Development and Application of a Sensitive Enzyme Immunoassay for 7-N-(p-Hydroxyphenyl)mitomycin C


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ABSTRACT

An antibody specific for 7-N-(p-hydroxyphenyl)mitomycin C (M-83) was developed and used in a simple and sensitive enzyme immunoassay for detection of this anticancer drug in serum. The imino group of M-83 was covalently coupled to mercaptosuccinyl groups introduced into bovine serum albumin with a cross-linker, m-maleoylbenzoic acid. The resulting conjugate was then used for the production of a specific antibody to M-83 in rabbits. Enzyme labeling of M-83 was performed using β-o-galactosidase (EC 3.2.1.23) via m-maleoylbenzoic acid. Antibody production was of sufficiently high titer in rabbits to allow the development of a highly sensitive enzyme immunoassay for the free drug which accurately measures 15 pg of M-83 per assay tube. This assay was monospecific to M-83 and showed almost no cross-reactivity with a variety of other mitomycin analogues (mitomycin A, B, and C; porfiromycin; and acetylmitomycin C). Using this assay, preliminary pharmacokinetic study was undertaken using serial serum samples obtained from a patient who received the drug i.v., revealing a biphasic fashion of the kinetics, with an α-serum half-life of 9.7 min and a β-serum half life of 80 min. An i.v. injection of M-83 into rats and assay of serum concentration revealed a similar biphasic response with an α-serum half-life of 8.3 min and a β-serum half-life of 62 min.

INTRODUCTION

M-83 is a derivative of MMC with higher antitumor activity against Hirosaki ascite sarcoma (18) and P388 ascite leukemia (8, 9, 15) and lower bone marrow toxicity (12) as compared with MMC. These advantages may allow M-83 to have an antitumor effect superior to that of the present MMC, and the drug has been on clinical trial in Japan.

It is considered that dosing of anticancer drug for clinical efficacy is aimed at achieving those concentrations of the drug within body fluids which selectively inhibit tumor cell growth without producing side effects. Thus, pharmacokinetic data will be useful for its optimal clinical usage. Previous pharmacokinetic studies of M-83 were undertaken by a laborious microbiological assay technique that lacks sensitivity and specificity or by the use of the 14C-labeled drug (16), thus using radioactivity as an indirect measure of the drug itself.

Quite recently, we developed an EIA for MMC, parent compound of M-83, using the bifunctional cross-linking agents MABA and MBA for preparing the immunogen and for the β-o-galactosidase labeling of the drug, respectively (5, 6). The assay was highly sensitive and specific to MMC, and it hardly detected its chemical derivative M-83.

This paper describes the methodology for the antibody production, the labeling of M-83 with β-o-galactosidase to act as a tracer, the initial characterization of antibody specificity, and the technique developed for the measurement of M-83 by EIA. Initial application of the assay to measure M-83 levels in a patient and rats demonstrates its utility in the assessment of basic pharmacokinetic distribution.

MATERIALS AND METHODS

Materials. M-83, MMA, MMB, and MMC were obtained from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. Ac-MMC and porfiromycin were prepared according to the method of Matsui et al. (13). β-o-Galactosidase (EC 3.2.1.23) from Escherichia coli was obtained from Boehringer/Mannheim, Mannheim, Germany. MABA and MBA were synthesized by the methods of Rich et al. (17) and Kitagawa and Aikaawa (11), respectively.

Introduction of Maleimide Group Into M-83. M-83 was acetylated with MABA through an imide bond by the mixed carbonic anhydride method of Anderson et al. (1). A solution containing 1 mg (5.46 μmol) of MABA and 0.7 μmol of N-methylmorpholine in 500 μl of tetrahydrofuran (freshly distilled off calcium hydride) was cooled at -5° and mixed with 1 μl of isobutyl chloroformate under vigorous stirring. Ten min later, 2.33 mg (5.46 μmol) of M-83 dissolved in 1.0 ml of tetrahydrofuran were further added, and the reaction mixture was stirred for the dark for 15 hr at 4°. After the solvent was removed under vacuum, 5 ml of ethyl acetate and 5 ml of 0.33 M citric acid were added to the residue, and the mixture was shaken vigorously. The water layer was discarded, and the ethyl acetate layer was further washed twice with the citric acid, subsequently 3 times with 1 N sodium bicarbonate, and finally with saturated sodium chloride. The ethyl acetate solution was dried over anhydrous sodium sulfate and was concentrated under vacuum. The resulting MABA-acetylated M-83 gave a single spot when chromatographed on a Silica Gel 60F254 plate.

