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Competitive Enzyme Immunoassay for Anti-ulcer Agent, (–)-*cis*-2,3-Dihydro-3-(4-methylpiperazinylmethyl)-2-phenyl-1,5-benzothiazepin-4(5*H*)-one Hydrochloride (BTM-1086)

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An enzyme immunoassay for an anti-ulcer agent, (–)-*cis*-2,3-dihydro-3-(4-methylpiperazinylmethyl)-2-phenyl-1,5-benzothiazepin-4(5*H*)-one hydrochloride (BTM-1086) was established. It was based on the double antibody solid phase procedure. The antisera to BTM-1086 were raised in rabbits by immunization with BTM-1086–ovalbumin conjugate. The formation of sulfoxide and the lack of a methyl group in the thiazepine ring and the piperazine ring, respectively, of the BTM-1086 skeleton markedly decreased the binding affinity for the antibody. Horseradish peroxidase was conjugated with hydrazinomethyl-BTM-1086 by the use of glutaraldehyde. The first and second immuno reactions require 20 min and 2.5 h, respectively. The assay allows determination of 2.5—100 pg of BTM-1086. This enzyme immunoassay has high sensitivity, and the results are reproducible. The time course of serum BTM-1086 level was also examined after a single intravenous administration in rats.

Keywords——enzyme immunoassay; anti-ulcer agent; ovalbumin; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; peroxidase; glutaraldehyde

(–)-*cis*-2,3-Dihydro-3-(4-methylpiperazinylmethyl)-2-phenyl-1,5-benzothiazepin-4(5*H*)-one hydrochloride (BTM-1086) is a potent anti-ulcer and gastric secretion-inhibiting agent possessing a benzothiazepine skeleton.[1] In animal experiments, BTM-1086 strongly suppresses the secretion of gastric juice and acid in the stomach induced by stimulation with tetragastrin or carbachol.[2] The main pharmacological function of BTM-1086 is thought to be an inhibition of acethylcholine release.[3] Since BTM-1086 is administered to human in very low dose, because of its high pharmacological activity, serum levels of BTM-1086 after oral administration of BTM-1086 are very low. Therefore, a sensitive and specific analytical method for trace amounts of BTM-1086 is required in order to investigate in detail the mechanism of pharmacological function of the drug. Recent development of a variety of immunological assay procedures has made possible the highly sensitive, specific, precise, rapid, and convenient measurement of the concentration of drugs.[4] In this paper, we describe an application of a highly sensitive enzyme immunoassay for BTM-1086, which possesses asymmetric carbon atoms.

Materials and Methods

Materials——BTM-1086 and its metabolites (chemical structures are shown in Fig. 1) were synthesized by Maruko Seiyaku Co., Ltd. (Nagoya, Japan). Horseradish peroxidase (POD Type VI) was obtained from Sigma Chemical Co. (U.S.A.), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride(EDAC-HCl) and glutaraldehyde were from Nakarai Chemicals, Ltd. (Kyoto). Ovalbumin (OVA) and immunoglobulin G (IgG) fraction of

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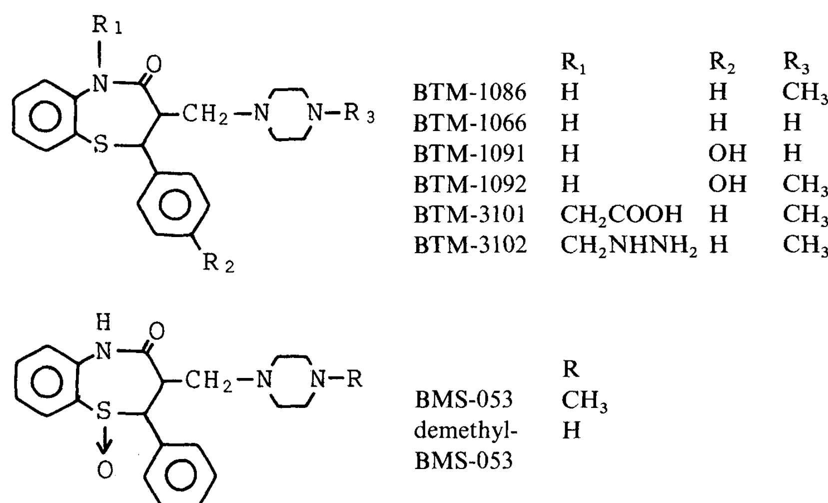

