A SENSITIVE ANALYTICAL METHOD FOR ACLACINOMYCIN A AND ITS ANALOGS BY THIN-LAYER CHROMATOGRAPHY AND FLUORESCENCE SCANNING

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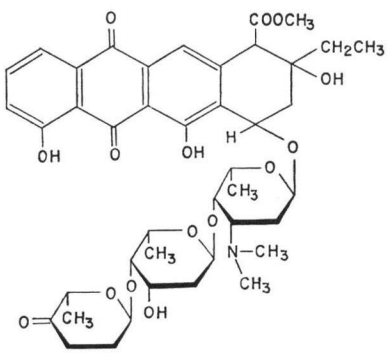
The anthracycline antibiotic aclacinomycin A (ACM-A, Fig. 1) isolated from a culture broth of *Streptomyces galilaeus* MA144-M1 (ATCC 31133)[1,2] is effective in treating various experimental animal tumors[3] and human cancer.[4] To elucidate the metabolism and disposition of ACM-A in animals, a sensitive and specific analytical method for the drug and its metabolites is essential. Several such methods, using high pressure liquid chromatography, radioimmunoassay[5] and fluorescence[6,7], have recently been reported for adriamycin and daunorubicin. Although the characteristic tissue distribution of ACM-A in animals has been observed by spectrophotometry at 430 nm and bioassay using *Bacillus subtilis*,[8] a more sensitive method is necessary to identify and quantify low concentrations of the drug and its metabolites, especially in human physiological fluids.

In this paper, we describe a method in which ACM-A and its metabolites are separated on a silica gel thin-layer plate and the amounts measured with a fluorescence chromatoscanner.

Plasma or blood cells (1.0 ml) were added to a centrifuge tube containing 1.0 ml of 0.1 M phosphate buffer (pH 7.0) and ACM-A and its metabolites were extracted with 8.0 ml of chloroform - methanol mixture (1 : 1, v/v) by shaking on a Thermomixer (Thermonics Co., Ltd.)for one minute. After centrifugation, the aqueous phase was re-extracted with 4.0 ml of chloroform. The organic phases were combined and concentrated under reduced pressure. The oily extract thus obtained was dissolved in 100 μl of chloroform - methanol mixture (1 : 1) and 50 μl was spotted on a silica gel plate (Kieselgel 60, 20 × 20 cm, 0.25 mm thick, without fluorescent indicator, E. Merck Co., Ltd.).

Fig. 1. Structure of aclacinomycin A.



Usually the adsorbent was scored to give 10 channels in which 7 unknown samples and 3 standard samples were spotted on a line 2.5 cm from the end of the plate. The chromatogram was first developed in chloroform by ascending flow to 12.5 cm from the origin and was dried briefly under air flow. In the determination of ACM-A alone, the chromatogram was then saturated 20 minutes in a vessel containing chloroform - methanol mixture (20 : 1), and was developed in this second solvent until ACM-A had migrated to near the middle of the plate. To separate all aglycone- and glycosidic metabolites, it was necessary to use four successive developing steps in chloroform - methanol mixtures of 65 : 1, 20 : 1, 10 : 1 and 5 : 1, as described below.

Fluorescence scanning of ACM-A and related compounds was performed with a Shimadzu dual wavelength chromatoscanner, Model CS-910, equipped with 75W xenon lamp, autozero-suppressor AZS-1 and two-pen recorder Model U225 MCS. It was carried out in the direction of chromatographic development at a speed of 20 mm per minute, with a 10 -mm wide slit collimated to 1 mm. The excitation wavelength was adjusted at 440 nm, and a 550-nm interference filter was used for the emission maximum.

Recent studies have suggested that ACM-A is metabolized mainly by two pathways in animals. One involves a reductive glycosidic cleavage to give 7-deoxyaklavinone and MA144-E1[9]; in the other reduction at the C-4‴ position of the terminal sugar, L-cinerulose, gives MA144-M1 and MA144-N1.[8] One of the main problems in developing the *in situ* TLC fluorescence scanning method was the separation of ACM-A and these metabolites from endogenous fluorescent materials present in plasma. This was achieved by multi-

Table 1. Rf values of aclacinomycin analogues in chloroform-methanol mixtures.

Compound	Solvent system: Chloroform - methanol mixture				
	1 $CHCl_3$	2 65 : 1	3 20 : 1	4 10 : 1	5 5 : 1
F: Bisanhydroaklavinone	**0.64**	0.77	0.89	0.93	0.87
C1: 7-Deoxyaklavinone	0.21	**0.54**	0.78	0.86	0.83
D1: Aklavinone	0.13	**0.45**	0.77	0.86	0.83
E1: MA144-E1	0.03	**0.31**	0.74	0.86	0.83
A1: Aclacinomycin A	0	0.03	**0.49**	0.83	0.83
M1: MA144-M1	0	0	0.15	**0.54**	0.72
N1: MA144-N1	0	0	0.11	**0.47**	0.66
S1: MA144-S1	0	0	0.02	0.17	**0.42**
T1: 1-Deoxypyrromycin	0	0	0.01	0.11	**0.22**

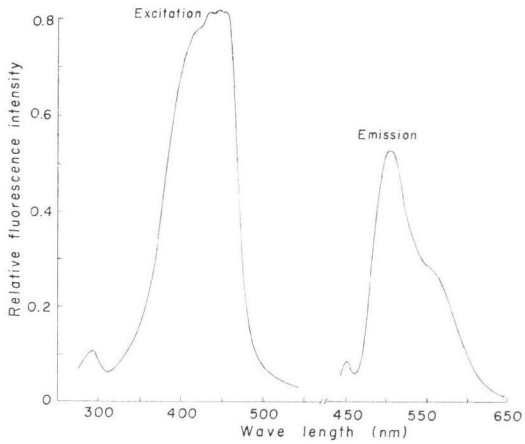
Bold-faced values indicate suitable solvent systems for TLC-fluorescence scanning of each compound.

ple-step separation in the chloroform-methanol mixtures. Nonpolar fluorescent materials and a large quantity of oily substances in plasma which interfere with proper separation of the metabolites were removed to the solvent front area by developing first in chloroform. Then the plate was briefly dried and again developed in the second solvent system. The procedure was repeated with increasingly polar solvent mixtures until all compounds were resolved. Rf values and the most suitable solvent mixture for TLC fluorescence determination of each compound are shown in Table 1.

