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Non-isotopic receptor assay for benzodiazepines using a biotin-labeled ligand and biotin-immobilized microtiter plate

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ABSTRACT

A non-isotopic receptor assay for benzodiazepine drugs was developed using a biotin-labeled ligand, biotin–1012S. Biotinylated bovine serum albumin (biotin–BSA) was immobilized onto the wall of microtiter plate wells by simple adsorption. Avidin peroxidase conjugate could be extracted from solution owing to its strong interaction with biotin. The amount of avidin peroxidase taken up on the wall was then determined by measuring the enzyme activity. The competition between immobilized biotin on the wall and free biotin for avidin provided the basis for a solid-phase avidin–biotin binding assay. By this binding assay, not only biotin but also biotin-1012S could be measured sensitively. Because 1012S is a ligand with high affinity to benzodiazepine receptors, biotin–1012S could be utilized as a probe ligand for a non-isotopic receptor assay. Based upon the competition between biotin–1012S and various benzodiazepine drugs for the receptor binding sites, a non-isotopic receptor assay was demonstrated.

INTRODUCTION

Many biological molecules have excellent molecular recognition properties. For example, enzymes recognize a substrate at the active site to catalyze a specific reaction and antibodies bind antigens very specifically. Neurotransmitters or hormone receptors, which are involved in the controle and/or modulation of every cellular and physiological process, are also triggered or modulated by the biospecific binding of a ligand at the binding sites [1,2]. Both enzymes and receptors are very large and complex protein molecules and the complex steric structure of these macromolecules provides an environment for excellent molecular recognition commonly referred to as biological affinity. Although artificial enzymes and receptors have been built up from more simple molecules to mimic biological molecular recognition systems [3,4], these are thus far inferior to natural biological systems. Therefore, analytical methods utilizing biological molecular recognition systems have been attempted in the fields of biospecific affinity chromatography [5], biosensors [6,7] and receptor binding assays [8–11].

A receptor binding assay is based on molecular recognition of a ligand by a receptor's binding site. Highly selective and sensitive assays are possible due to the great affinity of ligands to their respective receptor. Radio-isotopic ligands have been the most widely used probes for receptor assays [12,13]. Because the density of receptor binding sites is very low in most tissues [14], the ligand label must have a very low limit of detection. However, radio-isotopes have serious problems associated with their handling and disposal. Fluorophore- and enzyme-labeled ligands have been developed as alternatives to radio-labeled ligands [15,16]. Labeling with macromolecules such as an enzyme, however, sometimes alters the physical and chemical state of the ligand and leads to a reduction in both its specificity and affinity.

Previously, we reported that a biotin-labeled li-

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gand had potential usefulness as a probe ligand in a benzodiazepine receptor assay [17]. Labeling with biotin does not alter the affinity of the ligand to the receptor binding site and no increase in non-specific binding was observed with this method. The concentration of the free biotin-labeled ligand could be sensitively measured by an avidin–biotin binding assay using enzyme-labeled avidin along with biotin-immobilized polystyrene beads [18]. However, the method requires a rather tedious and time-consuming procedure for the preparation and washing of the biotin beads with occasional poor reproducibility. Thus it is not easy to apply this method to the determination of a large number of samples as a screening test or for the analysis of actual samples such as human serum and urine. To solve these problems, a new solid-phase avidin–biotin binding assay was investigated using microtiter plates and a plate reader to be incorporated into the non-isotopic receptor assay. The association of enzyme-labeled avidin with the biotin immobilized on a wall of a microtiter plate well was observed. The competitive reaction between immobilized biotin and free biotin to the biotin binding sites on avidin allowed for a sensitive determination of *d*-biotin and biotin-labeled benzodiazepine. This solid-phase avidin–biotin binding assay using microtiter plates was then applied to the benzodiazepine receptor assay.

