Radioimmunoassay for the Novel Platelet Activating Factor Receptor Antagonist E5880

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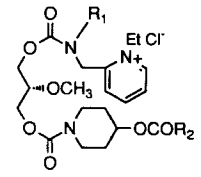
Abstract □ A direct radioimmunoassay for E5880, 1-ethyl-2-[[*N*-(2-methoxybenzoyl)-*N*-[[(2*R*)-2-methoxy-3-[[[4-[(octadecylcarbamoyl)-oxy]piperidino]carbonyl]oxy]propoxy]carbonyl]amino]methyl]pyridinium chloride, a novel analogue-type antagonist of platelet activating factor (PAF), was developed. In this procedure, [^{3}H]E5880 was used as the radioligand, and the antiserum was obtained from rabbits immunized with hapten covalently bound to bovine serum albumin. The hapten represents a structural analogue of E5880, with a carboxyl group on the terminal carbon of the 3-position side chain. A metabolite of E5880, deacyl-E5880, cross-reacted weakly (1.8%) with this antiserum. The assay buffer for the radioimmunoassay consisted of PBS, pH 6.5, containing 1% BSA to prevent the degradation of E5880 in aqueous solution and its adsorption to the tube. The detection limit of the assay was 200 pg/mL when a 0.1-mL plasma sample was used. The radioimmunoassay was used for the direct analysis of E5880 in dog plasma. The validity of the radioimmunoassay in dog plasma was demonstrated by comparative analysis of a number of samples by HPLC (r = 0.995, slope = 0.9425). The radioimmunoassay was also used to determine the pharmacokinetics of E5880 in the dog. After the intravenous administration of E5880 (0.2 mg/kg), plasma levels declined biexponentially. The initial plasma half-life, including the distribution phase, was 0.26 h, and the plasma half-life of elimination was 9.96 h.



1 is E5880

R1: R2: $-NHC_{18}H_{37}$

2 is the hapten

R1: R2: $-NHC_{18}H_{36}COOH$

3 is the radioligand [^{3}H] E5880

R1: R2: $-NH(CH_2)_8(CHT)_2CH_2(CHT)_2(CH_2)_4CH_3$

T: ^{3}H

4 is the metabolite of E5880 (Deacyl-E5880)

R1: $-H$ R2: $-NHC_{18}H_{37}$

5 is the internal standard

R1: R2: $-NHC_{20}H_{41}$

Scheme 1—Chemical structures of E5880 and related compounds

Introduction

Platelet activating factor (PAF) is a potent lipid mediator of various inflammatory and allergic reactions.[1] E5880 (1-ethyl-2-[[*N*-(2-methoxybenzoyl)-*N*-[[(2*R*)-2-methoxy-3-[[[4-[(octadecylcarbamoyl)oxy]piperidino]carbonyl]oxy]propoxy]carbonyl]-amino]methyl]pyridinium chloride, CAS Reg. No. 128420-61-1),[2,3] a novel analogue-type receptor antagonist of PAF, has been reported[4] to have potent PAF antagonistic activity *in vitro* and inhibitory effects in various experimentally induced *in vivo* shock models. Because the circulating drug concentration after the administration of therapeutic doses of E5880 is low, it is necessary to develop a highly sensitive and specific analytical technique for the determination of this agent in biological fluids. For this purpose, we selected radioimmunoassay because of its sensitivity, specificity, and simplicity; this type of procedure has already been employed for the quantitation of drugs.[5-7] There were a few difficulties, however, in sample treatment, i.e., the degradation of E5880 in aqueous solutions and its adsorption to tubes. In our development of a radioimmunoassay procedure for E5880, we therefore investigated its chemical stability and its adsorption to the tubes. We also synthesized the basic reagents. The application of this assay to pharmacokinetic studies of E5880 in dogs is also described.

Experimental Procedures

Chemicals—E5880 (1), the deacyl metabolite of E5880, deacyl-E5880 (4), the internal standard 5, and PAF were synthesized in-house (Scheme 1). All other chemicals were commercially obtained and were of analytical reagent grade.

