Establishment of an Enzyme-Linked Immunosorbid Assay for Measurement of Sotalol

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We have established an enzyme-linked immunosorbid assay suitable for routine monitoring of serum levels of sotalol. Anti-sotalol antibody was obtained by immunizing rabbits with sotalol conjugated with bovine serum albumin using the N-succinimidyl ester method. An enzyme marker was similarly prepared by coupling sotalol with β-D-galactosidase. The detection limit of sotalol by the enzyme-linked immunosorbid assay was approximately 32 ng/ml with 50-μl samples. This assay was specific for sotalol because of very slight cross-reactivity with 4-(methanesulfonylamino)benzonitrile (1.6%), but none with n,1-isoproterenol. Using this assay, drug levels were easily measured in the serum of rabbits after oral administration of sotalol at a single dose of 3 mg/kg. The enzyme-linked immunosorbid assay should be a valuable tool in therapeutic drug monitoring and pharmacokinetic studies of sotalol.

Key words sotalol; enzyme-linked immunosorbid assay (ELISA); antiarrhythmic drug

Sotalol, 4-(2-isopropylamino-1-hydroxyethyl)methanesulfonylanilide, is a nonselective β-adrenoceptor blocker with additional class III properties which is used for the treatment of supraventricular and ventricular arrhythmias.1,2 Its side effects are chiefly related to β-adrenergic blockade and prolonged myocardial repolarization (e.g., torsades de pointes).3 This drug is eliminated by renal excretion, and the elimination half-life is prolonged in patients with renal hypofunction.4 A correlation is assumed between serum sotalol concentrations and prolongation of the ventricular repolarization time (QT interval).5,6 Therefore it is necessary to adjust the administration interval and dosage according to renal function.

Previous therapeutic drug monitoring (TDM) and pharmacokinetic studies of sotalol were undertaken using HPLC.7–9 The enzyme-linked immunosorbid assay (ELISA) also appears to be an analytical method suitable for these studies. Recently, we have developed several ELISAs for some antiarrhythmic drugs, each of which was sensitive and specific for preparing the conjugates with bovine serum albumin (BSA) and β-Gal, respectively, as the sotalol immunogen and the tracer in the ELISA. The yield of sotalol hemisuccinate was tentatively estimated to be 33% according to HPLC measurements of the quantity of nonreacted sotalol.

1-Ethyl-3,3-dimethylaminopropyl-carbodiimide hydrochloride (EDPC) (6.2 mg, 32 μmol) and N-hydroxysuccinimide (3.7 mg, 32 μmol) were added to a solution of sotalol hemisuccinate (approximately 6 mg, 16 μmol) in 90% dioxane (0.5 ml), and the resulting solution was allowed to stand at room temperature for 2 h. The reaction mixture was immediately mixed with BSA (10 mg) in 1.5 ml of 0.1 M phosphate buffer (pH 7.0), and incubated at 30 °C for 2 h with stirring. The reaction mixture was dialyzed successively for 48 h against 50 and 1 mM phosphate buffer (pH 7.0) and H2O. The purified conjugate was lyophilized and used as an immunogen for the ELISA. Using the trinitrobenzene sulfonic acid method for the determination of the primary amine,10 22 molecules of sotalol were found to be coupled with one molecule of BSA on the basis of the decrease in the primary amine.

Antibody Production in Rabbits One milliliter of a saline suspension of 1 mg sotalol-BSA conjugate was emulsified with an equal volume of Freund’s complete adjuvant. Two white female rabbits were each given multiple subcutaneous injections over sites along both sides of their backs. Booster injections were then given three times at biweekly intervals, using one-half the amount of the dose of the first immunization. The rabbits were bled from an ear vein 10 weeks after immunization began. The sera (10 ml) were separated by centrifugation and heated at 55 °C for 30 min. Fractions of IgG were extracted from the sera with 50% saturated ammonium sulfate and chromatographed on a column of DEAE-Sephaloc (2.1×23 cm) using 17.5 mM phosphate

MATERIALS AND METHODS

Reagents D,L-Sotalol (sotalol) hydrochloride was purchased from Sigma (St. Louis, MO, U.S.A.). β-D-Galactosidase (β-Gal; EC 3.2.1.23) from Escherichia coli and 4-methylumbelliferyl-β-D-galactopyranoside were obtained from Boehringer Mannheim (Mannheim, Germany).