EXPERIMENTAL

Reagents

Biotinamidocaproic acid sulfosuccinimide ester (BAC-Sulfo-NHS) was purchased from Pierce (Rockford, IL, USA). Horseradish peroxidase–avidin D (Av-P, 1.5 mol of peroxidase per mol of avidin) was obtained from Vector Labs. (Burlingame, CA, USA). Biotinamidocaproyl–bovine serum albumin (biotin–BSA, *ca.* 9.6 mol of biotin per mol of BSA), bovine serum albumin fraction V (BSA). 3,3′,5,5′-tetramethylbenzidine (TMB), and sodium azide were obtained from Sigma (St. Louis, MO, USA). Hydrogen peroxide was purchased from Aldrich (Milwaukee, WI, USA). Benzodiazepine drugs, including 1012S, were provided by Shionogi & Co., (Osaka, Japan). Lyophilized benzodiazepine receptor from cow brain is purchased from Research Biochemicals (Natick, MA, USA). Bio-Rad (Richmond, CA, USA) protein assay reagent was used to measure the protein concentrations. Other chemicals were purchased from Fisher Scientific (Kent, WA, USA) and used without further purification.

Preparation of biotin–1012S conjugate

Biotin-labeled 1012S conjugate was prepared by mixing 500 μl of 2 m*M* BAC-Sulfo-NHS in N,N-dimethylformamide and 250 μl of 1 m*M* 1012S, which gives a 4:1 molar ratio, in 50 m*M* phosphate buffer (pH 7.4). Incubated overnight at 4°C, the mixture containing biotin–1012S conjugate was then purified by reversed-phase high-performance liquid chromatography (HPLC), using a column (5 cm × 4 mm I.D.) packed with Develosil ODS-5 (Seto-shi, Japan) and a eluent of acetonitrile–50 m*M* acetate buffer (pH 4.5) (26:74, v/v). The purified product showed a single HPLC peak with the retention time apparently different from that of non-labeled 1012S (Fig. 1), while the absorption spectrum of the product solution was identical to that of 1012S. The concentration was determined by using its apparent molar absorptivity of $2.6 \cdot 10^4$ M^{-1} cm^{-1} (0.02 *M* acetic acid at pH 4.5). The stability of the conjugate was investigated by measuring the absorbance at 236 nm, with no apparent change over a 3-month period as shown in Fig. 2.

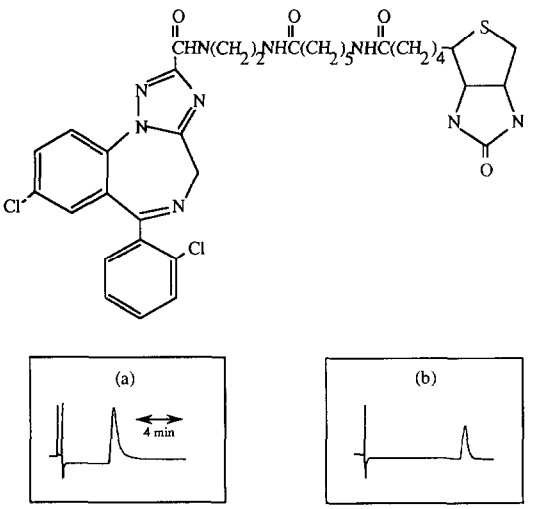

Fig. 1. The structure of the biotin–1012S conjugate and the chromatograms from reversed-phase HPLC. (a) 10 μl of 3 μ*M* 1012S standard solution; (b) biotin-1012S conjugate solution. Detection wavelength, 254 nm.

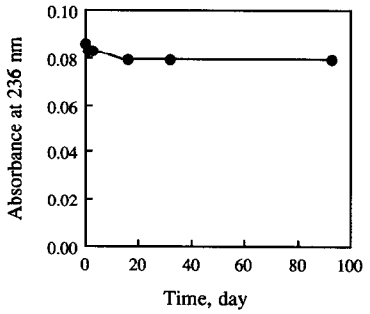

Fig. 2. The stability of the biotin–1012S conjugate. Absorbance at 236 nm of 3 μM biotin–1012S conjugate in 0.02 M acetate buffer solution (pH 4.5) was measured.

The conjugate solution after 3 months still showed the single peak of biotin–1012S by using reversed-phase HPLC, which confirmed that the conjugate was stable in 0.02 M acetic acid (pH 4.5) for at least 3 months.