The fluorescent characteristics of ACM-A determined in a Hitachi fluorescence spectrophotometer Model 240 are shown in Fig. 2. Excitation and emission maxima were observed at 440~445 nm and 505 nm, respectively. All of the glycosidic metabolites which, like ACM-A, contain aklavinone as their aglycone, and the aglycone-type analogs, 7-deoxyaklavinone and bisanhydroaklavinone, exhibited very similar fluorescence characteristics.

Estimation of known concentrations of ACM-A in rabbit plasma by this procedure showed a close linear relationship between the amount of drug present and the integrated value of the peak area in the range of 5~200 ng. Reproducibility, determined by repeated analysis of a standard solution on the same TLC plate was excellent. However, the usual recovery of ACM-A from physiological fluids such as plasma, blood cells, urine or tissue homogenate was about 85%, so that a standardization procedure was desirable

Fig. 2. Excitation and emission spectra of aclacinomycin A in *n*-butanol.



for quantitative determinations. This was achieved by adding known amounts of the standard compounds to a plasma sample obtained from the animal before the drug was administered. These samples were later analyzed on the same TLC plate as the known samples and used to prepare a standard curve. Usually three levels of a standard and seven unknown samples were determined on each silica gel thin-layer plate.

As an example of the use of this method, plasma and blood cell levels of ACM-A and its metabolites in rabbits were determined. After intravenous administration of 10 mg/kg of ACM-A, 2-ml blood samples were collected at timed intervals from 5 minutes to 26 hours. The

Fig. 3 TLC-fluorescence scannograms of rabbit blood 30 minutes after intravenous administration of 10 mg/kg aclacinomycin A.

The chromatogram was developed with chloroform - methanol mixture (20 : 1).

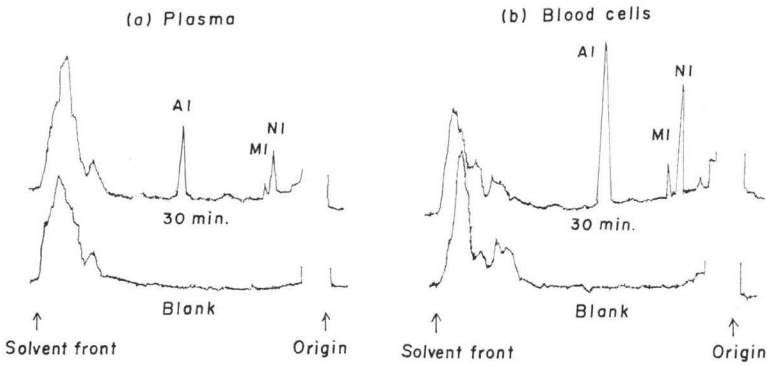
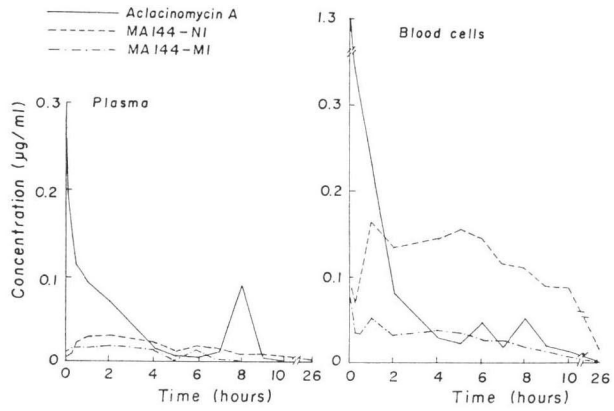


Fig. 4. Blood levels of aclacinomycin A and its metabolites in rabbits administered 10 mg/kg of alacinomycin A intravenously.



scannograms for plasma and blood-cell extracts which were collected 30 minutes after drug administration are shown in Fig. 3. The peak for ACM-A was observed in the middle of the chromatogram and 4‴-hydroxy metabolites MA144-M1 and MA144-N1 were also detected. In addition, large quantities of endogenous fluorescent material were observed in the solvent front area and around the origin. As shown in Fig. 4, the amount of ACM-A in blood decreased very rapidly and was less than 0.1 μg/ml after 1 hour in plasma and after 2 hours in blood cells. It then decreased gradually, but by 26 hours both plasma and blood-cell concentrations were below the detectable limit of this method. It is of interest that small rebound peaks of ACM-A were observed at 6~8 hours, suggesting the existence of entero-hepatic circulation of the drug. The main metabolites were MA144-M1 and MA144-N1, both of which are 4‴-hydroxy isomers reduced at the C-4‴ position of ACM-A. Two other spots corresponding to MA144-S1 and 1-deoxypyrromycin were found in small quantities, and trace amounts of the aglycone-type metabolites, 7-deoxyaklavinone and bisanhydroaklavinone were detected in the early period.

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