Preparation of the Immunogen for Sotalol A solution of sotalol hydrochloride (20 mg, 64.8 μmol) and succinic anhydride (6.5 mg, 65 μmol) in pyridine (300 μl) was stirred overnight at room temperature. After removing pyridine by passing nitrogen through the reaction mixture, 2 ml of ethyl acetate and 2 ml of 0.33 M citric acid were added to the residue, and the mixture was shaken vigorously. The ethyl acetate layer was washed with saturated sodium chloride, dried over anhydrous sodium sulfate, and evaporated to give sotalol hemisuccinate (8 mg) as a white solid. The resulting sotalol hemisuccinate was used without further purification for preparing the conjugates with bovine serum albumin (BSA) and β-Gal, respectively, as the sotalol immunogen and the tracer in the ELISA. The yield of sotalol hemisuccinate was tentatively estimated to be 33% according to HPLC measurements of the quantity of nonreacted sotalol.

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buffer (pH 6.8) as an eluant. The fraction passed through the column was lyophilized and used as anti-sotalol IgG for ELISA.

Enzyme Labeling of Sotalol Sotalol was labeled by binding to β-Gal, essentially by the same principle as used for the preparation of the sotalol immunogen. In brief, EDCP (2.5 mg, 13 μmol) and N-hydroxysuccinimide (1.5 mg, 13 μmol) were added to a solution of sotalol hemisuccinate (approximately 2 mg, 5.4 μmol) in 90% dioxane (0.5 ml), and the resulting solution was allowed to stand at room temperature for 2 h. Next, 50-μl portions of the above reaction mixture containing succinimidyl sotalol hemisuccinate (ca. 0.54 μmol) was added directly to β-Gal (156 μg, 0.28 nmol) in 1 ml of 0.1 M phosphate buffer (pH 7.0), followed by 1-h incubation at room temperature. The mixture was chromatographed on a Sepharose 6B column (2.0×40 cm) using 20 mM phosphate buffer (pH 7.0) containing 0.1 mM NaCl, 1 mM MgCl₂, 0.1% BSA, and 0.1% NaN₃ (buffer A) to remove any remaining small molecules. Four-milliliter fractions were collected, and fractions 14 to 16, representing the main peak showing enzyme activity, were combined and used as a label in the ELISA.

ELISA Method ELISA is based on the principle of competition between enzyme-labeled and unlabeled drugs for an immobilized antibody, followed by measurement of the marker enzyme activity of the immunocomplex bound to the solid phase. Briefly, the wells in microtiter plates (Nunc F Immunoplates I, Nunc, Reskilde, Denmark) were coated by the solid phase. Briefly, the wells in microtiter plates (Nunc F Immunoplates I, Nunc, Reskilde, Denmark) were coated by loading 150 μl of anti-sotalol IgG (0.5 μg/ml) in 10 mM Tris–HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM Na₂HPO₄ and allowed to stand for 1 h at 37°C. After the plates had been washed twice with 60 mM phosphate buffer (pH 7.4) containing 10 mM ethylenediaminetetraacetate, 0.1% BSA, and 0.1% NaN₃ (buffer B), they were incubated with 200 μl of 10 mM Tris–HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM Na₂HPO₄ containing 2% BSA for 20 min at 37°C to prevent nonspecific adsorption. The anti-sotalol IgG-coated wells were then filled with 50 μl of either sotalol-treated samples, or buffer B as a control, followed immediately by 50 μl of the pooled sotalol-β-Gal conjugate (diluted 1:5000 in buffer B). The wells were then incubated for 3 h at room temperature and once again washed thoroughly with buffer B.

The activity of the enzyme conjugate bound to each well was then measured by the addition of 125 μl of 0.1 mM 4-methylumbelliferyl-β-D-galactopyranoside in buffer A, followed by incubation of the wells at 37°C for 60 min. The enzyme reaction was stopped by the addition of 75 μl of 0.5 M glycine–NaOH buffer (pH 10.3) to each well, and the resulting 4-methylumbelliferone was measured using spectrofluorometry at wavelengths of 355 nm for excitation and 460 nm for emission using a fluorescence microplate reader (Fluoroskan Ascent, Labsystems, Helsinki, Finland).