Preparation of biotin-immobilized microtiter plate (biotin plate)

The microtiter plates (Falcon 3915, polystyrene Probin Plate), purchased from Becton Dickinson (Lincoln park, NJ, USA), were first washed with detergent [dilution solution (30:1) of Versa-Clean, Fisher Scientific] and water. A 200-μl volume of 0.002% biotin–BSA phosphate buffer solution was added to wells of the plate and incubated overnight at 4°C. After washing the plate with 10 mM phosphate buffer containing 0.1 M sodium chloride at pH 7.4 containing 0.1% BSA (PBS-B) solution three times, 200 μl of 1% BSA solution were added to each well and incubated at 4°C for 1 h in order to eliminate all remaining hydrophobic binding sites, and then washed three times with PBS-B. The biotin plates were stored at 4°C in PBS-B containing 0.1% sodium azide and again washed once with PBS-B before use.

Solid phase avidin–biotin binding assay

A sample (100 μl) and PBS-B (50 μl) were pipetted into each well of the biotin plate and Av-P (0.12 nM, 50 μl) was then added. The mixture was incubated overnight at 4°C with continuous shaking 80 oscillations/min by a Precision Model 25 shaker bath (Chicago, IL, USA). Following the incubation the biotin-plate was washed five times with 10 mM phosphate buffer containing 0.1 M sodium chloride at pH 7.4 (PBS), then TMB solution (100 μl) was added to each well. The enzyme reaction was started by the addition of 0.01% hydrogen peroxide (50 μl) and this mixture was incubated for 5 to 15 min with shaking. The reaction was then stopped by the addition of 0.1 M sulfuric acid (50 μl). The absorbance at 492 nm was measured with a plate reader (EAR 340AT; SLT-Labinstruments, Salzburg, Austria).

Receptor assay for benzodiazepine drugs

A 400-mg amount of benzodiazepine receptor preparation was suspended in 8 ml of 25 mM phosphate buffer at pH 7.4 (PB) and homogenized by a glass tissue grinder. The receptor suspension was then centrifuged at 22 000 g at 4°C for 15 min with an IEC HT centrifuge (Needham, MA, USA) and the supernatant was discarded. After washing the precipitate with PB solution three times, it was resuspended in 8 ml of PB solution. A mixture containing 400 μl of the receptor suspension, 50 μl of 20 nM biotin–1012S conjugate and 50 μl of various concentrations of benzodiazepine drugs in PB was incubated for 1 h at 4°C in a microcentrifuge tube. It was then centrifuged at 22 000 g for 15 min at 4°C. A 100-μl aliquot of the supernatant was removed for the avidin–biotin binding assay described above.

RESULTS AND DISCUSSION

Uptake of avidin on the biotin plate

The amount of biotin–BSA immobilized onto the wall of the microtiter well was determined by measuring the concentration of BSA in biotin–BSA solution before and after incubation using the Bio-Rad protein assay based on the Bradford method [19]. The average value of biotin-BSA immobilized on the wall of a well was 0.58 ± 0.04 μg, corresponding to about 10^{-10} mol biotin per well. Although not all of the immobilized biotin is available for the binding of Av-P, the amount of biotin immobilized in each well is large enough to be in excess compared to the amount of Av-P, about 10^{-14} to 10^{-15} mol, found in the samples.

The uptake behavior of avidin peroxidase onto the biotin plate was investigated by changing the concentration of Av-P. For this study, 50μl of

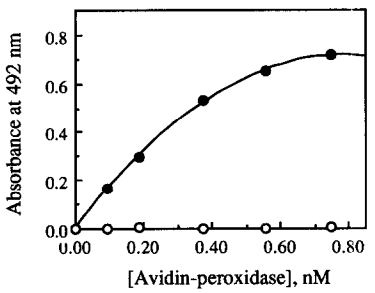

Fig. 3. Uptake of avidin peroxidase onto the biotin plate. The enzyme acitivity after overnight incubation was measured in a TMB/H_2O_2 substrate solution. ● = Biotin plate; ○ = non-biotinplate.

