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

II. REVERSE PHASE HPLC DETERMINATION OF ACLACINOMYCIN A AND ITS METABOLITES IN BIOLOGICAL FLUIDS USING FLUORESCENCE DETECTION

TATSUO OGASAWARA, YOSHIRO MASUDA, SHINJI GOTO, SHUNRO MORI and TOSHIKAZU OKI

Central Research Laboratories, Sanraku-Ocean Co., Ltd., Johnan, Fujisawa, Kanagawa, Japan

(Received for publication August 13, 1980)

A sensitive, simple and selective reverse phase high performance liquid chromatographic method was applied to the determination of aclacinomycin A (ACM) and its metabolites in plasma. The chromatography was performed on a microparticulate phenethyl derivative of silica using a mobile phase of acetonitrile-0.03 M ammonium formate buffer (pH 5.0), (50: 50 ,v/v) and monitoring with fluorescence detector (excitation 435 nm-emission 505 nm). High reproducibility and linear calibration curves were obtained from 20 ng/ml to 100 ng/ml of ACM and its metabolites in plasma by use of internal standard methods.

Aclacinomycin A (ACM)[1~5] is a cancer chemotherapeutic agent under clinical trial. In order to pursue ACM and its metabolites in such biological materials as serum, plasma and tissue obtained from clinical trials, it has been necessary to develop a rapid and efficient analytical method for the determination of these compounds. In the previous paper[6], we reported a normal phase high performance liquid chromatography (HPLC) for the quantitative determination of ACM and its related compounds which was suitable to monitor fermentation and purification processes and pharmaceutical products. Several papers[7~10] have been recently published in which reverse phase HPLC was used for determing adriamycin, daunomycin and their metabolites. These analytical conditions could not be applied to resolve ACM and its metabolites from endogenous materials in serum and plasma.

In this paper we describe a reverse phase HPLC monitoring with a flow fluorescence detector for the quantitative determination of ACM and its metabolites in plasma samples.

Experimental

Chemicals and reagents

Acetonitrile was of HPLC grade. Ethyl acetate and methanol were of analytical grade and used after distillation. Potassium phosphate (monobasic), sodium phosphate (dibasic), aqueous ammonia and formic acid were all of analytical grade, and used without further purification. All reagents were purchased from Wako Pure Chemical Co., (Tokyo, Japan). Deionized and distilled water was used for preparation of buffers. ACM and related compounds were prepared by isolating and purifying from the cultured broth of ACM-producing microorganism, *Streptomyces galilaeus* MA144-M1 (ATCC 31133), according to the method described in the previous paper[5].

Apparatus

A Shimadzu Liquid Chromatograph Model LC-3A with RF-500LC fluorescence detector monitoring at the excitation wavelength of 435 nm and at the emission wavelength of 505 nm was used with a

Shimadzu Model C-R1A Chromatopak as both a recorder and an integrator. This system was equipped with a stainless-steel column (Waters Assoc., U.S.A., 30 cm × 3.9 mm i.d.) packed with reverse phase μ-Bondapak alkyl phenyl (phenethyl derivative of silica).

The operating conditions were as follows: mobile phase, acetonitrile - 0.03 M ammonium formate buffer (pH 5.0), (50: 50, v/v); flow rate, 1.0 ml/min.; pressure, about 1,000 psi; room temperature controlled at 21±1°C; fluorescence sensitivity, gain 100 and range 8.

Internal standard solution

Aclacinomycin B, which has not been found in mammalian metabolites, was used as an internal standard, and this standard solution was prepared by dissolving and diluting with methanol to concentration of 100 ng/ml.

Standard solutions

Standard solutions of each concentration of ACM and its analogues were prepared by dissolving and diluting with the mobile phase. Concentrations of each analogue (ACM, MA144 S1, MA144 T1 and MA144 M1) were adjusted at five levels from 200 to 1,000 ng/ml. These standard solutions were used for determination of precision and reproducibility of the HPLC method without extraction procedure.

Preparation of spiked plasma samples

One ml of plasma was spiked with 100 μl of the standard solution diluted with water to obtain the five concentration levels of ACM and its analogues from 20 to 100 ng/ml.

Extraction procedure

One ml of plasma, 1 ml of 0.2 M phosphate buffer (KH_2PO_4-Na_2HPO_4, pH 7.0) and 3 ml of ethyl acetate were added to a 20-ml test tube with stopper. The test tubes were gently shaken for 10 minutes and then centrifuged for 5 minutes at 3,000 rpm under room temperature to obtain the ethyl acetate layer. The aqueous layer was reextracted with 3 ml of ethyl acetate, and then the ethyl acetate layers were combined together and evaporated to dryness. The residue was dissolved in 1 ml of the methanolic internal standard solution. This solution was also evaporated to dryness. The residue was redissolved in 100 μl of the mobile phase and then injected onto the HPLC column.

