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Drug Sensor: An Enzyme Immunoelectrode for Theophylline

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An enzyme immunoelectrode system for monitoring theophylline has been developed. The immunoelectrode was composed of an oxygen electrode and an antibody-coupled membrane. Anti-theophylline antibody was covalently immobilized on a nylon net with dimethyl sulfate, 1,6-hexanediamine, and glutaraldehyde. The reported method for obtaining antibody-coupled membrane was modified and the binding capacity of the membrane was characterized by using [8-^{3}H]theophylline as a tracer. The assay procedure involved the competitive immunochemical reaction of the membrane-bound antibody with catalase-labelled and nonlabelled theophylline. The amount of labelled theophylline bound specifically on the membrane was determined electrochemically from the reducing current of the oxygen generated enzymatically. Under optimum conditions, theophylline could be determined in the concentration range from 9.0 to 90 ng/ml (5×10^{-8} to 5×10^{-7} M). This immunoelectrode is highly specific for theophylline, and may be applicable as a drug sensor for clinical use.

Keywords——drug sensor; enzyme immunoelectrode; oxygen electrode; *O*-alkylated nylon net; antibody-coupled membrane; theophylline; catalase-labelled theophylline; immunoassay

The enzyme immunoelectrode system,[1] which combines the advantages of immunoassay and electrochemical determination, appears to be applicable to the assay of many drugs in clinical use. In a previous report,[2] we described an enzyme immunoelectrode system for insulin using insulin antibody coupled with acetylcellulose membrane and catalase-labelled insulin. However, the acetylcellulose membrane is fragile and not suitable for repeated use. To overcome this defect, we describe here the use of nylon net for immobilizing antibody and present a modified method for the quantitation of theophylline by the use of an enzyme immunoelectrode system.

Experimental

Materials——5,6-Diamino-1,3-dimethyluracil was purchased from Aldrich Chemical Company (Milwaukee, Wisc.), 7-(2,3-dihydroxypropyl)-theophylline, 3-methylxanthine, 1,3-dimethyluric acid, bovine serum albumin (BSA) and catalase were from Sigma Chemical Company (St. Louis, Mo.), theophylline, theobromine, glutaric anhydride, *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide were from Nakarai Chemicals, Ltd. (Kyoto, Japan), dimethyl sulfate, hexamethylenediamine, glutaraldehyde, 1,4-bis-[2-(5-phenyloxazolyl)]benzene (POPOP), 2,5-diphenyloxazole (DPO) and Triton X-100 were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), caffeine was from Kanto Chemicals Company, Inc. (Tokyo, Japan), hypoxanthine and uric acid were from Tokyo Kasei Kogyo Company, Ltd. (Tokyo, Japan), xanthine was from Kojin Company, Ltd. (Tokyo, Japan), and [8-^{3}H]theophylline was from the Radiochemical Centre (Amersham, England). Complete Freund's adjuvant was obtained from Iatron Laboratories (Tokyo, Japan). Sephadex G-25 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Nylon net was from Gunze Company, Ltd. (Tokyo, Japan).

Preparation of Antigen——8-(3-Carboxypropyl)-1,3-dimethylxanthine was synthesized according to the method of Cook *et al.*[3] A solution of 144.7 mg of 8-(3-carboxypropyl)-1,3-dimethylxanthine in 1.5 ml of *N*,*N*-dimethylformamide was treated with 62.6 mg of *N*-hydroxysuccinimide and 112.2 mg of DCC.[4] The mixture was stirred at room temperature for 1 h, then a freshly prepared solution of 200 mg of BSA in 10 ml of 0.03 M phosphate buffer (pH 7.0) was added. The mixture was maintained for 4 h at room temperature and then dialyzed for 24 h against the same buffer at 4°C. The 8-(3-carboxypropyl)-1,3-dimethylxan-

thine-BSA conjugate was introduced onto a column of Sephadex G-25 (2.8×40 cm) and the column was eluted with water. The conjugate was lyophilized. The extent of conjugation of 8-(3-carboxypropyl)-1,3-dimethylxanthine to the protein was determined by differential ultraviolet spectrophotometry (Erlanger *et al.*[5]) at 274 nm. The number of 8-(3-carboxypropyl)-1,3-dimethylxanthine residues per mole of BSA was about 18.

