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Sensitive enzyme immunoassay for the quantification of aclacinomycin A using β-D-galactosidase as a label

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Summary. *A sensitive enzyme immunoassay method (EIA) for an anticancer drug, aclacinomycin A (ACM), has been developed. With a double-antibody technique, ACM at a concentration as low as 100 pg/tube can be detected. An antibody to ACM was obtained by immunizing rabbits with an antigen prepared by coupling ACM with mercaptosuccinylated bovine serum albumin via N-maleoyl aminobutyric acid (MABA) as a coupling agent. Enzyme labeling of ACM was performed with β-D-galactosidase (β-Gal; EC 3.2.1.23) via m-maleoyl benzoic acid (MBA). The standard curve of the assay was linear on a logit-log plot over a concentration range of 30 pg to 10 ng. The antibody detected ACM and its metabolites, MA144 M1 (M1), MA144 N1 (N1), MA144 S1 (S1), and aklavin (T1) equally well, but was only minimally reactive with aklavinone (D1) and 7-deoxyaklavinone (C1), thus suggesting that this EIA can detect the total amounts of ACM and its biologically active glycosides among metabolites of ACM. This EIA is practically free from interference by any other anticancer drugs. Using this assay, serum levels of ACM equivalents can be determined accurately after administration of the drug to rats at a single dose of 10 mg/kg. Since ACM is now undergoing clinical trial, the EIA of the drug will be a valuable tool in clinical pharmacological studies.*

Introduction

A new anthracycline antibiotic aclacinomycin A (ACM), isolated from the culture of *Streptomyces galilaeus* MA144-M1 [21], exhibits antitumor activity against a variety of animal [9, 19] and human tumors [7]. This drug is an aklavinone trisaccharide, cinerulosyl-2-deoxyfucosyl-rhodosaminyl-aklavinone, and is metabolized in mammals or tissue in several ways, giving rise to the glycosidic metabolites, MA44 M1, N1, S1, or aklavin (T1) and some nonglycosidic metabolites, 7-deoxyaklavinone (C1), its dimer (E1), aklavinone (D1), or bisanhydroaklavinone (F1) (Fig. 1) [4, 16, 22, 23].

The development of a simple and sensitive assay method for the quantification of ACM will greatly facilitate the design of dose and treatment schedules for use with cancer patients and in pharmacokinetic studies in human and animal systems. Previous pharmacokinetic studies of ACM have been undertaken with isotopically labeled ACM [11], microbiological assay [20] and fluorometric techniques [13], or high-performance liquid chromatography [18]. A radioimmunoassay for ACM has recently been reported [17].

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Recent studies have shown that an enzyme immunoassay (EIA) may have a comparable sensitivity to the radioimmunoassay [28] and overcome drawbacks inherent in radiolabeling [12, 24]. However, an EIA system for ACM has not previously been developed.

This paper describes the methodology for the antibody production, the labeling of ACM with β-D-galactosidase to act as a tracer, the charaterization of antibody specificity, and the technique developed for the measurement of ACM by EIA. The initial application of the assay to the measurement of ACM levels in rats demonstrates its usefulness in the assessment of basic pharmacokinetic distribution.

Materials and methods

ACM hydrochloride and its metabolites, MA144 M1, N1, S1, aklavin, aklavinone, and 7-deoxyaklavinone were kindly supplied by Dr. T. Oki, Sanraku-Ocean Co., Ltd, Tokyo, Japan. β-D-Galactosidase (β-Gal; EC 3.2.1.23) from *Escherichia coli* was obtained from the Boehringer Mannheim Co., W. Germany. *N*-Maleoyl aminobutyric acid (MABA) and *m*-maleoyl benzoic acid (MBA) were synthesized by the methods of Rich et al. [25] and Kitagawa and Aikawa [12], respectively.