Fig. 1. Chemical Structures of BTM-1086, Its Metabolites, and Its Derivatives

BTM-1066, BTM-1091, BTM-1092, BMS-053, and demethyl-BMS-053 are metabolites of BTM-1086. BTM-3101 and BTM-3102 are derivatives of BTM-1086.

anti-rabbit IgG (goat) were purchased from Miles Laboratories (U.S.A.). Bovine serum albumin (BSA fraction V) was from Wako Pure Chemical Industries, Ltd. (Osaka). Polystyrene beads (diameter 6.4 mm) were obtained from Nissho Co. (Osaka). All other solvents and chemicals were of special grade.

Buffer Solution——The buffers used in this work were as follows: 0.05 M phosphate buffer (pH 7.4) containing 0.3 M sodium chloride, 0.02% Triton X-405, 0.02% merthiolate sodium (buffer A), 0.01 M phosphate buffer (pH 7.0) containing 0.15 M sodium chloride, 0.1% BSA, 0.01% merthiolate sodium (buffer A-H), and 0.01 M phosphate buffer (pH 7.0) containing 0.15 M sodium chloride, 0.05% Tween 20 (buffer R).

Standard Solution——BTM-1086 and its derivatives were dissolved in buffer A before use.

Preparation of Immunogen——Carboxymethyl-BTM-1086(BTM-3101) was conjugated to OVA according to the method of Michiels *et al.*,[5] with a minor modification. BTM-3101 (10 mg) was dissolved in 0.5 ml of distilled water and the solution was added dropwise to EDAC-HCl (40 mg) dissolved in 0.5 ml of distilled water. OVA (14 mg) in 2 ml of 0.05 M phosphate buffer (pH 6.0) was added dropwise to the mixture and the whole was stirred overnight at 4 °C. The resulting mixture was applied to a column (1.5 × 85 cm) of Sephadex G-25 equilibrated with 0.05 M phosphate beffer (pH 7.5), and the BTM-1086–OVA conjugate fraction was lyophilized and stored at −20 °C.

Immunization[6]——Two albino female rabbits were injected into each foot pad and into several intradermal sites on the back with the conjugate dissolved in 1 ml of saline (approximately 1 mg protein) emulsified in an equal volume of Freund's complete adjuvant. Thirteen booster injections were given at biweekly intervals. The rabbits were bled from earveins and from the carotid one week after the final injection, and the serum was stored at −20 °C.

Preparation of BTM-1086–POD Conjugate——POD was conjugated to hydrazinomethyl-BTM-1086 (BTM-3102) by using glutaraldehyde.[7] POD (5 mg) was dissolved in 0.1 M sodium phosphate buffer (pH 6.8) containing 1.25% glutaraldehyde (0.3 ml), then incubated overnight at room temperature. The mixture was applied to a column (1.1 × 60 cm) of Sephadex G-25 equilibrated with saline, and the brown fractions were collected (3.0 ml). The BTM-3102 (5 mg) was dissolved in 0.5 ml of 0.1 M phosphate buffer (pH 6.8) and then added to the POD fraction. The mixture was incubated at 4 °C for 24 h and dialyzed against phosphate-buffered saline (PBS), then L-lysine (30 mg) was added, and the whole was allowed to stand at 4 °C for 24 h, then dialyzed against PBS. The dialysate was mixed with the same volume of glycerol containing merthiolate sodium (0.04%) and stored at 4 °C.