Preparation of the Hapten—The hapten **2** was prepared as shown in Scheme 2. The starting material was prepared from 1,2-*O*-isopropylidene-(*S*)-glycerol as an intermediate to synthesize E5880. A hydroxy group of **6** was protected with a *tert*-butyldimethylsilyl group to obtain **7** in quantitative yield. After the catalytic hydrogenation of **7** with 10% palladium on carbon in ethyl acetate, the newly generated primary alcohol was converted to carbamate **9** by heating its phenylcarbonate with 2-(aminomethyl)pyridine in 89% yield from **7**. Reaction with 2-methoxybenzoyl chloride and subsequent acidic deprotection gave the secondary alcohol **10** in 89% yield. Introduction of the carboxylic moiety was performed by heating **11** with silyl-protected carboxylic acid isocyanate, prepared from the monosilyl ester of 1,18-octadecanedicarboxylic acid and diphenyl phosphorazidate, to obtain **13** in 69% yield. By successive quaternization of the pyridine moiety with iodoethane, ion exchange, and acid treatment, **13** was converted into hapten **2** in 25% overall yield.

Preparation of [^{3}H]E5880—The synthetic pathway of [^{3}H]E5880 as a radioligand is shown in Scheme 3. The intermediate **12** was used as the starting material in the hapten synthesis. A mixture of 3.95 mg of **12a**, 11 mg of 4-DMAP, and 20 mCi of [^{3}H]octadecylamine in 1 mL of pyridine was refluxed for 5 h; the mixture was evaporated, purified by thin-layer chromatography (acetone/*n*-hexane = 1:2), and eluted with 10% acetone/EtOAc to obtain **15**. The pyridine moiety of **15** was quaternized with iodomethane/DMF, ion exchanged with IRA-410(Cl^-), and purified on HPLC to obtain 9.9 mCi of **3**.

Preparation of Hapten–BSA Conjugate—Since E5880 (1) is not immunogenic, a hapten–protein conjugate was proposed by coupling the hapten to bovine serum albumin (BSA), using carbodiimide[8] as the activator. The molar ratio of hapten to BSA in 50% ethanol aqueous solution was calculated by a UV difference spectral method.[9,10]

Immunization Procedure—Three male Japanese white rabbits were used for immunization. One milligram of antigen was dissolved in 0.5 mL of sterile isotonic saline and emulsified with 0.5 mL of complete

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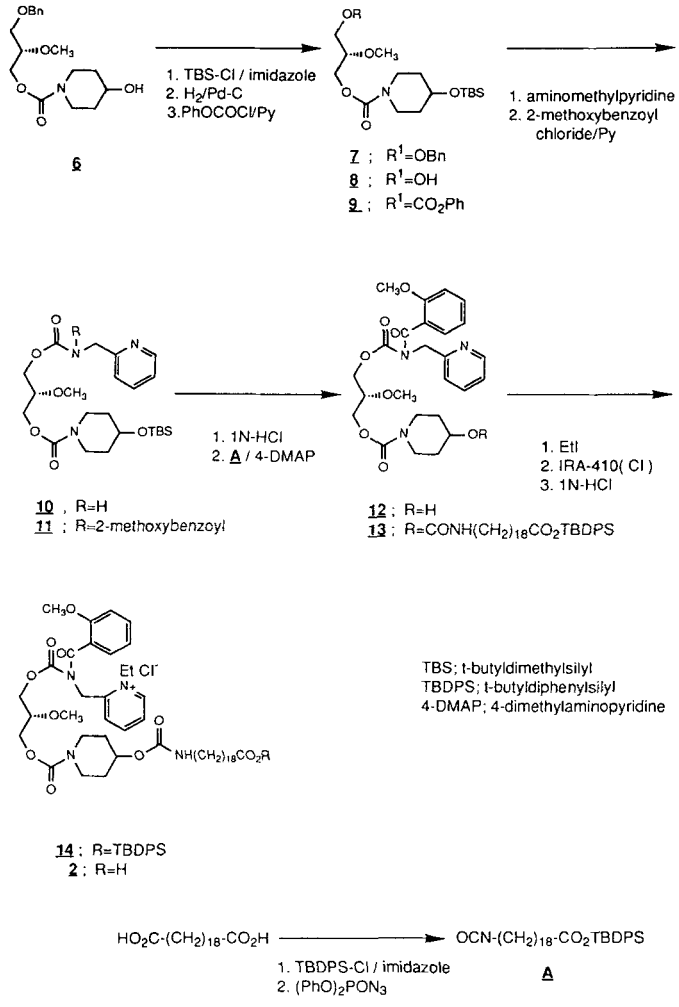