Coupling Reaction for Haptens. The preparative process was similar to our previous method of synthesizing immunogen for MMC (6). Acetyl-MSBSA (10 mg), containing 17 acetylmercaptosuccinyl groups per BSA molecule, was incubated in 0.2 ml of freshly prepared 0.1 M hydroxylamine, pH 7.2, at 25° for 30 min to remove the protecting acetyl group. After dilution with 2.0 ml of 20% phosphate buffer, pH 7.0, containing 3 M urea, the resulting MSBSA was added immediately to the MABA-acetylated M-83 in 50 μl of tetrahydrofuran and then incubated for 30 min with vigorous stirring. The mixture was chromatographed on a column of Sephadex G-100 (2.8 x 42 cm) with 0.1 M phosphate buffer, pH 7.0, containing 3 M urea. Then, the purified conjugate was examined spectrophotometrically and was estimated to contain 3.4 molecules of M-83 per BSA molecule assuming the molar extinction coefficients of M-83...
to be 6,000 at 280 nm and 11,800 at 380 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 M-83-BSA complex, was emulsified with an equal volume of Freund’s complete adjuvant. Three white female rabbits received the complex by i.m. and s.c. injections. This was followed 3 times by biweekly booster injections of 0.5 mg of the complex. The rabbits were bled from an ear vein 2 weeks after each injection. The samples of serum were separated by centrifugation, heated at 55° for 30 min, and stored at −30°.

These serum samples were used to examine the levels of anti-M-83 antibody produced by the following procedure. The M-83-β-Gal conjugate prepared in the next section was incubated with solutions of antisem for 14 hr. This was followed by incubation for 4 hr with the second antibody (goat anti-rabbit IgG and normal rabbit serum). The uptake of M-83-β-Gal in the precipitate was measured as a quantitative index of bound antibody (6). As a result, the antibody titer in the serum increased gradually with the booster injections and peaked 2 weeks after the final injection (data not shown).

Preparation of M-83-β-Gal. Labeling of M-83 with β-o-galactosidase was performed by the help of MBA as a bifunctional cross-linker which might avoid a cross-reaction with the antibody produced against the immunogen linkage group of MABA (14). MBA-acylated M-83 was synthesized from MBA (1 mg; 4.61 μmol) and M-83 (2.13 mg; 5 μmol) and purified by what is essentially a repetition of the procedure for MBA-acylated M-83. The MBA-acylated M-83 (approximately 5 μg; 8.0 nmol) in 50 μl of tetrahydrofuran was added directly to a solution of β-o-galactosidase (78 μg; 0.14 nmol) (3) in 1.0 ml of 0.1 M phosphate buffer (pH 7.0), and incubated for 30 min with stirring. The mixture was then chromatographed on a column of Sepharose 6B (2 x 38 cm) with 20 mM (pH 7.0), and incubated for 30 min with stirring. The elution profile of the enzyme and immunoreactive enzyme activities are shown in Chart 1, revealing that the immunoreactive enzyme activity of the conjugate was eluted in parallel with the pure enzyme activity; however, the immune specificity of the conjugate, defined as the immunoreactive enzyme activity in the presence of free M-83 (36.8 ng), is better differentiated from MABA-acylated M-83 in the latter peak fractions than in the early peak fractions. Fractions 16 to 18 of the main peak were thus chosen for the EIA. The conjugate was stable for within 3 months in Buffer A at 4° without any loss of immunoreactive enzyme activity. The Km of the substrate (β-o-galactopyranosylxylo-4-methylcoumarin) against the M-83-β-Gal conjugate was found to be 0.50 μm, which was slightly larger than that of the pure enzyme (0.33 μm).

