Enzyme Immunoassay for 6-Amino-5-chloro-1-isopropyl-2-(4-methyl-1-piperazinyl)benzimidazole, a Novel 5-HT$_3$ Receptor Antagonist

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An enzyme immunoassay (EIA) has been developed for determination of 6-amino-5-chloro-1-isopropyl-2-(4-methyl-1-piperazinyl) benzimidazole (KB-6806), a novel 5-HT$_3$ receptor antagonist. Anti-KB-6806 antiserum was elicited against the KB-6806-bovine serum albumin (BSA) conjugate prepared by a diazo coupling reaction through the inherent 6-amino group. $\beta$-Galactosidase-labeled 6-amino-5-chloro-1-isobutyl-2-(4-methyl-1-piperazinyl)benzimidazole was similarly prepared by diazo coupling reaction as an enzyme-labeled antigen with a hapten heterologous combination of antiserum. The modification at the 4-methyl group of the piperazine moiety of KB-6806 significantly decreased the binding affinity to the antibody. This method could quantitate KB-6806 in dog plasma in the concentration range of 0.078—10 ng/ml with good accuracy and precision. The EIA method has been successfully applied to the determination of KB-6806 in plasma after intravenous administration of KB-6806 to dogs.

Key words enzyme immunoassay; KB-6806; pharmacokinetics

The nausea and vomiting associated with cancer chemotherapy can cause severe distress. Research on the mechanisms involved in cytostatic-induced nausea and vomiting has implicated activation of the 5-hydroxytrptamine-3 (5-HT$_3$) receptor. The discovery of 5-HT$_3$ antagonists and their effect on emesis appears to be radically changing the outlook on cancer chemotherapy.

6-Amino-5-chloro-1-isopropyl-2-(4-methyl-1-piperazinyl)-benzimidazole (KB-6806, Table 1), is a novel 5-HT$_3$ receptor antagonist, a candidate drug for depressing emesis derived from cisplatin.1)

In order to study the pharmacokinetics of KB-6806, we developed the enzyme immunoassay (EIA) for KB-6806 based on a hapten heterologous combination of antiserum and enzyme-labeled antigen. And the EIA method was applied to the determination of KB-6806 in plasma after intravenous administration of KB-6806 to beagle dogs.

MATERIALS AND METHODS

Materials KB-6806 and related compounds (Table 1) were prepared by R & D Laboratories of Nippon Organon K.K. $\beta$-Galactosidase (E.C.3.2.1.23) from Escherichia coli (Grade VIII) was supplied by Sigma (St. Louis, MO, U.S.A.). Goat anti rabbit IgG antiserum was obtained from Daichi Radioisotope Labs., Ltd. (Tokyo, Japan). Blocking reagent, Block Ace$^\text{TM}$ powder, was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Immunogen A solution of KB-6806 (24.7 mg) in 0.5 ml HCl (0.5 M) was added dropwise to aqueous NaNO$_2$ (6.3 mg/0.5 ml) on ice. The mixture was stirred for 30 min and added slowly into a solution of bovine serum albumin (BSA) (100 mg) in 1 ml of sodium phosphate buffer (pH 9.0; 0.2 M), the pH being adjusted to 8—9 with 1 M NaOH. The whole was stirred for 18 h at 4°C. The BSA fraction was precipitated out by addition of ethanol and ethyl acetate followed by centrifugation. The precipitate was dissolved in aqueous pyridine and dialyzed against cold running water for 2 d. Lyophilization of the resultant solution gave the KB-6806-BSA conjugate (approx. 74 mg).

Preparation of Antiserum The hapten-BSA conjugate (2 mg) in sterile isotonic saline (0.5 ml) was emulsified with complete Freund’s adjuvant (1.0 ml). Every second week the emulsion was injected into domestic male albino rabbits subcutaneously at multiple sites along the back. Blood was withdrawn from the jugular vein at one week after the last injection. The blood was clotted at room temperature and an antiserum was separated by centrifugation at 3000 rpm for 10 min. The antiserum was stored at 4°C in the presence of 0.1% (w/v) sodium azide.