Scheme 2—Preparation of hapten

Freund's adjuvant (Difco Laboratories) with a sonifier (cell disruptor 185E, Branson).

The emulsion was injected into rabbits subcutaneously at multiple sites. Booster immunizations were repeated five times, at intervals of 2–4 weeks, over a 3-month period. The rabbits were bled 10 days after the final booster injection, and the serum was separated by centrifugation at 3000 rpm for 10 min (KR-500, Kubota) and stored at −20 °C in small aliquots until analysis.

Antiserum Titer Determinations—Various dilutions of rabbit antiserum in assay buffer (0.1 mL) were incubated with [^{3}H]E5880 (30 pg/100 000 dpm in 0.1 mL assay buffer) and additional assay buffer (0.1 mL) in polypropylene tubes at 37 °C for 2 h. Tubes containing only [^{3}H]E5880 (0.1 mL) and buffer (0.2 mL) served to determine the total count and nonspecific binding, respectively. After 2 h, 0.3 mL of iced ethanol was added to all assay tubes, except for the total count tubes, and the contents were vortex-mixed and left to stand at 0 °C for 60 min. All tubes were then centrifuged at 4 °C for 10 min, and the supernatant of each tube was decanted into a vial. After the addition of 15 mL of ACS-II (Amersham), radioactivity was determined with a liquid scintillation counter (Aloka LSC-3500, Tokyo, Japan). The antiserum titer was defined as the dilution that bound 50% of the added radioligand.

Chemical Stability in Aqueous Solutions and Adsorption of E5880—The effects of pH on the chemical stability of E5880 were investigated as follows: 1-mL aliquots of E5880 solution (100 μg/mL), mixed with Britton-Robinson buffer[11] at various pH values, were transferred to 2-mL ampules, and the ampule tips were heat-sealed. One ampule containing E5880 solution in distilled water was refrigerated at 4 °C to serve as the "zero-time" sample, and the others were stored at 37 °C for 24 h, after which they were stored at 4 °C until HPLC analysis.

The effects of temperature on reaction rate were investigated as follows: 1-mL aliquots of E5880 solution (100 μg/mL), buffered at pH 6.5, were transferred to 2-mL ampules, which were sealed and divided into sets of 10. Each set of ampules was heated in constant temperature baths at 37, 45, and 55 °C. The ampules were then removed from the baths at appropriate intervals, cooled, and stored at 4 °C until HPLC analysis.

The adsorption of [^{3}H]E5880 to glass, polystyrene, and polypropylene, tubes was investigated as follows: 1-mL of [^{3}H]E5880 saline solution (250 pg/mL) was vortex-mixed in a 10-mL polypropylene tube, and the solution was then transferred to a glass, polystyrene, or polypropylene tube. The transfer was repeated five times, and the radioactivity in each solution was determined. The inhibitory effect of BSA on adsorption was investigated as follows: an acetonitrile solution containing 3 ng of [^{3}H]E5880 was dried under a stream of nitrogen gas in a 10-mL polypropylene tube. One milliliter of assay buffer, containing of 0%, 0.5%, 1%, or 5% BSA was added to the tube, and the contents were vortex-mixed. Each solution was then diluted with each assay buffer 10- and 100-fold. The radioactivity of each solution was determined immediately and after the solution had been allowed to stand for 14 h.

