.9 mm i.d., μ-Porasil; mobile phase, chloroform - methanol - acetic acid - water - triethylamine (68: 20: 10: 2: 0.01, v/v); pressure, about 1,000 psi; flow rate, 1.0 ml/min.; detector, Vis (436 nm); sensitivity, 1.0 AUFS.

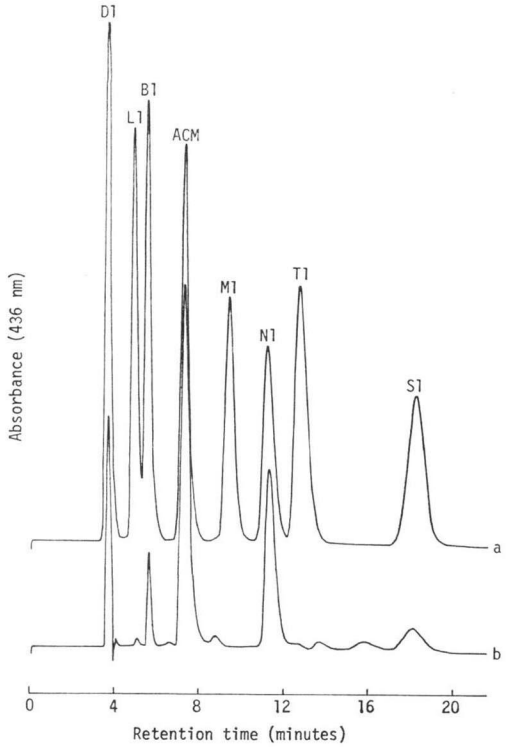


Fig. 2. A calibration curve of aclacinomycin A on HPLC with an internal standard, MA144 M1.
Conditions: same as in Fig. 1 except that MA144 M1 was added to the concentration of 0.5 mg/ml.

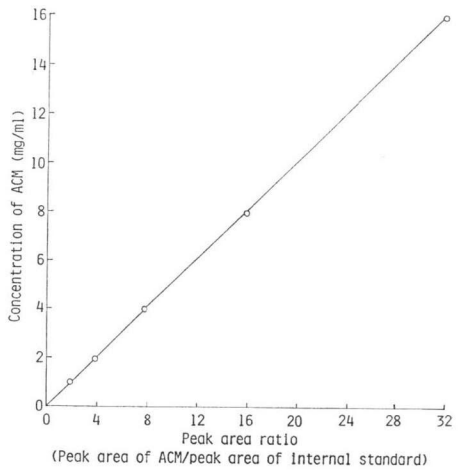
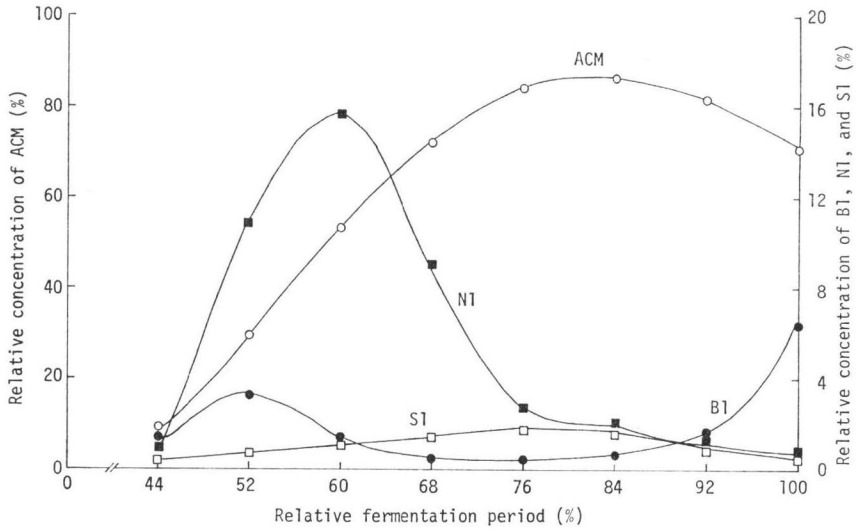


Fig. 3. The HPLC monitoring of aclacinomycin A and its analogues during the fermentation of *S. galilaeus* MA144-M1.

Conditions: same as in Fig. 2.



of regression coefficient, 0.11%.

The calculation of ACM concentration in a sample could be made as follows:

$$\text{Concentration of ACM (mg/ml)} = \frac{\text{peak area of ACM}}{\text{peak area of internal standard}} \times \frac{\text{concentration of internal standard (mg/ml)}}{\text{relative weight response}}$$

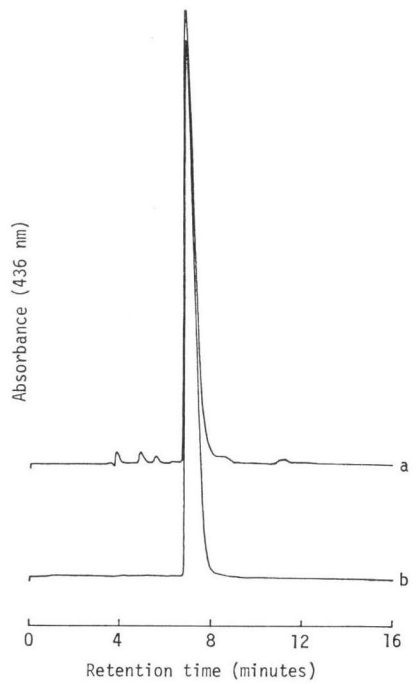
where: relative weight response

$$= \frac{\text{peak area of ACM}/\mu\text{g}}{\text{peak area of internal standard}/\mu\text{g}}$$

The relative weight response of ACM to M1 used as the internal standard was found to be 0.990. The analogues could be analyzed by using the same equation replacing ACM by each analogue since the relative weight response of each analogue to the internal standard as determined to be as follows: B1=0.990, L1=0.990, N1=0.990, S1=1.149 and T1=1.409.

Fig. 4. Chromatogram of samples taken at the early (a) and the final (b) steps of aclacinomycin A purification process.

Conditions: same as in Fig. 1.



Application of HPLC Method to Fermentation and Purification Processes

A typical fermentation process monitored by the HPLC method with the internal standard is graphically illustrated in Fig. 3. Samples taken from the early and final steps of the purification

processes could be subjected to HPLC without the internal standard for purity determination as shown in Fig. 4. When monitored at 436 nm, the relative ratio of the peak area of ACM analogues to that of ACM gave molar-base relative impurity contents on the basis of total analogues (ACM+ analogue impurities) since the analogues including ACM, B1, L1, M1, N1, S1, T1 and D1 had the same molecular absorbancy index (ε).

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