Coupling reaction for hapten. The preparation was similar to our method of synthesizing immunogens for mitomycin C [6] (Fig. 2). The carbonyl group of the cinerulose A moiety of ACM was chemically aminated by the method of Tanaka et al. [26], and the resulting 4‴-deoxo-4‴-aminoaclacinomycin A HCl (4‴-NH_2ACM HCl) was acylated with MABA through an amide bond by a mixed carbonic anhydride method of Anderson et al. [1]: A solution containing 2.25 mg (12.3 μmol) MABA and 1.24 μl *N*-methylmorpholine in 1.0 ml tetrahydrofuran (freshly distilled from calcium hydride) was cooled at 0° C and mixed with 1.68 μl isobutyl chloroformate under vigorous stirring. After 1 min, 10 mg (11.6 μmol) 4‴-NH_2ACM HCl dissolved in 400 μl tetrahydrofuran was also added and the reaction mixture was stirred for 2 h at room temperature. After removal of the solvent under vacuum, 4 ml chloroform and 4 ml 0.33 *M* citric acid were added to the residue and the mixture was shaken vigorously. The water layer was discarded and the chloroform layer was washed with water, then with 1 *N* sodium bicarbonate, and finally with saturated sodium chloride. The chloroform solution was dried over anhydrous sodium sulfate and then in a vacuum. At the same time, acetylmercaptosuccinyl bovine serum albumin

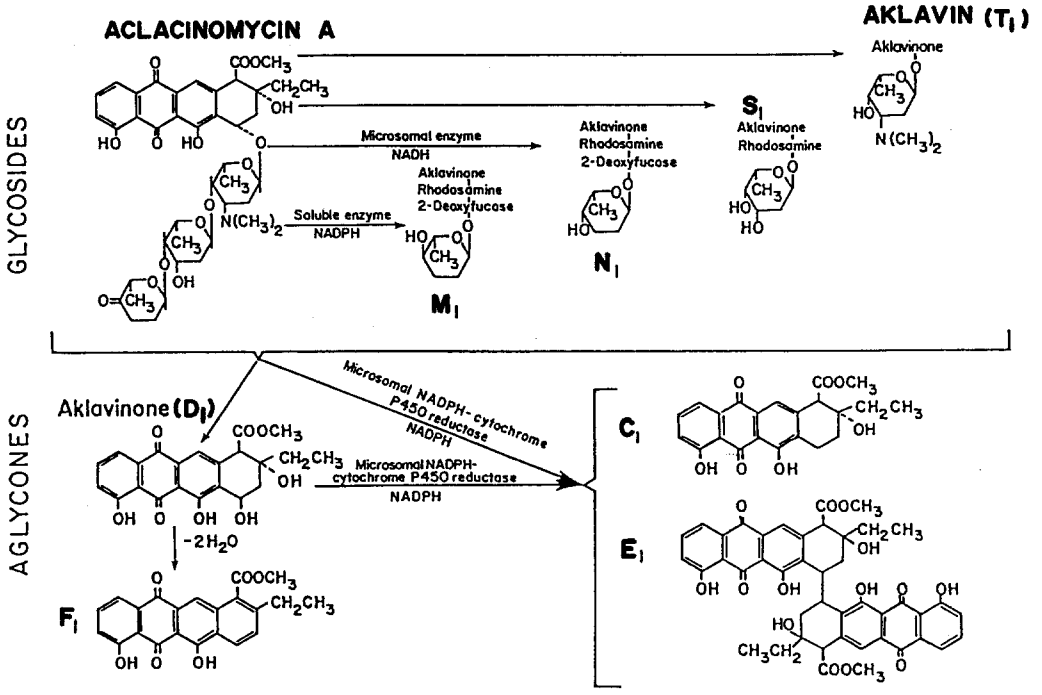

Fig. 1. Formula of aclacinomycin A and its metabolites. (Egorin et al. [4])