Standard Assay Procedure for Radioimmunoassay—Scheme 4 shows the standard assay procedure for the radioimmunoassay. The assay buffer consisted of 0.067 M potassium–sodium phosphate buffer, pH 6.5, containing 0.9% NaCl and 1% BSA. This assay buffer was used to prepare all subsequent working solutions. Standard E5880 stock solution (10 μg/mL) was prepared in 90% acetonitrile aqueous solution and stored at −20 °C until used for the assay. In the assay, a standard curve was prepared from 10 pg to 100 ng E5880 per sample in a total of 12 steps. Each sample (plasma, blank, and standards) was run in duplicate in a polypropylene round-bottomed tube. Plasma samples were diluted 1:2, 1:9, 1:99, and 1:999 with assay buffer.

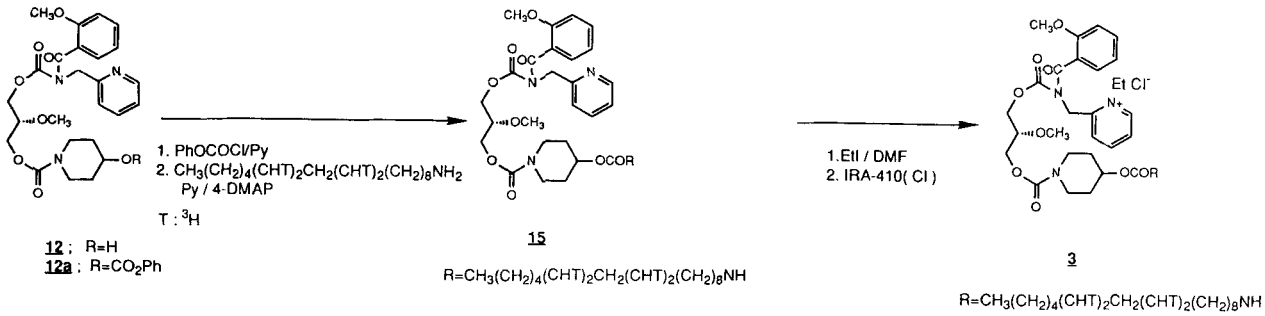
The assay was performed with a standard solution of E5880 in assay buffer (0.1 mL) or assay buffer (0.1 mL), normal dog plasma or assay sample, or [^{3}H]E5880 solution (0.1 mL = 30 pg of [^{3}H]E5880 − 10 000 dpm) or antibody serum (0.1 mL; dilution 1:2000 with assay buffer). Equilibrium was obtained by allowing the samples to incubate for 2 h at 37 °C. After the addition of rabbit γ-globulin (4 mg in 0.1 mL assay buffer) and 1 mL of iced ethanol, the resulting solution was vortex-mixed and then allowed to stand for at least 30 min in an ice bath. The supernatant of each sample was separated by a 10-min centrifugation at 3000 rpm (4 °C) and decanted into a vial. Fifteen milliliters of ACS-II (Amersham) was added, and the radioactivity was determined with a liquid scintillation counter.

A standard curve was constructed on each day of analysis. Calculations were made by the logit–log procedure,[12] by plotting the logit values of B/B_0 on the y-axis and the log values of the E5880 plasma concentration on the x-axis, where B and B_0 were the antibody-bound radioactivity in the presence and absence of unlabeled E5880, respectively. Logit B/B_0 is defined as

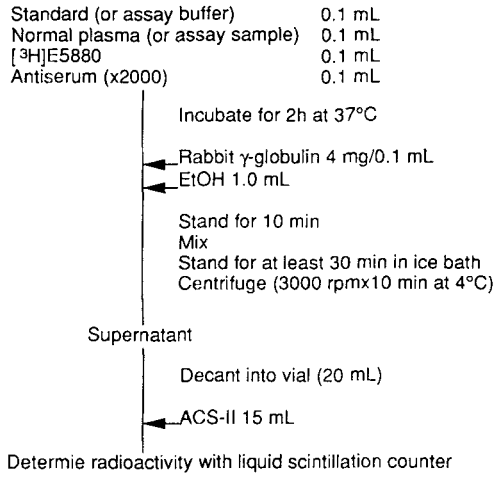
$$\ln\left[\frac{B/B_0}{1-(B/B_0)}\right]$$