Pharmacokinetic Evaluation Two white female rabbits in the weight range of 3.5 to 4 kg were used in this study. Sotalol hydrochloride was orally administered at a dose of 3 mg/kg to the rabbits. The drug was dissolved in isotonic sodium chloride at concentrations of 3 mg/ml. Blood samples were collected at 0.25, 0.5, 1, 2, 3, 4, 6, and 8 h postadministration, and the serum was stored at −20°C until assay for sotalol concentration. The serum was diluted 4-fold with buffer B to obtain a sotalol concentration appropriate for measurement by ELISA.

RESULTS AND DISCUSSION Sotalol has an amino group and hydroxy group at the molecular level. Therefore we chose to introduce a carboxylic group at the amino group or the hydroxy group with succinic anhydride. For the linear conjugation of sotalol with BSA, a molar ratio of sotalol to succinic anhydride of 1:1 was chosen for the first step to introduce the carboxylic group of succinic anhydride into either the amino group or hydroxy group of sotalol. In the second step, the sotalol hemisuccinate was coupled to BSA using the N-succinimidyl ester method. The sotalol-BSA conjugate, with 22 mol of sotalol per mol of BSA, induced the formation of specific antibodies in each of the two rabbits immunized.

The sotalol-β-Gal conjugate was also prepared by essentially the same procedure. The conjugate thus obtained was stable in eluted buffer (pH 7.0) at 4°C for more than 6 months, during which no loss of enzyme activity or immunoreactivity was seen.

Using anti-sotalol antibody and sotalol-β-Gal as a tracer, an ELISA for the quantification of sotalol was developed. The dose–response standard curve of sotalol obtained in the serum is shown in Fig. 1. The range of sotalol detection by ELISA was between 6.4 ng and 20 μg/ml of sotalol. For practical purposes, the working range was arbitrarily set between 32 and 4000 ng/ml based on the precision and accuracy findings for the ELISA in serum (Table 1), which showed this ELISA to be a reproducible technique. Recoveries of four different sotalol levels ranging from 32 ng to 4000 ng/ml were satisfactory (95.0 to 103.8%, n=5). The coefficients of variation for intra- and interassays at four different sotalol levels between 32 ng to 4000 ng/ml were 7.2 to 11.7% and 5.3 to 9.0% (each n=5), respectively. The detection limit of sotalol by ELISA is 32 ng/ml (Student’s t-test, n=3, p<0.001 compared with the B₀ value). Studies for dilution recovery were performed using 5-fold double dilutions of known con-
centrations of control sample, and the mean recovery was 100.5% (Table 2). The therapeutic range of sotalol was reported to be 1—3 mg/ml.\textsuperscript{15,16} Therefore this ELISA may be sufficiently sensitive to quantify sotalol for TDM.

The antibody specificity was determined by the cross-reactivity with two types of similar compounds, which were defined as the percentage of each compound to sotalol in the concentrations required for 50\% replacement of bound $\beta$-Gal activity. The anti-sotalol antibody showed 1.6\% cross-reaction with 4-(methanesulfonylamino)benzonitrile. No detectable cross-reaction, however, was found with D,L-isoproterenol (Table 3). These findings suggest that the antibody well recognizes both the methanesulfonanilide and the 1-hydroxy-2-(isopropylamino)ethyl moieties, and thus is sufficiently specific to the structure of sotalol.

Figure 2 shows the time course of the serum concentrations of sotalol following its oral administration at a dose of 3 mg/kg to the rabbits. Sotalol was rapidly absorbed, reached a peak concentration in the serum of 3.66 mg/ml (average in two rabbits) 60 min after dosing, and then slowly decreased. In humans, sotalol has the advantage of relatively simple pharmacokinetics.\textsuperscript{17} It is nearly completely absorbed after oral administration and has no first-pass metabolism. Approximately 80—90\% of the drug is excreted unchanged in the urine. To the best of our knowledge, there is no report in which the metabolite of sotalol is identified. Therefore this ELISA may be sufficiently specific to quantify sotalol for TDM and pharmacokinetic studies.

In conclusion, the ELISA procedure for sotalol reported here is sensitive, specific, reproducible, simple, and adaptable for analyses of a large number of samples. This ELISA will be a valuable tool in TDM and pharmacokinetic studies.

REFERENCES