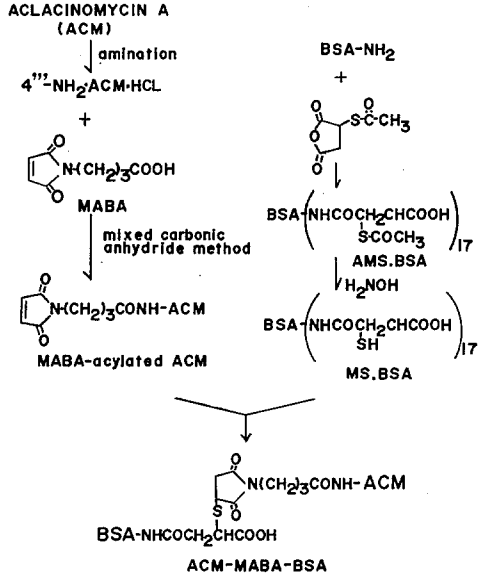

Fig. 2. Schematic for synthesic of immunogen for ACM

(AMS.BSA 10 mg) prepared by the method of Klotz and Heiney [14] was incubated in 0.2 ml 0.1 *M* hydroxylamine, pH 7.2, at 25° C for 30 min to remove the protecting acetyl group. The resulting mercaptosuccinyl bovine serum albumin (MS.BSA), estimated to contain 17 thiol groups per BSA molecule [5], was diluted with 3 ml 0.1 *M* phosphate buffer, pH 7.0, and added immediately to the MABA-acylated ACM solution, and then incubated for 30 min with vigorous stirring. The mixture was applied to a 2.8 × 45 cm column of Sephadex G-100 equilibrated with 0.1 *M* phosphate buffer, pH 7.0, containing 3 *M* urea and developed with the same buffer. Then the purified conjugate was examined spectrophotometrically and estimated to contain about 6.8 molecules of ACM per BSA molecule, assuming the molar extinction coefficients of ACM to be 11,800 at 280 nm and 13,100 at 440 nm, and those of BSA to be 43,600 at 280 nm.

Antibody production in rabbits. An aliquot containing about 1.0 mg of the ACM-BSA complex was emulsified with an equal volume of Freund's complete adjuvant. Two white female rabbits were each given ten SC injections over sites along both sites of their back. Booster injections were then given three times at biweekly intervals, using one-half the amount of the dose of the first immunization. Each rabbit was bled from the ear vein every 2 weeks, and serum was separated by centrifugation at 500 *g* and analyzed as described in the following section.

Preparation of ACM-β-Gal conjugate. ACM-β-Gal was prepared by essentially the same two-step procedure as the immunogen for ACM, using a cross-linker MBA which differs from MABA in the substitution of a benzoic acid radical for an *n*-butyric acid radical. First MBA-acylated ACM was synthesized from MBA (1 mg; 4.61 μmol) and 4'''-NH_2ACM (5.0 mg; 5.8 μmol) by a method similar to that for the preparation of MABA-acylated ACM. The MBA-acylated ACM (approximately 10 μg; 9.51 nmol) in 50 μl methanol was added directly to β-Gal solution containing 78 μg (0.14 nmol) of the enzyme [2] in 1.0 ml 0.1 *M* phosphate buffer, pH 6.0. The mixture was incubated for 30 min with stirring and then applied to a Sepharose 6B column (1.5 × 42 cm) equilibrated with 0.02 *M* phosphate buffer, pH 7.0, containing 0.1 *M* NaCl, 1 m*M* $MgCl_2$, 0.1% BSA, and 0.1% NaN_3 (Buffer A) and eluted with the same buffer. The elution profiles of both enzyme and immunoreactive enzyme activities in Fig. 3 show that the immune activity of the conjugate eluted in parallel with enzyme activity. However, the immune specificity, defined as the immunoreactive enzyme activity in the presence of free ACM (1 ng), showed that the conjugate was better differentiated from ACM in the latter peak fractions than in the early peak fractions. Fractions 18–20 of the main peak

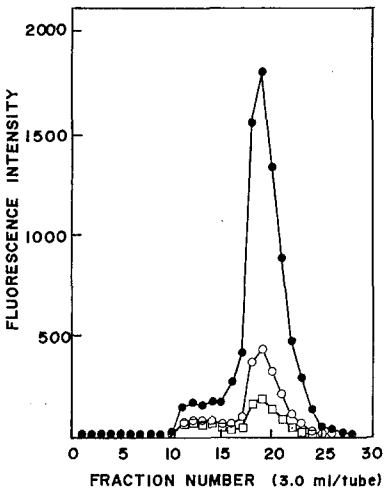

Fig. 3. Elution profiles of ACM-β-Gal from a Sepharose 6B column. ●, Enzyme activity of the conjugate, measured by the use of 5 µl from each fraction at 30° C for 5 min; ○, immunoreactive enzyme activity, determined by the EIA described in *Materials and Methods* except that 5 µl of the conjugate and a 1/1000 solution of anti-ACM serum were used in the absende of ACM; □, immune specificity, determined in the same way in the presence of 1 ng of ACM