Preparation of plasma samples

A mongrel dog was given a single rapid intravenous injection of 5 mg/kg of ACM hydrochloride. Blood samples (4 ml) were collected by use of syringe added with heparin at intervals of 2, 5, 10, 15, 30, 45, 60, 120 and 180 minutes after injection, and then centrifuged to separate plasma from blood cells. The plasma samples were treated in duplicate according to the above extraction procedure.

Results and Discussion

By the optimized reverse phase HPLC method in conjunction with the extraction procedure, ACM and its analogues were clearly separated from each other and resolved not only from the internal standard but also from other coextracted endogenous biological materials, as shown in Fig. 1. The mobile phase consisted of acetonitrile and 0.03 M ammonium formate buffer (pH 5.0). Replacing acetonitrile by other organic solvents such as acetone, dioxane, methanol or tetrahydrofuran, resulted in a poor separation. In addition, while the elution time of each analogue was faster as the pH was lower, ACM was rather unstable in acidic state and the separation selectivity was independent of the pH of the ammonium formate buffer.

The detection limit of ACM and its analogues was a few ng per injection on an absolute mass basis.

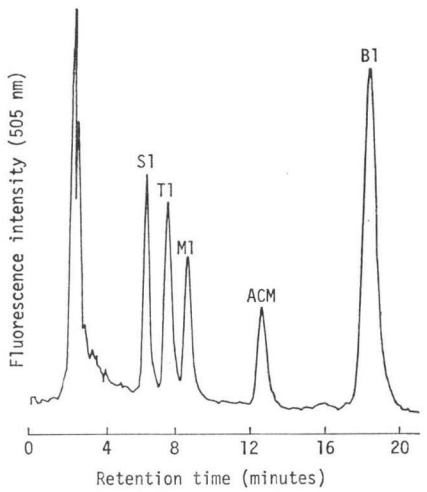
The recovery of ACM and its analogues in the extraction procedure was calculated by comparison of peak areas of ACM and its analogues in the standard solution with those of the extracted sample from the spiked dog plasma. Table 1 shows the average percent recovery and their standard deviation (S.D.)

for spiked dog plasma with ACM and its analogues at the concentrations of 20 ng/ml and 100 ng/ml, giving high recovery and reproducibility to this extraction procedure.

Fig. 1. Chromatogram of extracted dog plasma spiked with aclacinomycin A, its metabolites and aclacinomycin B (Bl) as the internal standard.

Column: 30 cm×3.9 mm i.d., μ-Bondapak alkyl phenyl; mobile phase, acetonitrile - 0.03 M ammonium formate buffer (pH 5.0), (50: 50, v/v); pressure, about 1,000 psi; flow rate, 1.0 ml/min; detector, fluorescence (excitation 435 nm - emission 505 nm).

Metabolites used: ACM, aclacinomycin A; Ml, MA144 Ml; Sl, MA144 Sl; Tl, MA144 Tl.



For the reproducibility analysis, the following procedure was conducted. Dog plasma samples spiked with ACM and its analogues with four duplicates of five levels of the concentrations from 20 ng/ml to 100 ng/ml were extracted. The extracts were added with the internal standard

Fig. 2. A calibration curve of aclacinomycin A in dog plasma.

Peak area ratio=peak area of aclacinomycin A/peak area of the internal standard, aclacinomycin B (100 ng/injection).

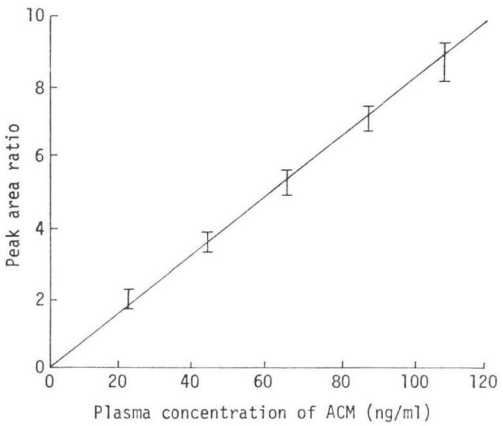


Table 1. Extraction recoveries of aclacinomycin A and its metabolites from dog plasma at the concentration of 20 and 100 ng/ml.

Concentration (ng/ml)	Percent recovery*			
	Aclacinomycin A	MA144 Sl	MA144 Tl	MA144 Ml
20	84.7±2.6	76.2±4.2	78.0±3.7	79.1±2.7
100	92.5±0.9	88.9±1.2	91.8±1.3	85.9±1.1

* Mean±S.D., n=4.