PBS-B, 100 μl of PB and 50 μl of PB solution containing Av-P were added to a well of the biotin plate and incubated overnight at 4°C. After washing the plate with PBS five times, the enzyme activity was determined with the TMB/H_2O_2 substrate solution. The relationship between the absorbance at 492 nm of the solution and the concentration of avidin peroxidase is shown in Fig. 3. The amount of Av-P that accumulates on the biotin plate, due to the strong avidin–biotin binding, increases with increasing concentration of Av-P and is directly proportional up to 0.4 n*M* but deviates from linearity above this concentration. This Av-P uptake behavior suggests that the amount of immobilized biotin available for the binding of Av-P is not any more than the amount calculated from the protein assay data. Av-P is not taken up on a non-biotin plate, *i.e.* not incubated with the biotin–BSA solution but only with 1% BSA for 1 h (Fig. 3). These results confirm that the uptake of Av-P onto the biotin plate is almost totally due to the specific interaction between avidin and biotin, with non-specific binding being extremely low for this method. The Av-P concentration (0.12 n*M*) utilized in this assay is in the linear range for Av-P binding to the biotin plate.

Avidin–biotin binding assay for biotin and the biotin–1012S conjugate

The uptake of avidin peroxidase onto the biotin plate is inhibited by the addition of biotin into the solution, because of the competition between immobilized biotin and free biotin for the limited number of biotin binding sites of the avidin conjugate. Once all four of the biotin binding sites on avidin [20] are occupied by free biotin, the avidin is unable to complex with immobilized biotin, therefore uptake of avidin onto the biotin plate does not take place. These results demonstrate the utility of a solid phase avidin–biotin binding assay for the determination of biotin and biotin-labeled conjugates. The principle of the method is schematically illustrated in Fig. 4. The dose–response curve for *d*-biotin obtained by this assay is well defined as shown in Fig. 5. The detection limit for *d*-biotin is about 0.1 n*M* ($5 \cdot 10^{-15}$ mol) and the relative standard deviation at 1 n*M* *d*-biotin was about 5% ($n = 5$). Bayer *et al.* [21] have reported a sensitive avidin–biotin binding assay for biotin using a biotin–alkaline phosphatase conjugate and immobilized streptavidin on biotin–BSA adsorbed microtiter plates. However, their method requires a large amount of streptavidin (0.1 μg of streptavidin per sample) and a very long incubation time for the enzyme reaction. In our method, only 1 ng of avidin per sample is necessary and an incubation time of only 5 to 15 min is required for the enzyme reaction. The biotin–1012S conjugate, which has a strong affinity for the benzodiazepine receptor [22] and has potential usefulness as a probe ligand in a receptor assay, can

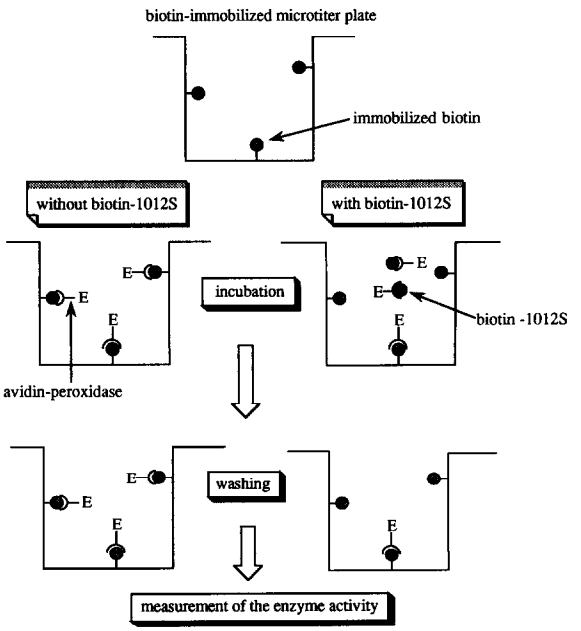

Fig. 4. Abbreviated schematic diagram of the solid-phase avidin–biotin binding assay.

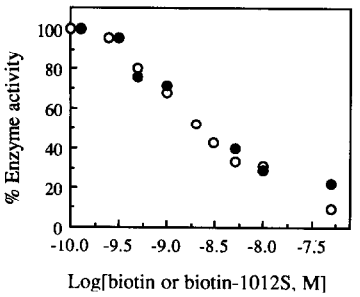

Fig. 5. Dose–response curves for biotin (●) and the biotin–1012S conjugate (○) by the solid-phase avidin–biotin binding assay. The ordinate shows the percent of enzyme acitivity in the solution with biotin to that without biotin.