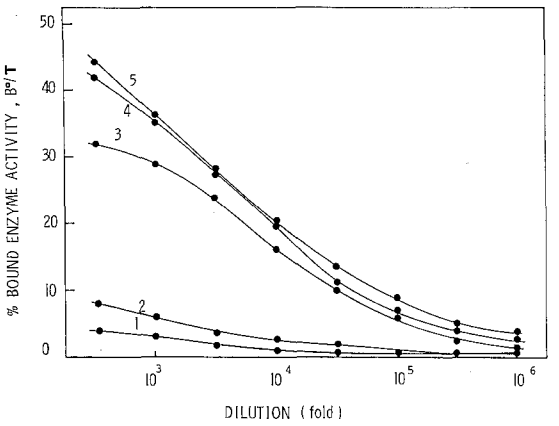

Fig. 4. Quantitative determination of anti-ACM antibody. Samples 2–5 are the antisera collected 2 weeks after each injection. Sample 1 was prebled before immunization. The ACM-β-Gal (110 µU) (T) was incubated with solutions of the antiserum (or prebled serum) for 6 h; this was followed by further incubation for 3 h with the second antibody (goat anti-rabbit IgG and normal rabbit serum). The uptake of ACM-β-Gal in the precipitate was measured (B°) and expressed as B°/T (% bound enzyme activity versus the total enzyme activity)

were thus chosen for the EIA. The amount of immunoreactive enzyme activity of the conjugate formed was 24.3% of the enzyme activity. The conjugate was stable for greater than 3 months in Buffer A at 4° C without any loss of immunoreactive enzyme activity. A Km value of the substrate (7-β-D-galactopyranosyloxy-4-methylcoumarin) against the ACM-β-Gal conjugate was found to be 0.33 m*M*, which was not different from that of the pure enzyme.

Measurement of β-Gel activity. Enzyme activity was measured by the method reported previously [6]: 50 µl diluted enzyme solution was incubated with 0.15 ml 0.1 m*M* 7-β-D-galactopyranosyloxy-4-methylcoumarin in Buffer A at 30° C for 1 h. The reaction was stopped by the addition of 2.5 ml 0.2 *M* glycine-NaOH buffer, pH 10.3, and the 7-hydroxy-4-methylcoumarin released was measured by spectrofluorometry at wavelengths of 365 nm for excitation and 448 nm for emission. The amount of the conjugate was expressed in units of β-Gal activity, defining 1 unit of enzyme activity as the amount that hydrolyzed 1 µmol substrate/min.

Enzyme immunoassay method (EIA). The assay was performed by the double-antibody method [27]. Experimental conditions for the EIA were extensively examined [7], and an effective assay system was established as follows. An antiserum, ACM-β-Gal, and unlabeled ACM were diluted in Buffer B (0.06 *M* sodium phosphate, pH 7.4, containing 0.01 *M* EDTA, 0.1% BSA and 0.1% NaN_3). Then 50 µl ACM-β-Gal conjugate (110 µU), 50 µl ACM or sample, as appropriate, and 50 µl 1/10,000 solution of the antiserum were mixed, giving a final reaction volume of 150 µl, and the mixture was incubated at 25° C for 8 h. Then 50 µl 10% solution of goat anti-rabbit IgG and 50 µl 0.33% solution of normal rabbit serum were added. After further incubation for 3 h, the immune precipitate was washed twice by the addition of 1.0 ml Buffer A and centrifuged at 2,500 rpm for 15 min in a refrigerated centrifuge. The supernatant was decanted, and the enzyme activity in the immune precipitate was measured.