Table 2. Regression analysis between peak area ratio and concentration of aclacinomycin A and its metabolites in dog plasma.

	Aclacinomycin A	MA144 Sl	MA144 Tl	MA144 Ml
Intercept	0.0	0.0	0.0	0.0
Regression coefficient*	0.815×10^{-2}	1.179×10^{-2}	1.414×10^{-2}	1.025×10^{-2}
Standard error of regression coefficient	0.013×10^{-2}	0.018×10^{-2}	0.023×10^{-2}	0.011×10^{-2}
Correlation coefficient	0.997	0.997	0.997	0.999

* Regression coefficient=peak area ratio (peak area of each metabolite divided by peak area of the internal standard, 100 ng/injection)/concentration of each metabolite in dog plasma (ng/ml).

Table 3. Precision and reproducibility in the determination of aclacinomycin A (ACM) and its metabolites in dog plasma.

ACM (ng/ml*)		MA144 S1 (ng/ml*)		MA144 T1 (ng/ml*)		MA144 M1 (ng/ml*)	
Theoretical	Calculated**	Theoretical	Calculated**	Theoretical	Calculated**	Theoretical	Calculated**
21.8	25.2±0.8	20.9	22.7±3.4	19.6	22.6±3.3	21.7	22.6±1.2
43.6	44.5±2.3	41.8	42.0±5.4	39.2	40.2±4.8	43.5	42.9±3.5
65.4	64.4±3.1	62.7	66.2±3.2	58.8	60.7±2.6	65.2	66.4±1.4
87.0	89.6±8.4	83.6	81.9±4.8	78.0	76.7±3.5	87.0	86.1±3.6
109.0	105.3±6.2	104.5	101.6±4.3	98.0	94.9±3.9	108.7	107.6±5.4

* Concentration in dog plasma.
** Mean±S.D., n=4.

Table 4. Regression analysis between the absolute mass of injection and the peak area in the determination of aclacinomycin A and its metabolites of standard solutions.

	Aclacinomycin A	MA144 S1	MA144 T1	MA144 M1
Intercept	0.0	0.0	0.0	0.0
Regression coefficient	0.785×10^{-2}	1.275×10^{-2}	1.321×10^{-2}	1.164×10^{-2}
Standard error of regression coefficient	0.007×10^{-2}	0.006×10^{-2}	0.008×10^{-2}	0.012×10^{-2}
Correlation coefficient	0.999	0.999	0.999	0.999

* Regression coefficient=peak area ratio (peak area of each metabolite divided by peak area of the internal standard, 100 ng/injection)/absolute mass of each metabolite in standard solutions (ng/injection).

Table 5. Precision and reproducibility in the determination of aclacinomycin A (ACM) and its metabolites in standard solutions.

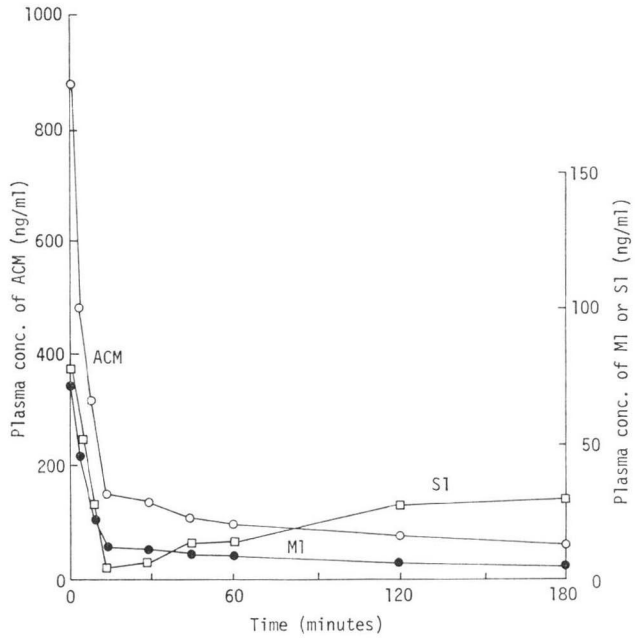
ACM (ng/inj.*)		MA144 S1 (ng/inj.*)		MA144 T1 (ng/inj.*)		MA144 M1 (ng/inj.*)	
Theoretical	Calculated**	Theoretical	Calculated**	Theoretical	Calculated**	Theoretical	Calculated**
22.8	21.8±1.3	21.9	21.0±0.3	19.8	16.8±0.4	23.2	17.6±0.6
45.6	43.9±3.3	43.7	42.9±0.2	39.5	38.7±0.1	46.4	44.9±0.5
68.3	67.7±2.0	65.6	63.5±0.3	59.3	58.0±0.4	69.6	69.9±1.9
91.1	92.4±5.0	87.4	87.3±0.7	79.0	78.6±0.5	92.8	95.2±3.7
113.9	114.4±2.1	109.3	111.3±0.8	98.8	100.8±0.7	116.0	115.8±0.8