Cross-Reaction Study—The specificity of the antiserum against the hapten–BSA conjugate was assessed by cross-reaction studies with related compounds. The relative amounts required to reduce the initial binding of [^{3}H]E5880 by half, where the mass of unlabeled E5880 was arbitrarily taken as 100%, were calculated from the standard curve.

Determination of E5880 by HPLC with UV Detection—A 0.5-mL plasma sample was acidified with 1 mL 1 N HCl and added to 0.1 mL of internal standard (5) in methanol; extraction was performed twice, with 5 mL of ethyl acetate, on a reciprocating shaker for 10 min. The combined ethyl acetate extracts were evaporated to dryness in a 10-mL glass tube at 30 °C under a gentle stream of nitrogen gas. The dried residue was then disolved in 1 mL of methanol. The extraction column (Bond Elut C18; Varian Associates, Inc., cat. no. 1210-2028) was activated by two washings in 3 mL of methanol, and the sample was applied to the top of the column. The column was then washed with 3 mL of methanol and 3 mL of methanol/distilled water = 80:20 containing 0.10 N HCl, and E5880 was eluted with 3 mL of methanol containing 0.01 N HCl. The effluent was evaporated to dryness at 30 °C under a gentle stream of nitrogen gas. The dried residue was dissolved in 0.2 mL of acetonitrile/water = 95:5 and the solution was injected into the HPLC column. The HPLC system consisted of a Model M600 pump (Waters) a Unidec Model 100-IV UV spectrophotometer (Jasco), and a Model 710B WISP autosampler (Waters). A reversed-phase column (Inertsil ODS, 5 μm, 4.6 i.d. × 250 mm, GL Science) was used as the stationary phase; the mobile phase, acetonitrile, containing 5% (v/v) water and 0.1% (v/v) perchloric acid, was pumped at the rate of 1.5 mL/min.



Scheme 3—Preparation of [^{3}H]E5880



Scheme 4—Standard assay procedure for radioimmunoassay of E5880

Absorbance at 266 nm was monitored. The detection limit for plasma was 50 ng/mL. The CV value was 5.0%.

Preparation of Assay Samples—Samples were obtained from three male beagle dogs after they had received single intravenous injections of E5880 (0.2 mg/kg) administrated in 0.9% NaCl solution. All blood samples were drawn from a peripheral vein into heparinized blood-collecting tubes. Plasma, obtained by centrifugation of the samples at 3000 rpm for 10 min, was stored at −20 °C until analysis.

Pharmacokinetic Calculations—The plasma concentration data following intravenous administration are expressed as the mean and standard deviation for three beagle dogs. The data were fitted to a two-compartment model, using the MULTI program.[13] The plasma concentrations of E5880 were fitted to a biexponential curve, using nonlinear regression. The half-lives of the distribution ($t_{1/2\alpha}$) and elimination phase ($t_{1/2\beta}$) were calculated as $0.693/k$ from the rate constants of the rapid (k_α) and slow (k_β) phases, respectively. The area under the plasma concentration–time curve for infinite time ($AUC_{(0-\infty)}$), calculated by the trapezoidal rule up to the last measured plasma concentration (C_{last}), was extrapolated to infinity by adding the quotient C_{last}/k_β.

Results and Discussion

Development of Radioimmunoassay—*Synthesis of Immunogen and Radioligand*—UV analysis of the hapten–BSA conjugate indicated that 2.7 mol of hapten was covalently linked to 1 mol of BSA. [^{3}H]E5880 was finally purified by HPLC, and the radiochemical purity was >99%. The specific radioactivity was estimated to be 127 Ci/mmol. The radioligand was stable for at least 3 months at −20 °C in acetonitrile solution.