Results

Enzyme immunoassay of a hapten ACM requires chemical modifications of the drug for the preparations of the immunogenic form and of enzyme labeling as the tracer. 4'''-NH_2ACM, a chemical derivative of ACM possessing a primary amino group at C-4''' position, was prepared and conjugated with a sulfhydryl group of a protein (MS.BSA or β-Gal) by thiolation using a heterobifunctional agent, MABA or MBA (see Fig. 2 for immunogen preparation).

Immunogen preparation

4'''-NH_2ACM was acylated with MABA through an amide bond by a mixed carbonic anhydride method and the resulting MABA-acylated ACM was extracted in acidic pH and basic pH with chloroform separating unreacted ACM and the coupler. This process of purification of MABA-acylated ACM was very important for the subsequent conjugation reaction with the thiol groups of MS.BSA to prepare a homogeneous conjugate of ACM-MABA-BSA, not giving unwanted MABA-BSA conjugate. Gel filtration of the ACM-MABA-BSA on Sephadex G-100 showed only one UV absorbing peak apart from excess ACM, the conjugate appearing immediately after the void volume from the column. The conjugate fraction contained all the orange color of ACM. The conjugate contained 6.8 mol ACM per mol BSA.

Antiserum

ACM antisera were obtained following the priming injection from two rabbits immunized. Subsequent booster injections increased the antiserum titers considerably. Preimmunization sera did not bind ACM-β-Gal conjugate. Figure 4 shows the typical antibody response to immunogen in one of the two rabbits immunized with ACM-BSA. Samples of serum collected 8 weeks after the first immunization was used to develop and validate the EIA and could be used an initial dilution of approximately 1 : 10,000.

EIA for ACM

The dose-response standard curve of ACM in a buffer system was essentially linear on a logit-log plot between 30 pg and 10 ng (Fig. 5). For practical purposes, the working range was arbitrarily set at 100 pg to 10 ng/assay tube. Assay precision, defined as the ratio between the standard deviation of Y and the slope of the regression line between mean values of logit Y and the corresponding log X, was $\lambda = 0.09$ at 0.33 ng. In these experiments the specific binding of the ACM-β-Gal to the anti-ACM antibody in the absence of nonlabeled ACM (B°) was 20.5 ± 0.34 (mean ± SD).

Standard curves were also constructed with 20 and 50 µl normal pooled drug-free serum and 5 and 10 µl normal human pooled urine added. The maximum volume of sample that would be added to the assay is represented by 50 µl, although most samples would be assayed at considerably high dilutions. There was no effect of either serum or urine on the shape of the standard curve or the percentage of binding.

Experiments were also designed to measure the change in immunoreactivity of ACM following incubation in normal human serum or urine. Known concentrations of ACM were incubated in serum or urine for up to 25 h at 37° C, and aliquots of the mixture were periodically withdrawn, diluted 100 times with Buffer B and measured by the EIA using the buffer system. No significant decrease of ACM immunoreactivity caused by the incubation was observed, there being an overall mean recovery of 96.8% ± 4.5% (SE) and 95.8% ± 3.9%, respectively, at six different levels of ACM.

The antibody specificity was determined by measuring the displacement of bound ACM-β-Gal by ACM, its glycosidic and nonglycosidic metabolites. The values of the cross-reactivity were defined as a ratio of each compound to ACM in the concentrations required for 50% inhibition of ACM-β-Gal binding to the antibody. As shown in Table 1, the anti-ACM antibody cross-reacted with the biologically active metabolites [MA144 M1, N1, S1, and T1 (aklavin)] as strongly as the parent compound ACM, but was only minimally reactive with the biologically inactive nonglycosidic metabolites [aklavinone (D1) and 7-deoxyaklavinone (C1)].