Measurement of β-o-Galactosidase Activity. Fifty μl of diluted enzyme solution were incubated with 0.15 ml of 0.1 M 7-o-galactopyranosylxylo-4-methylcoumarin in Buffer A at 30° for 1 hr. The reaction was stopped by the addition of 2.5 ml of 0.2 mM glycine-NaOH buffer (pH 10.3), and the 7-hydroxy-4-methylcoumarin liberated was measured spectrophotometrically at wavelengths of 365 nm for excitation and 448 nm for emission. The amount of the conjugate was expressed in units of β-o-galactosidase activity, defining 1 unit of the enzyme activity as the amount that hydrolyzes 1 μmol of the substrate per min.

EIA Method. The assay was performed by the double antibody method (19). An antiserum, M-83-β-Gal, and unlabeled M-83 were diluted in Buffer B (0.06 M sodium phosphate, pH 7.4, containing 0.01 M EDTA, 0.1% BSA, and 0.1% NaN₃). Fifty μl of M-83-β-Gal conjugate (60 units), 50 μl of M-83 or sample, as appropriate, and 50 μl of a 1/620,000 solution of the antiserum were mixed, giving a final reaction volume of 150 μl, and the mixture was incubated at 25° for 14 hr. Then, 50 μl of a 10% solution of goat anti-rabbit IgG and 50 μl of a 0.33% solution of normal rabbit serum were added. After further incubation for 4 hr, the immune precipitate was washed twice by the addition of 1.0 ml of Buffer A and centrifuged at 2500 rpm for 15 min in a refrigerated centrifuge. The supernatant was decanted, and the enzyme activity in the immune precipitate was measured.

Pharmacokinetic Analysis of M-83. The human subject studied was a 65-year-old male patient receiving treatment for cholangiocarcinoma at the Medical College of Nagasaki University. The patient received M-83 by i.v. bolus at a dose of 1.8 mg/kg via the femoral vein. Serial blood samples were obtained using an i.v. catheter from the opposite arm from which the drug was administered. Seven ml of blood drawn prior to dosing (Time 0) and 2 ml each at 1, 3, 5, 15, 30, 60, 120, 180, 240, and 300 min were centrifuged at 2500 rpm for 15 min, and the serum was separated and stored at −30°. Analysis of blood levels of M-83 was performed using 50 μl of each of those sera diluted 2.5, 10, or 100 times with Buffer B as described above. The unknown amount of M-83 present in the serum was quantitated by interpolation on the linearized standard curve performed with serum obtained prior to drug administration.

Two male Wistar rats, each weighing 250 g, received M-83 at a dose of 1.8 mg/kg via the femoral vein. Serial blood samples were collected at 3, 10, 30, 60, 120, 180, 240, and 300 min from the same femoral vein. Serum analysis was the same as described previously for the human subject.

RESULTS

M-83 was coupled to BSA with the intention of producing a specific anti-M-83 antibody. The coupling procedure is based on the reaction of a maleimide group coupled to a hapten and a sulfhydryl group introduced into a BSA by thiolation according to our previous method (6). However, the acylation reaction of M-83 with MABA was slightly modified and done by a mixed carbonic anhydride method, and the resulting MABA-acylated M-83 was extracted in acidic pH and basic pH with ethyl acetate separating unrelated MABA and M-83. This process of purification of MABA-acylated M-83 was important for the subsequent conjugation reaction with the thiol groups of MSBSA to prepare a homogeneous conjugate of M-83-MABA-BSA, not giving unwanted MABA-BSA conjugate. After gel filtration of the M-83-MABA-BSA conjugate on Sephadex G-100, it was determined...
that 3.4 mol of M-83 bound each mol of BSA.

The M-83-BSA conjugate elicited the production of anti-M-83 antibody in each of 3 rabbits, the highest titer of which was seen 8 weeks after the initial inoculation. The dilution curves of this antiserum were prepared to decide its concentrations suitable for EIA. As was shown in Chart 2, the binding potency of antiserum to M-83-β-Gal conjugate was relatively high in the absence of M-83 and decreased gradually with increasing dilutions, and it was better inhibited by a fixed amount of nonlabeled M-83 (36.8 ng), especially in rather higher diluted antiserum. The antiserum was thus decided to use an initial dilution of approximately 1/620,000 with a specific binding value in the absence of free drug of 32%. Using this concentration of antiserum, an optimal incubation time for the immune reaction of anti-M-83 antibody with the labeled M-83 (in the absence of unlabeled M-83) and subsequently with a second antibody was examined (Chart 3). The first-step and the second-step immune reactions almost reached the equilibrium in 14 and 4 hr, respectively, at 25°. Thus, we chose these incubation times for EIA.