Antiserum **Production**—After a 3-month administration of the immunization protocol, all three rabbits immunized with the hapten–BSA conjugate produced antisera capable of binding [^{3}H]E5880. These antisera was diluted 1:48, 1:240, and 1:2700, respectively, to obtain 50% binding of [^{3}H]E5880, using a standard assay procedure. The rabbit with the highest serum titer was selected for further studies.

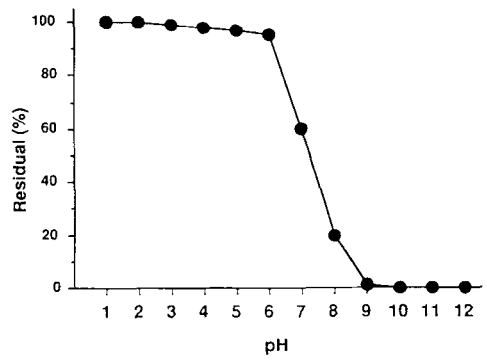


Figure 1—Degradation profile of E5880 in aqueous solutions at 37 °C over a 24-h period.

Table 1—First-Order Rate Constants for Degradation of E5880 in Buffer (pH 6.5)

temp (°C)	k_{obs} (h^{-1})	$t_{97\%}$ (h)
55	0.0438	0.69
45	0.0148	2.06
37	0.0064	4.78

Chemical Stability of E5880 in Aqueous Solutions—E5880 was stable in buffer solution below pH 6 but was degraded via hydrolysis in neutral or basic aqueous solutions, as shown in Figure 1. In neutral or weak basic solutions, the hydrolysate deacyl-E5880 (4) formed, resulting in the loss of pharmacological activity. However, the radioimmunoassay could no be performed when the pH of the assay buffer was lower than 6. In kinetic studies, degradation was shown as a pseudo-first-order reaction at pH 6.5. The observed rate constants at 37, 45, and 55 °C are presented in Table 1. From the Arrhenius plot of log k_{obs} versus $1/T$, the activation energy, calculated by the equation $E_a = 2.303 \times 1.987 \times$ (the slope of the line), was 21.7 kcal deg/mol. The calculated rate constants at 25 and 4 °C were 0.0015 and 0.000095, respectively. E5880 was thus considered to be sufficiently stable for sample treatment at room temperature and reaction incubation at 37 °C for 2 h. A pH of 6.5 for the assay buffer was therefore selected for the radioimmunoassay.

Adsorption of E5880 to Tubes—[^{3}H]E5880 in aqueous solution was strongly adsorped to glass, polystyrene, and polypropylene tubes at levels decreasing in that order. Addition of organic solvents, i.e., methanol, ethanol, or acetonitrile, prevented this adsorption. The adsorption of [^{3}H]E5880 in saline to polypropylene tubes, the combination which had the lowest adsorption, was 65%; no adsorption occurred in 50% (v/v) ethanol solutions. A high concentration of protein (BSA) also prevented adsorption of the E5880 to polypropylene tubes, as shown in Figure 2. Therefore, in the radioimmunoassay system, we used an assay buffer containing 1% BSA and an iced ethanol method for B/F separation. Polypropylene tubes were used for the running of all samples (plasma, blank, and standards).