We also examined the effect of other anticancer drug on the EIA for ACM. No cross-reaction was seen with the following drugs: 5-fluorouracil, vincristine, endoxan, bleomycin, and mitomycin C. However, doxorubicin and adriamycin

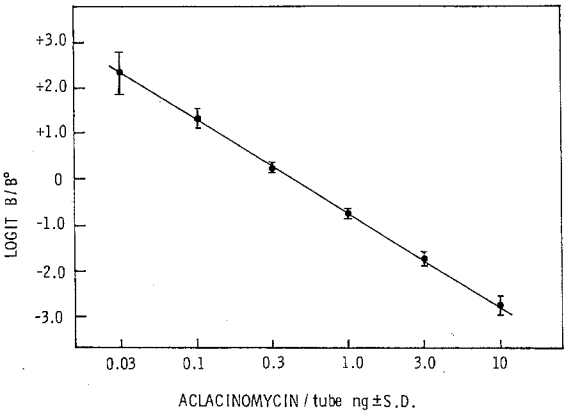

Fig. 5. Standard curve for the EIA for ACM. The response, Y, to the dose, X, can be defined as a logit function of Y so that logit Y = In (Y/l-Y). For experimental purpose, Y is further defined as the ratio between antibody-bound enzyme activity (B) and the enzyme activity bound at zero concentration of unlabeled ACM (B°). Thus, the response logit B/B° to dose, log ACM concentration is plotted. Each *point* represents the mean ± SD for six replicates

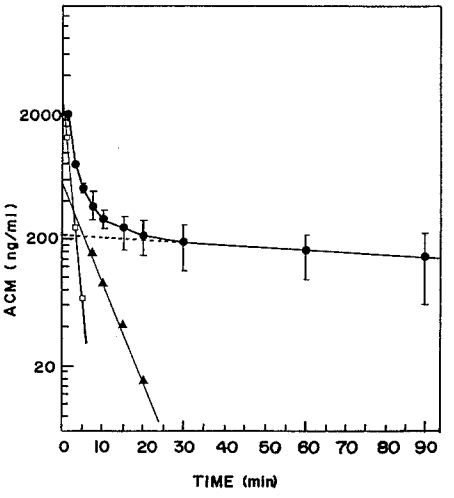

Fig. 6. Serum levels of ACM equivalents in rats after a single IV injection of the drug. Three rats, each weighing about 400 g, were given injections 10 mg ACM per kg. At each time interval, blood was collected and the serum ACM content was measured. The initial (□) and second (▲) phases of distribution were graphically calculated by the method of 'residuals'. The composite curve is also shown (●)

Table 1. Immunoreactivity of aclacinomycin metabolites with anti-aclacinomycin A antiserum[a]

Compounds	Mean concentration		SD		% Cross-reactivity
	ng	(pmol)	ng	(pmol)	
Aclacinomycin A	0.401	(0.495)	± 0.013	(0.017)	100
MA144 M1	0.422	(0.544)	± 0.016	(0.020)	91
MA144 N1	0.418	(0.515)	± 0.026	(0.032)	96
MA144 S1	0.288	(0.412)	± 0.010	(0.015)	120
Aklavin (T1)	0.370	(0.651)	± 0.014	(0.026)	76
Aklavinone (D1)	3.185	(7.73)	± 0.127	(0.31)	6.4
7-Deoxy-aklavinone (C1)	4.559	(11.51)	± 1.628	(4.11)	0.43

[a] Indicated quantity of aclacinomycin metabolites was required to produce a 50% inhibition of β-Gal-labeled ACM bound to antibody. Results are expressed as the mean concentration (picomol) of the aclacinomycin metabolite determined in five separate analyses

caused a slight reduction in ACM-β-Gal conjugate binding to the antibody, with values for cross-reactivity of 0.20% and 0.1%, respectively.

A comparison of the sensitivity of ACM detection by the EIA for ACM and a microbiological assay using *Bacillus megaterium* revealed that the lower limit of detection by the microbiological assay allowed 0.6 μg/ml in a 0.08 ml volume to be measured. Thus, this assay is 300 times less sensitive than the EIA.