Preparation of Second Antibody-Bound Polystyrene Beads——Second antibody was immobilized on polystyrene beads by physical adsorption according to the method of Sato and Yamamoto.[8] Beads were soaked in a solution of the goat IgG fraction of anti-rabbit IgG (0.1 mg/ml dissolved in 0.25 M phosphate buffer, pH 7.5), and rocked at 37 °C for 1 h, then incubated overnight at 4 °C. The anti-rabbit IgG antibody-coated beads were washed three times with buffer A-H and stored at 4 °C in the same buffer.

Assay Procedure——All solutions were diluted with buffer A. All standards and samples were assayed in duplicate.

Serum or serum diluted with buffer A (5 μl) was added to each assay tube which contained BTM-1086–free serum (diluted 1 : 6.6 with buffer A) (100 μl). For the standard curve, BTM-1086 standard solution (5 μl, 0—100 pg) was added to each assay tube, which contained BTM-1086 free rat serum (diluted 1 : 6.6 with buffer A) (100 μl). BTM-1086–POD conjugate (diluted 1 : 10000 with buffer A) (50 μl) and anti-BTM-1086 antiserum (diluted 1 : 70000 with buffer A) (50 μl) were added to each assay tube and the tube was incubated at room temperature for 20 min, then the second antibody-immobilized polystyrene bead was added to each assay tube and allowed to stand at 37 °C for 2.5 h

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with shaking. The bead was washed three times with buffer R (2 ml) and transferred to another tube. The washed bead was subjected to assay of POD activity by the following spectrophotometric method.

Assay of Peroxidase Activity[9]——A 0.1 M phosphate–citrate buffer (pH 6.5) containing 0.02% hydrogen peroxide and 16.6 mM *o*-phenylenediamine dihydrochloride (200 μl) was added to the tube containing the washed bead, and the mixture was incubated at 37 °C for 30 min. The enzyme reaction was stopped by the addition of 1 N HCl (2 ml). The absorbance at 492 nm was measured with a Hitachi model 100-20 spectrophotometer.

Correlation between Results Obtained by Enzyme Immunoassay (EIA) and Gas Chromatography (GC)——Male Wistar rats were bled from the descending aorta at 0.5, 1, 2, 4, 6, 9, and 24 h after a single oral administration of BTM-1086 (30 mg/kg). The serum samples were assayed for BTM-1086 by EIA and GC. GC was performed on a Hitachi 163 gas chromatograph equipped with a hydrogen flame thermionic detector, and the conditions used were as follows: 1 m × 3 mm glass column packed with 2% OV-17 Chromosorb W (AW, DMCS); carrier gas, He, 50 ml/min; injection port temp., 300 °C; column temp., 280 °C.[10]

Results

Preparation of Immunogen

The immunogen was prepared by conjugation of carboxymethyl-BTM-1086 to OVA using EDAC-HCl. The molar ratio of carboxymethyl-BTM-1086 to OVA was 9 determined by a spectrophotometric method.

Standard Curve for BTM-1086

Figure 2 shows a typical standard curve for BTM-1086, constructed for BTM-1086 concentrations of 2.5 to 100 pg/assay tube.

Recovery of BTM-1086 from Various Sera

Recovery studies were performed by the addition of 5.00, 10.00 or 50.00 pg of authentic BTM-1086 to various sera: three hemolytic sera and three high bilirubin sera. As shown in

TABLE I. Recovery of BTM-1086 from Serum

Added (pg/tube)	Found (pg/tube)	*n*	Recovery (%)
5.00	4.81 ± 0.50	6	96.17 ± 9.91
10.00	9.57 ± 0.54	6	95.67 ± 5.39
50.00	51.50 ± 1.49	6	103.00 ± 2.98