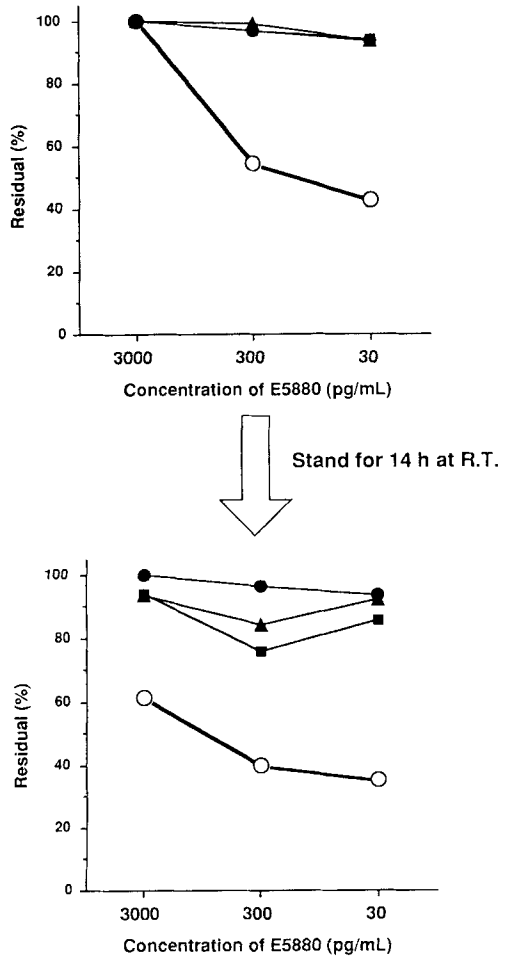


Figure 2—Inhibitory effects of BSA on adsorption of E5880 to polypropylene tubes. Key: (O) saline solution; (■) 0.5% BSA; (▲) 1% BSA; (●) 5% BSA.

Nonspecific Binding—The radioimmunoassay was performed with no preliminary purification of the E5880 in plasma. There was a difference, however, between the calibration curves of the assay buffer and the plasma samples (Figure 3A), this probably being due to nonspecific binding (NSB) to plasma protein. However, when maximum binding (B_0) and NSB were measured in E5880-free plasma instead of the assay buffer, the plasma and buffer calibration curves showed a close fit (Figure 3B). To minimize differences in the calibration curves, we found it necessary to correct B_0 and NSB by adding equivalent aliquots of E5880-free plasma, either diluted or undiluted, as required.

Specificity, Sensitivity, and Precision of Radioimmunoassay—The cross-reactivity of antiserum with related compounds is shown in Table 2. Percentage cross-reactivities were calculated from the drug concentrations which gave 50% B/B_0 antibody binding. A cross-reactivity of 1.8% was found with deacyl-E5880 (4). In studies of the metabolic fate of [^{14}C]-E5880 in rats and dogs, the major compound detected in the plasma and tissues was the unchanged drug, and the level of the main metabolite deacyl-E5880 in the plasma and tissues was very low. Thus, there is no problem with immunoreactive metabolites. The antiserum exhibited no cross-reactivity with intact compounds, i.e., PAF and lecithin. These results reflect the high specificity of the assay in relation to the primary metabolites of E5880.

The detection limit of the assay was 200 pg/mL when a 0.1-mL plasma sample was used. This sensitivity was 250 times that of the HPLC–UV method, in which the detection limit was 50 ng/mL.