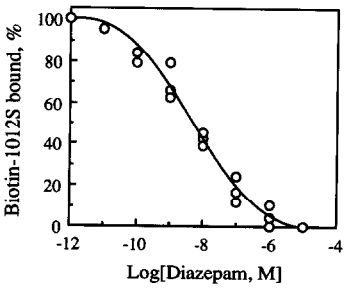

Fig. 6. Dose–response curves for diazepam by the receptor assay. The ordinate shows the percent of specifically bound biotin–1012S in the presence of diazepam to that in the absence of diazepam.

also be sensitively measured by this assay. The dose–response curve is almost identical to that of *d*-biotin as shown in Fig. 5. In fact it has the same detection limit and relative standard deviation as *d*-biotin. The agreement between the two curves strongly suggests that the conjugate has the same affinity for avidin as *d*-biotin, that is, the benzodiazepine moiety of the conjugate does not seem to affect the affinity between avidin and biotin.

Receptor assay for benzodiazepine drugs

Since it was confirmed that biotin–1012S conjugate could be sensitively measured by the solid-phase avidin–biotin binding assay using a biotin plate, the method was applied to the receptor assay for benzodiazepine drugs. Because the first supernatant of the benzodiazepine receptor suspension contains substances which interfere with the formation of the avidin–biotin complex [17], the receptor suspension was first spun down and the pellet was resuspended in fresh PB buffer for use in the receptor assay. After benzodiazepine–receptor samples had been centrifuged, a 100-μl aliquot of supernatant was used for the solid-phase avidin–biotin binding assay. The dose–response curve for diazepam obtained by this method is shown in Fig. 6. The curve is well-defined and has about a 7% relative standard deviation (n = 3), which corresponds closely to that obtained with the biotin-labeled bead method reported previously [17]. Assays for lorazepam and clonazepam also gave well-defined dose–response curves as shown in Fig. 7. The order of the IC_{50} values evaluated from these curves approximates that by radio-isotopic ligand method [23], where IC_{50} is the concentration of displacing ligand required to inhibit 50% of probe ligand binding.

Kominami *et al.* [24] reported a combined method of HPLC and enzyme immunoassay (EIA) for active metabolites of a benzodiazepine derivative. After the HPLC separation/fractionation of the active metabolites, the fractions were assayed by EIA. The replacement of EIA with the present receptor assay would provide higher selectivity for the pharmaceutical activities of metabolites, making the method more useful. Another approach to the use of interactions between the biotinylated benzodiazepine and the receptors would be affinity purification of γ-aminobutyric acid (GABA) receptors. It is well-accepted that GABA receptors are coupled with benzodiazepine receptors. Taguchi and Kuriyama [25] have already demonstrated the usefulness of 1012S as an immobilized ligand of affinity chromatography for the purification of GABA receptors. Because the preparation of 1012S-immobilized support could be easily obtained by using an

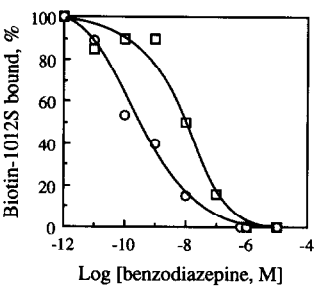

Fig. 7. Doze–response curves for clonazepam (○) and lorazepam (□).

avidin-immobilized support treated with biotin–1012S, these chromatographic applications would be achieved without any tedious support preparation processes.

In conclusion, the procedure for an avidin–biotin binding assay was simplified and made much faster by using biotin-labeled plates and a plate reader. The method is sensitive and was used to determine not only *d*-biotin but also a biotin–1012S conjugate, which is used as a probe ligand for the benzodiazepine receptor. Thus the method could also be applied to the receptor assay for benzodiazepine drugs. We expect that the receptor assay method will be widely used for the determination of benzodiazepine drugs in human serum and urine as well as for the drug development as an alternative method to radio-isotope labeled ligands.

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