A dose-response standard curve of M-83 obtained in a buffer system is shown in Chart 4. The curve was essentially linear on a logit-log plot between 6 pg and 3 ng. The lowest detectable amounts of M-83 which were significantly different from zero varied 1 to 10 pg. For practical purposes, the lower and upper limits of sensitivity were arbitrarily set at 15 pg and 2 ng per assay tube, respectively. Intrassay precision, defined as the ratio between the mean deviation of Y and the slope of the regression line between mean values of logit Y and the corresponding log X, was λ = 0.12, 0.040, and 0.082 at 15.5 pg, 140 pg, and 1.25 ng, respectively, whereas interassay precision (λ = 0.131, 0.052, and 0.088 at each of the same doses as above in 6 different assays) was also indicative of a reproducible EIA technique.

Quantification of the drug in samples of normal human serum (50 μl) was also performed by adding known concentrations. The standard curve yielded was essentially linear and parallel with that in the buffer system (Chart 4). For practical purposes, the lower and upper limits of the sensitivity were arbitrarily set at 30 pg and 7 ng per assay tube, respectively. Intrassay precision (λ = 0.096, 0.042, and 0.073) and interassay precision (λ = 0.139, 0.055, and 0.086; n = 6) were obtained at 28.7 pg, 259 pg, and 2.33 ng, respectively. The presence of 50 μl serum slightly reduced the inhibiton of M-83. Thus, instead of obtaining 50% inhibition with 150 pg of M-83, 400 pg were required. The specific binding (B°) was the same as that in a buffer system. A standard curve was also constructed with 5 and 20 μl of normal human pooled urine added. Urine had no effect on the shape of the standard curve or the percentage of binding.

The change in immunoreactivity of M-83 following incubation in normal human serum or urine was examined. Known concentrations of M-83 were incubated in serum or urine for up to 24 hr at 37°, and aliquots of the mixture were periodically withdrawn, diluted 100 times with Buffer B, and measured by EIA using the buffer system. By both incubations, no appreciable...

![Chart 2](image)

**Chart 2.** Dilution curves of anti-M-83 antiserum. Several dilutions of the antiserum collected 8 weeks after the initial inoculation were prepared with Buffer B, and 50 μl each were incubated with 50 μl of the M-83-β-Gal (50 μunits) for 14 hr at 25°. This was followed by further incubation for 4 hr with the second antibody (goat anti-rabbit IgG and normal rabbit serum). The uptake of M-83-β-Gal in the precipitate was measured (B) and expressed as B/T (•). Inhibition experiments for the immune reaction of anti-M-83 antiserum were prepared to decide its concentrations suitable for EIA. As was shown in Chart 2, the binding potency of antiserum to M-83-β-Gal conjugate was relatively high in the absence of M-83 and decreased gradually with increasing dilutions, and it was better inhibited by a fixed amount of nonlabeled M-83 (36.8 ng), especially in rather higher diluted antiserum. The antiserum was thus decided to use an initial dilution of approxi...
EIA of M-83

Charts. Standard curve for M-83 and cross-reactivity of some mitomycin analogues with anti-M-83 serum. The curves show the amount (percentage) of bound enzyme activity for various doses of M-83 or the mitomycin analogues (8) as a ratio of that bound using the M-83-β-Gal alone (B°). Antiserum used had been collected 8 weeks after starting immunization. O, M-83; □, Ac-MMC; △, porfiromycin; △, MMC; ■, MMA; □, MMB.

A decrease in M-83 immunoreactivity was caused with an almost complete recovery.