Preparation of $\beta$-Galactosidase-Labeled Antigen A solution of 6-amino-5-chloro-1-isobutyl-2-(4-methyl-1-piperazinyl) benzimidazole (compound A, Table 2) (1.1 mg) in 0.23 ml of 0.1 M HCl was added dropwise to aqueous NaNO$_2$ (2.3 mg/0.23 ml) on ice. After stirring for 30 min, 5 µl of the mixture was added to a solution of $\beta$-galactosidase (0.5 mg) in 0.2 ml of 50 mM sodium phosphate buffer (pH 7.3) containing 0.9% (w/v) NaCl and 1 mM MgCl$_2$ on ice. The whole was stirred on ice for 2 h. The resulting solution was dialyzed against 50 mM sodium phosphate buffer (pH 7.3) containing 0.9% (w/v) NaCl at 4°C for 2 d and diluted with the same buffer to 0.5 ml. A solution of $\beta$-galactosidase-labeled antigen was added to BSA (7 mg) and glycogen (0.5 ml), and was stored below −20°C until use. For immunoassay this stock solution was diluted to 1:2500 (v/v) with 50 mM sodium phosphate buffer (pH 7.3) containing 0.4% (w/v) Block Ace$^\text{TM}$ powder (assay buffer).

Procedure for EIA The goat anti rabbit IgG antiserum was diluted to 1:1000 (v/v) with sodium phosphate buffer (pH 7.3; 50 mM) and coated to the Maxisorp 96-well microtitre plate (FluoroNunc$^\text{TM}$ Modules, Nunc A/S, Denmark) by incubation of 100 µl per well overnight at 2—8°C. Then the plate was blocked with 300 µl per well of sodium phosphate buffer (pH 7.3; 50 mM) containing 1% (w/v) Block Ace$^\text{TM}$ powder at room temperature for 75 min. The plate was washed two times with 10 mM sodium phosphate buffer (pH 7.3) containing NaCl (0.9%, w/v) and 0.05% (v/v) polyoxyethylene sorbitan monolaurate (Tween$^\text{®}$ 20). The anti-
serum was diluted to 1:30000 (v/v) with assay buffer. Assay buffer was added to the blank wells and the diluted antiserum was added to the rest of the wells (100 µl per well). The plate was incubated at room temperature for 100 min and washed two times. The standard samples spiked with KB-6806 (0 and 0.078—10 ng/ml) (or related compounds for determination of the specificity) and testing samples were mixed with assay buffer and the solution of β-galactosidase-labeled antigen (1:2:1, v/v/v). The mixture was added to each well (100 µl per well) in triplicate. The plate was incubated at room temperature for 75 min and washed four times. The assay buffer containing 0.1 mM 4-methylumbelliferyl-β-D-galactopyranoside was added to each well (100 µl per well) and the plate was incubated at room temperature for 75 min. The enzymatic reaction was terminated by addition of 50 µl of 0.1 M glycine–NaOH buffer (pH 10.3).

The fluorescence intensity was measured with the excitation at 360 nm and emission at 450 nm using microplate reader MTP-32/F2 (Corona electric Co., Ltd., Ibaraki, Japan). A calibration curve was constructed by the modified equation of four parameters logistic model.

Specificity of EIA The specificity of the assay system was assessed by cross-reactivity of antiserum to eight compounds related to KB-6806. The cross-reactivity was expressed as the percentage of the amount, which reduced the enzymatic activity by half, of each compound to that of KB-6806 in Table 1.

Accuracy and Precision The accuracy and precision of the assay were assessed by blank dog plasma spiked with KB-6806 at five concentrations: 0.078, 0.1, 0.625, 1 and 10 ng/ml. The intra-assay variability was assessed with five replicates (each replicate analyzed in triplicate) at the same time. The inter-assay variability was assessed with five assays (each assay analyzed in triplicate).

Pharmacokinetic Study Blood was obtained from the cephalic vein after intravenous administration of KB-6806 at doses 0.01 and 1 mg/kg to male beagle dogs weighing 10.0—11.5 kg. The plasma was separated by centrifugation at 3000 rpm for 10 min and stored at −20 °C until assayed. Pharmacokinetic parameters were calculated with the time-plasma concentrations of KB-6806 profiles using Win Nonlin (version 1.0, Pharsight Corp., U.S.A.).

RESULTS AND DISCUSSION

KB-6806 proved to have a strong anti-5HT₃ antagonistic activity with an ED50 value at 5 min after intravenous administration of KB-6806 demaleate of 0.071 µg/kg. We assumed the plasma concentration of KB-6806 after dosing was low level and a sensitive method for determination of KB-6806 in biological fluids was required. In the present study, we developed an EIA determination method.