* ng per injection (injection volume=100 μl).
** Mean±S.D., n=4.

and then injected onto the column. The least-squares linear regression analysis was performed on the peak area ratio (peak area of each analogue divided by peak area of the internal standard) as the response to determine regression coefficients and correlation coefficients for each analogue. Table 2 shows the results suggesting that the relationship between analogue concentration and peak area ratio is linear with high reproducibility. The calibration curve for ACM is shown in Fig. 2.

The precision and reproducibility of this extraction-HPLC analysis was determined by calculating the mean concentration of ACM and its analogues. The results are given in Table 3. Furthermore, in order to determine the precision and reproducibility of this HPLC analysis itself without the extraction procedure, the standard solution of each analogue with the internal standard were directly injected onto

Fig. 3. Plasma concentration-time curve in a mongrel dog for aclacinomycin A and its metabolites after a 5 mg/kg intravenous dose of aclacinomycin A hydrochloride.



the column. The results were given in Table 4 for the least-squares linear regressions analysis and in Table 5 for the mean calculated concentration $\pm$S.D.

The pooled relative standard deviation is 5.6% for ACM, 8.6% for MA144 S1, 7.9% for MA144 T1 and 5.0% for MA144 M1 in the extraction-HPLC analysis of dog plasma. And in the HPLC analysis of standard solutions without the extraction procedure, the pooled relative standard deviation is 4.7% for ACM, 0.8% for MA144 S1, 0.9% for MA144 T1 and 2.4% for MA144 M1.

Plasma concentration-time curves for ACM and its metabolites in a mongrel dog were determined by the above HPLC-extraction procedure, as shown in Fig. 3.

Acknowledgements

We wish to express our sincere thanks to Prof. HAMAO UMEZAWA, Institute of Microbial Chemistry, Tokyo, for his kind advice and criticism, and also thanks to Mr. KENTARO OHMORI of our laboratory for providing dog plasma samples.

References

1) OKI, T.; T. MATSUZAWA, A. YOSHIMOTO, K. NUMATA, I. KITAMURA, S. HORI, A. TAKAMATSU, H. UMEZAWA, M. ISHIZUKA, H. NAGANAWA, H. SUDA, M. HAMADA & T. TAKEUCHI: New antitumor antibiotics, aclacinomycin A and B. J. Antibiotics 28: 830~834, 1975
2) OKI, T.: New anthracycline antibiotics. Jap. J. Antibiotics 30 (Suppl.): S 70~S 84, 1977
3) HORI, S.; M. SHIRAI, S. HIRANO, T. OKI, T. INUI, S. TSUKAGOSHI, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: Antitumor activity of new anthracycline antibiotics, aclacinomycin A and its analogues and their toxicity. Gann 68: 685~690, 1977
4) OKI, T.; T. TAKEUCHI, S. OKA & H. UMEZAWA: New anthracycline antibiotic aclacinomycin A. Experimental studies and correlations with clinical trials. Recent Results Can. Res. 76: 21~40, 1980

5) OKI, T.; I. KITAMURA, Y. MATSUZAWA, N. SHIBAMOTO, T. OGASAWARA, A. YOSHIMOTO, T. INUI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Antitumor anthracycline antibiotics, aclacinomycin A and its analogues. II. Structural determination. J. Antibiotics 32: 801~819, 1979
6) OGASAWARA, T.; S. GOTO, S. MORI & T. OKI: High performance liquid chromatographic determination of aclacinomycin A and its related compounds. I. Normal phase HPLC for monitoring fermentation and purification processes. J. Antibiotics 34: 47~51, 1981
7) MILLER, R. D. & N. NEUSS: High performance liquid chromatography of natural products: Direct biological correlation of components in the fermentation broth. J. Antibiotics 31: 1132~1136, 1978
8) EKSBORG, S. & H. EHRSSON: Liquid chromatographic determination of daunorubicin and daunorubicinol in plasma from leukemic patients. J. Chromatogr. 153: 211~218, 1978
9) PIERCE, R. N. & P. I. JATLOW: Measurement of adriamycin (doxorubicin) and its metabolites in human plasma using reversed-phase high performance liquid chromatography and fluorescence detection. J. Chromatogr. 164: 471~478, 1979
10) EKSBORG, S.; H. EHRSSON & I. ANDERSSON: Reversed-phase liquid chromatographic determination of plasma levels of adriamycin and adriamycinol. J. Chromatogr. 164: 479~486, 1979