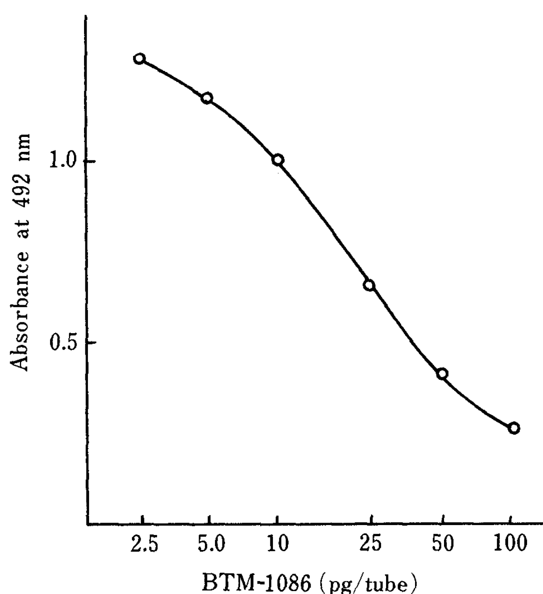

Fig. 2. Standard Curve for BTM-1086

TABLE II. Accuracy and Precision of EIA for BTM-1086

	Sample number	*n*	BTM-1086 (pg/tube)	CV (%)
Intra-assay	1	10	5.76 ± 0.45	7.81
	2	9	9.95 ± 0.59	5.93
	3	10	50.95 ± 2.04	4.02
Inter-assay	1	10	5.10 ± 0.41	8.05
	2	10	9.55 ± 0.55	5.76
	3	10	49.75 ± 3.98	4.03

TABLE III. Specificity of Antiserum against BTM-1086

Compound	Cross-reactivity (%)
BTM-1086	100
BTM-1066	12.85
BTM-1091	0.99
BTM-1092	20.60
BTM-1041	0.01
BTM-1013	0.67
BMS-053	0.63
Demethyl-BMS-053	0.21

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Table I, recoveries of 96.17±9.91% (n=6), 95.67±5.39% (n=6), and 103.00±2.98% (n=6) were obtained, respectively.

Accuracy and Precision

The accuracy and precision of this EIA were examined with duplicate assays of standard BTM-1086 solution added to normal rat serum (Table II). The intra-assay coefficient of variation (CV) ranged from 4.02% to 7.81%, while the inter-assay CV ranged from 4.03% to 8.05%.

Specificity

The results of the cross reaction study are summarized in Table III. The cross-reaction percentage was calculated at 50% displacement of the BTM-1086–POD conjugate in the EIA system. BTM-1066, BTM-1091, BTM-1092, BMS-053, and demethyl-BMS-053 are metabolites of BTM-1086. BTM-1041 and BTM-1013 showed very low cross-reactivity compared

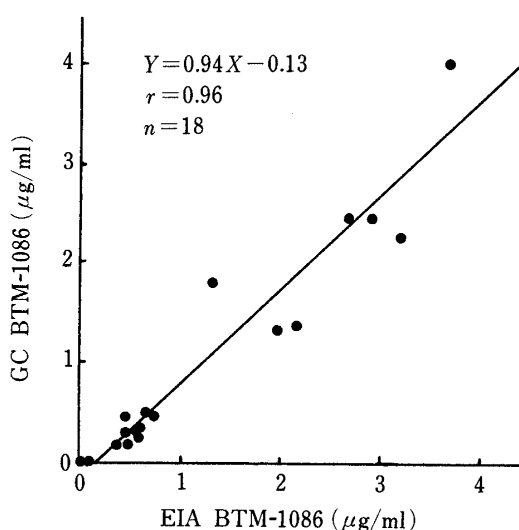

Fig. 3. Correlation between the Results of EIA and GC with Rat Serum Samples

Wistar male rats were bled at 0, 0.5, 1, 2, 4, 6, 9, 24 h after an oral administration of BTM-1086 (30 mg/kg). The serum BTM-1086 levels were determined by EIA and GC.

Table IV. Pharmacokinetic Parameters after an i.v. Administration of BTM-1086 to Rats

Parameter	Value
A (μg/ml)	5.0
B (μg/ml)	1.0
α (h^{-1})	3.84
β (h^{-1})	0.35
$t_{1/2}$ (h)	1.98
k_{el} (h^{-1})	1.44
AUG (μg·h/ml)	4.17
Cl (1/h per kg)	1.18
V_{dss} (1/kg)	2.28

The values are expressed as means from three experiments. A: intercept of the straight part at α phase to ordinate; B: intercept of the straight part at β phase to ordinate; α: slope of straight part at α phase; β: slope of straight part at β phase; $t_{1/2}$: half life at β phase; k_{el}: elimination rate constant from central compartment; AUG: area under a blood level *versus* time curve; Cl: clearance; V_{dss}: volume of distribution at the steady state.