Quantification of ACM in rat serum by EIA

The drug levels were quantitated in serum of rats following administration of ACM in a single dose of 10 mg/kg IV and expressed as μg equivalents of ACM/ml in an average of three rats (Fig. 6). Within 90 min after the administration, the decline of ACM in serum was well described by three-compartment model pharmacokinetics defined by the equation, serum concentration

$$C_s = 2.6e^{-0.74t} + 0.56e^{-0.179t} + 0.22e^{-0.0048t}.$$

From this equation serum half-lives were tentatively calculated at 0.9, 3.9, and 144 min for the α, β, and γ phases, respectively, showing that the serum drug level initially declined very rapidly but then more slowly at later times after administration. As shown in the composite curve (Fig. 6) there was close agreement among the individual rats studied, and there was close agreement in the area under the curve ($C \times t$) in the 0–90 min portion for each rat studied (22.3 ± 5.3 μg/ml · min, mean ± SD). As the sensitivity of the assay was known to be at least 100 pg/tube, it appears that additional values beyond the 90-min time point could be obtained.

Discussion

A sensitive EIA for ACM, which is suitable for pharmacokinetic studies on the drug, has been developed. The antiserum used in the assay was produced in response to ACM-BSA conjugate, making use of a cross-linker MABA such as has previously been used in the development of EIA for mitomycin C [6].

In the present study we modified the procedure slightly so that MABA-acylated ACM prepared by a mixed carbonic anhydride method was separated from the nonreacted MABA and the hapten drug by extracting with chloroform; it was exclusively conjugated with a carrier protein MS.BSA, giving a homogeneous conjugate of the hapten ACM with high coupling efficiency.

ACM-β-Gal conjugate was also prepared, with a full retention of enzyme activity, by essentially the same procedure as used in the preparation of immunogen for ACM, using MBA structurally different from MABA, to eliminate any antibody binding to the cross-linkage region of β-Gal. This enzyme labeling of ACM and its subsequent purification by a Sepharose 6B column chromatography could be performed more easily, rapidly, and safely within 1 day than radioactive labeling of ACM for ACM radioimmunoassay [17]. Furthermore, the enzyme conjugate thus obtained was very stable and was found to lose little reactivity when stored for at least 6 months at 4° C in Buffer A.

With ACM-β-Gal as the tracer, a sensitive double-antibody EIA for ACM could quantify as little as 100 pg/assay tube. This assay appears to be 300 and 8 times more sensitive than the corresponding microbiological assay and a recently reported radioimmunoassay [17] for ACM, respectively.

The anti-ACM antibody showed fairly high cross-reactivity with the metabolites of ACM, such as MA144 M1, N1, S1, and aklavin, to the same extent as that of parent compound ACM; however, its cross-reactivity with the metabolites of aklavinone and 7-deoxyaklavinone was low, suggesting that the antibody does not recognize cinerulose A and 2-deoxyfucose but does recognize the *N,N*-dimethylamino sugar (rhodosamine) in the ACM structure (Fig. 1). Thus, this EIA is unsuitable for specific estimation of ACM in the presence of its metabolites, but remains nonetheless a very useful tool for the total amounts of ACM and its biologically active metabolites. This means that this EIA for ACM may become rather important not only in the clinical evaluation of ACM chemotherapy, but also in metabolic studies of ACM, especially in combination with other conventional assay methods such as higher-performance liquid chromatography or radioimmunoassay for ACM. Also, the antibody specificity appears to be in good agreement with that reported for the ACM radioimmunoassay [17].

Using this EIA, preliminary pharmacokinetic studies were undertaken in rats, showing that ACM equivalents disappears from serum in a triphasic fashion, with very small values of half-lives estimated in the α and β phases in comparison with that in the γ phase. This finding might presumably imply that only a minute amount of the metabolites of ACM that does not influence the ACM kinetics would occur during a 90-min infusion in rats, although ACM is known to be metabolized extensively in animals rather longer after dosing [3, 15, 16, 22, 23]. This rapid decline in serum level of ACM equivalents in a triphasic fashion was also observed with ACM plasma spectrofluorometry studies in humans [4].

The EIA procedure for ACM presented here is highly sensitive, reproducible, simple, nonisotopic and safe to perform, and adaptable for analyses of a large number of samples. This EIA will be a valuable tool in clinical pharmacological studies during clinical trials of ACM.

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