A heterologous EIA system is generally more sensitive than a homologous EIA system. The utility of a heterologous combination of the enzyme-labeled antigen and antibody (hapten heterology, bridge heterology and site heterology) has been demonstrated for the development of sensitive EIA for estrogen, cortisol, clenbuterol, tryptophan pyrolysate and 4-hydroxy-2-(4-methylphenyl)benzothiazole.

It is sufficiently substantiated that the specificity of the antibody raised against a small molecule is remarkably influenced by the position on the hapten molecule used for conjugation to the carrier protein and significant cross reactivity with compounds homologous around the bridge portion is usually observed. The metabolism of KB-6806 was not well established. However, it seems that the main metabolic pathway of KB-6806 is oxidation or glucuronidation at the 4-methyl group on the piperazine moiety of KB-6806 because it was reported that the piperazine-containing drugs like clozapine, loxapine, olanzapine and mianserin were metabolized to N-demethylate, N-oxide and N-glucuronide of piperazone rings. According to a preliminary metabolism study of KB-6806, compound F (N-oxide) and G (N-demethylate) in Table 1 have already been identified. On the other hand, the metabolites modified at the 6-amino group of KB-6806 have not yet been found (unpublished data). Therefore, KB-6806 was conjugated with BSA through the 6-amino group by diazo coupling reaction for immunization.

The immunogen was administered to rabbits with complete Freund’s adjuvant subcutaneously. An appropriate antiserum was obtained from rabbits at six months after the first administration.

The typical calibration curve of EIA for KB-6806 prepared in dog plasma is shown in Fig. 1. The correlation coefficient (r) of the calibration curve was 0.998 in the concentration range of 0.078—10 ng/ml of KB-6806.

The specificity of the EIA system was assessed to measure

<table>
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<tr>
<th>Compound</th>
<th>R₁</th>
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<th>R₃</th>
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<tr>
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Fig. 1. The Typical Calibration Curve for KB-6806
The 50% displacement of bound enzyme-labeled antigen by related benzimidazoles and expressed as percent cross-reactivity in Table 1. The cross-reactivities of the related compounds modified at the 4-methyl group on the benzimidazole moiety (N-oxide, \( R_3 = \text{NH}_2 \) and H) were less than 1%, suggesting that the metabolites of KB-6806, compound F and G, don’t affect the determination of KB-6806 by this EIA method.

The cross-reactivity of compound A used as an enzyme-labeled hapten was 27.1%. Similarly, the cross-reactivities of compound B and H modified at the 1-alkyl group on the benzimidazole moiety \(( R_1 = \text{CH}(\text{CH}_2\text{CH}_3)_2 \) and \( \text{CH}_2\text{CH}_3 \), respectively) were 23.9% and 54.4%, respectively. On the other hand, the deaminated and acetylated compounds \(( R_1 = \text{H} \) and \( \text{CH}_2\text{CONH} \)) exhibited high cross-reactivity (99.5% and 80.3%, respectively). Therefore, the present EIA system for KB-6806 is fairly specific except for the derivatives at the 6-amino group.

To assess the precision and accuracy of the assay, spiked KB-6806 in plasma at five concentrations representing the entire range of the calibration curve were studied. Table 2 shows that the intra-assay precision (% RSD) was under 2.3%, and the intra-assay accuracy (% deviation) was less than 9.3% for all five concentrations. As shown in Table 3, the inter-assay precision and accuracy were in the range from 1.0 to 5.3% and from -5.4 to 7.9%, respectively. From these results, the precision and accuracy were sufficient to determine KB-6806 in dog plasma in the concentration range of 0.078—10 ng/ml.

The EIA method was applied to the determination of KB-6806 in plasma after intravenous administration of KB-6806 to dogs. The profiles of plasma concentration of KB-6806 and the pharmacokinetic parameters are shown in Fig. 2 and Table 4, respectively. Plasma concentrations of KB-6806, \( C_0 \) and \( AUC_{0-\infty} \) after intravenous administration of KB-6806 to dogs increased dose dependently. And the elimination half-lives (\( T_{1/2} \)), plasma total body clearance (CL) and steady-state distribution volume (\( V_{dss} \)) at a dose of 0.01 mg/kg were similar to those at doses of 1 mg/kg.

Consequently, the developed EIA is well suited for the routine analysis of KB-6806 in plasma samples.

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