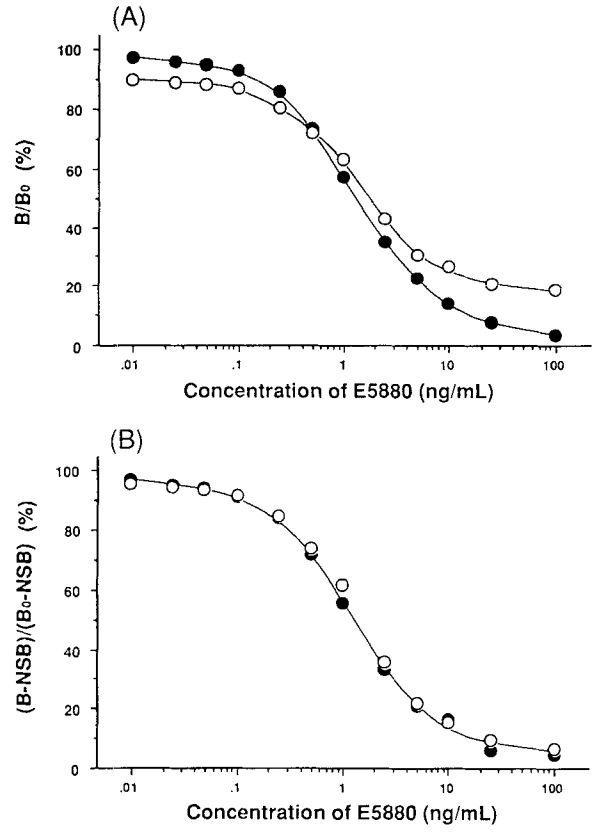


Figure 3—Radioimmunoassay standard curves for E5880 in assay buffer (●) and dog plasma (O) (a) without nonspecific binding correction (b) with nonspecific binding correction.

Table 2—Cross-Reactivity of Antiserum with Related Compounds

Compound	% Cross-Reactivity
E5880	100
PAF	<0.001
Lecithin	<0.001
Deacyl-E5880	1.8

The coefficient of variation for the replicate analyses of various concentrations of E5880 from 0.2 to 10 ng/mL was 5.7%, and the assay had good accuracy (94.8%–114.8%).

Pharmacokinetics in Dogs—The mean plasma concentration, determined by radioimmunoassay after the intravenous administration of E5880 (0.2 mg/kg) in three beagle dogs, is shown in Figure 4. Five minutes after intravenous administration of E5880, the plasma concentration was 1.70 ± 0.24 μg/mL (means ± SD); this was followed by a two-phase decline. The data fitted well to a two-component model, and the kinetic parameters were obtained from the model. The initial plasma half-life ($t_{1/2\alpha}$), including the distribution phase, was 0.26 ± 0.01 h, and the plasma elimination half-life ($t_{1/2\beta}$) was 9.96 ± 1.12 h. The area under the plasma concentration-time curve for infinity was 0.873 ± 0.121 μg·h/mL. The distribution volume at steady state and the total body clearance were 860 ± 350 mL/kg and 232 ± 31 mL/kg per h, respectively.

Correlation between Radioimmunoassay and HPLC–UV Method—The correlation between the radioimmunoassay and the HPLC-UV method was demonstrated in the analysis of dog plasma after the administration of E5880 (0.2 mg/kg). The regression equation was $Y = 0.9425X - 0.0006$. Excellent correlation ($r = 0.995$) and good agreement (slope = 0.9425)

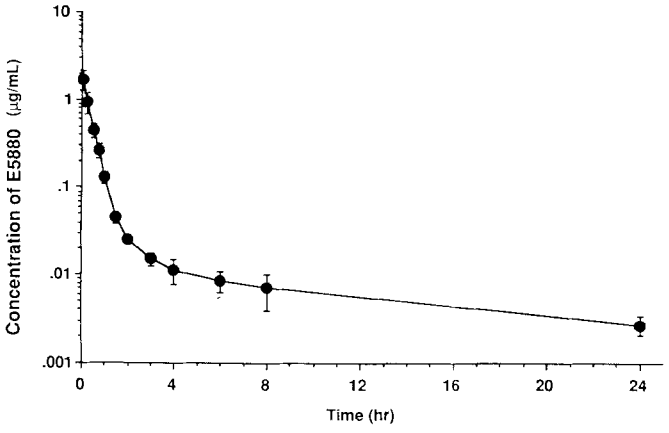


Figure 4—Plasma concentration after intravenous administration of E5880 (0.2 mg/kg) to beagle dogs. Each point represents the mean ± SD of three animals.

were found for these two analytical methods, confirming the validity of the radioimmunoassay for the determination of E5880 in plasma.

Conclusion

We described here a new radioimmunoassay procedure for E5880 which was sufficiently sensitive and specific for the analysis of the drug in dog plasma samples up to 24 h after the injection of a therapeutic dose. The assay is fast and easy to perform, and an extraction procedure is not required. We are currently employing this method in human pharmacokinetic and pharmacodynamic studies of E5880 after intravenous administration. These pharmacokinetic studies of E5880 in humans will be reported in the near future.

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