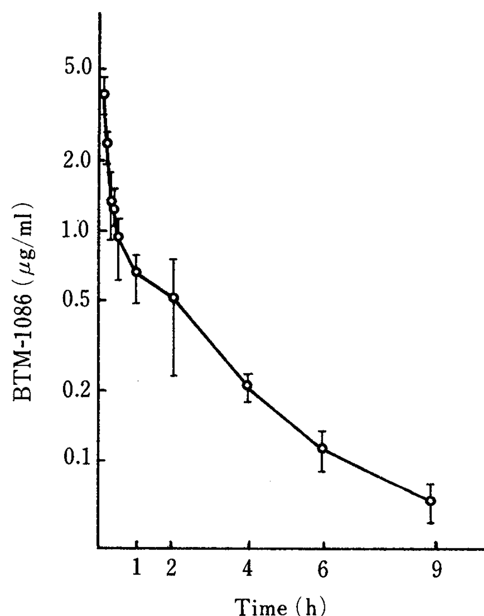

Fig. 4. Serum BTM-1086 Levels after an i.v. Administration

Five mg/kg of BTM-1086 was intravenously administered to three Wistar male rats. The values are expressed as mean±S.D. from three experiments.

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with the metabolites. The structures of BTM-1041 and BTM-1013 are the same as that of BTM-1086, but they have *cis* (+) and *trans* (+) configuration, respectively.

Correlation between EIA and GC

BTM-1086 concentrations in serum samples were determined from the standard curve. As shown in Fig. 3, the values correlated well with those obtained by the GC method; and the correlation coefficient was 0.96.

Serum Level of BTM-1086 in Rats after a Single Intravenous Administration

By using this EIA, the time course of serum levels of BTM-1086 was examined. Serum levels of BTM-1086 after a single intravenous injection (5 mg/kg) decreased in a biphasic fashion (Fig. 4). The pharmacokinetic parameters are listed in Table IV.

Discussion

We have developed a sensitive double-antibody solid-phase EIA for BTM-1086. This assay allows the determination of 2.5-100 pg of BTM-1086.

BTM-1086 is metabolized mainly by *N*-demethylation, glucuronidation, and ring hydroxylation in rats (Fig. 1). In order to obtain antiserum which reacts with BTM-1086 and not with its metabolites, the imino group in the benzothiazepine ring of BTM-1086 was carboxymethylated (BTM-3101). The amino group of OVA and the carboxy group of BTM-3101 were conjugated by the carbodiimide method.[5] Since high sensitivity was required, we employed a bridge heterologous combination.[11] The imino group in the benzothiazepine ring of BTM-1086 was hydrazinomethylated and conjugated to POD by the glutaraldehyde method.

Although cross reactivity with BTM-1092, which is one of the metabolites of BTM-1086, was observed, it is already known that the concentration of BTM-1092 that appears in the blood is very low in rats. Moreover in the BTM-1086 assay, the results of EIA correlated well with those of GC (Fig.3). This suggests that the cross reactivity of BTM-1092 is not a practical problem in the determination of the serum level of BTM-1086. The specificity of the antiserum was high for structure around the sulfur atom, piperazine ring, and phenyl group. The cross reactivity with BTM-1041 or BTM-1013 (isomers of BTM-1086) was very much lower than those with the metabolites. The difference of affinity of the antiserum in this study may be due to not only the structural difference of the antigens but also stereochemical factors. The recovery tests and the intra- and inter-assay precision indicated satisfactory assay performance. The EIA for BTM-1086 described here is therefore considered to be valid and reliable.

Serum levels of BTM-1086 after a single intravenous administration were fitted to a two-compartment open model. The volume of distribution at the steady state (V_{dss}) was large (2.28 l/kg). This may suggest that BTM-1086 tends to pass into tissues.

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