Since several mitomycin analogues have been identified (7, 10, 13), we sought to characterize a specific part of the molecule which contains the antigenic determinant. Calibration curves showing the relationship between concentrations of unlabeled agents and the percentage of bound M-83-β-Gal were plotted (Chart 5). When MMA, MMB, MMC, or porfiromycin was substituted for unlabeled M-83 in the EIA, it can be seen that very little competition with M-83 was observed. Ac-MMC, which possesses the acetyl group on the aziridine moiety of MMC, produced only very slight cross-reaction of 0.017% at the amount of the drug for a 50% inhibition.

Initial application of the EIA was made to measure M-83 levels in serum of a patient with cholangiocarcinoma following administration of M-83 in a single dose of 0.35 mg/kg i.v. The disappearance of M-83 immunoreactivity from the sera is given in Chart 6. Results were analyzed according to a 2-compartment model following an i.v. bolus injection. Estimated from the curve, the α-phase serum half-life was 9.7 min, whereas the β-phase half-life was 80 min.

Animal experiments were also designed to follow the serum levels of M-83 by the EIA. Two rats were given 1.8 mg of M-83/kg by rapid injection into the femoral vein. The results in Chart 7 show a similar biphasic fashion of the kinetics with a rapid initial clearance (distribution) followed by a slower elimination phase. The rat α-phase serum half-life was 8.3 min, whereas the β-phase half-life was 62 min as an average of 2 rats. Because the sensitivity of the assay was known to be at least 1.4 pmol/ml, it would appear that additional values beyond the 5-hr time point are possible to obtain.

DISCUSSION

Numerous analogues of MMC have been prepared in the hope of obtaining compounds with improved therapeutic properties (2), since the toxicity of MMC, particularly its myelosuppressive effect, has prevented it from gaining wider and more rapid acceptance in cancer chemotherapy. M-83 has recently emerged as a clinical agent with such a hopeful possibility (8, 9, 12, 15, 18) and has been undergoing clinical trial in Japan.

Nonisotopic EIA for the quantification of M-83 will contribute extensively to an understanding of the pharmacology.

A heterobifunctional cross-linker, MABA, was used to prepare the immunogen as could be done in the EIA for MMC (6). In the September 1984 issue, the EIA of M-83 was discussed in detail.
order to obtain a homogeneous preparation of M-83-BSA conjugate which would be expected to produce a sensitive and specific antibody directed toward the drug, we modified the procedure slightly so that MABA-acetylated M-83 prepared by a mixed carbodiimide anhydride method was separated from the non-reacted MABA and the hapten drug by extracting with ethyl acetate, and this was followed by reaction with a carrier protein, MSBSA. The M-83-BSA conjugate thus synthesized elicited in rabbits the production of a considerably high titer of anti-M-83 antibody, such that the 620,000-fold diluted assayed conjugate still bound about 30% of the added M-83-β-Gal in control tubes containing no labeled M-83 and made possible a sensitive assay for M-83.

The present extraction technique in synthesizing M-83-β-Gal using MBA as a cross-linker led also to a preparation of the conjugate which rather better competed with a small amount of acetate, and this was followed by reaction with a carrier protein, in order to obtain a homogeneous preparation of M-83-BSA conjugate in which M-83 is bound by peptide bonds via the imino group of M-83. It was also found that with this EIA no cross-reactivity existed with other anticancer drugs (Adriamycin, daunomycin, 5-fluorouracil, and actinomycin D), indicating that the EIA for M-83 is appropriate for use in clinical evaluation of combination chemotherapy since anticancer drugs are often used with other chemotherapy agents.

The utility of the EIA was demonstrated with its application to measure serum levels of M-83 in a patient and rats. Despite species differences between human and rat, a similar biphasic pharmacokinetic pattern was observed with a rapid initial clearance followed by a slower elimination phase. Fujita (4) has recently reported using microbiological assay that the clearance of M-83 from the sera of patients obeys a 2-compartment model with a β-phase half-life of about 18 min, which was, however, calculated only from the serum concentrations of M-83 within 90 min after the dosing. By the present EIA following the serum levels of M-83 in a patient during 5 hr after i.v. administration, it was revealed that the β-phase serum half-life was 80 min. Obviously, these results must be confirmed in more patients before a firm conclusion can be made about actual half-lives and species variation.

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