Immunization——Male New Zealand white rabbits were immunized with antigen. Antigen (2 mg) was dissolved in 0.9% NaCl solution (1 ml) and emulsified with an equal volume of Freund's complete adjuvant. The immunogen was given subcutaneously every 2 weeks. Blood was collected 4 months after the initial immunization. Immunological potencies were determined by measuring binding of [8-^{3}H]theophylline. A mixture of diluted serum solution (100 μl), [8-^{3}H]theophylline solution (50 μl), normal rabbit serum (100 μl), and 0.2 M Tris-HCl buffer pH 7.6 (250 μl) was incubated at 4°C for 24 h, then free ligand and antibody-bound ligand were separated by the addition of saturated ammonium sulfate solution (500 μl). After centrifugation at 1500×*g* for 30 min at 4°C, the precipitate was dissolved in 500 μl of distilled water. The solution was dissolved in 6 ml of scintillation fluid (6 g of DPO, 0.5 g of POPOP, 333 ml of Triton X-100, 667 ml of toluene) and the radioactivity was measured in a liquid scintillation counter (Beckman LS 7000). Final dilutions of antibody from 1: 800 to 1: 1300 could bind 50% of the [8-^{3}H]theophylline.

Preparation of Antibody-coupled Membrane——A modification Kageyama's method[6] was used to couple the enzyme with nylon membrane.

Forty pieces of nylon net (1×1 cm) were dipped into 100 ml of dimethyl sulfate for 5 min, then washed with 100 ml of ice-cold water for 10 min, and with 100 ml of ice-cold absolute ethanol for 10 min. The activated nylon nets were treated with 100 ml of 0.5 M hexamethylenediamine in water (pH 9.0) with moderate stirring for 3 h at room temperature. The pH was maintained at 9.0 by the addition of concentrated HCl. After termination of the reaction, the membranes were washed with 100 ml of 1 M NaCl and mixed with 100 ml of 12.5% glutaraldehyde solution in 0.05 M borate buffer (pH 8.5) with moderate stirring at 4°C for 30 min. The membranes were then taken out, washed with 100 ml of 0.1 M borate buffer (pH 8.5) and allowed to react with 50 mg of anti-theophylline antibody in 100 ml of 0.1 M borate buffer (pH 8.5) with moderate stirring for 24 h at 4°C. The membranes were washed with 100 ml of 0.1 M borate buffer (pH 8.5) and stored in the same buffer. A postulated scheme for the immobilization of antibody is presented in Fig. 1.

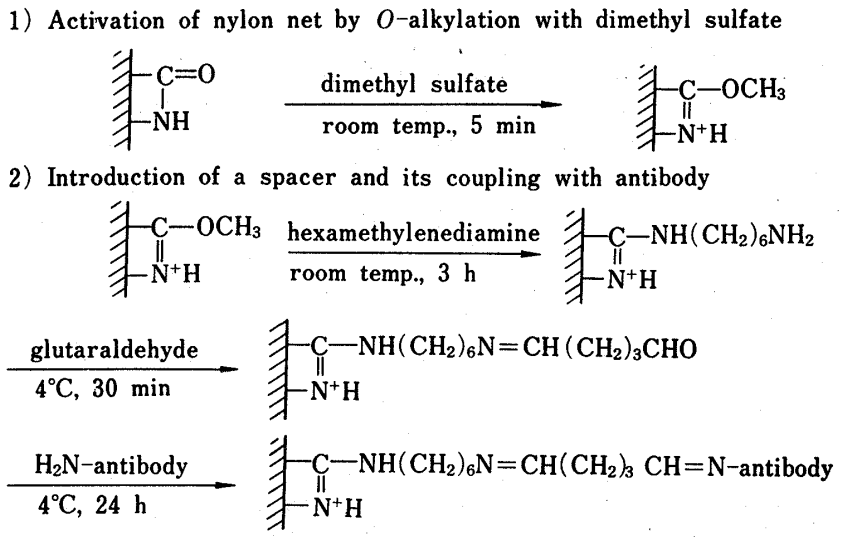

Fig. 1. A Postulated Scheme for Immobilization of Antibody onto Nylon Net Surface

Determination of the Binding Capacity of Antibody-coupled Membrane——A 100 μl aliquot of [8-^{3}H]-theophylline (20.3 Ci/mmol) (about 20000 dpm), 900 μl of buffered solution and then a piece of antibody-coupled membrane were placed in a test tube, and the mixture was incubated for a definite time at a definite temperature. The membrane was taken out, washed with 100 ml of 1 M NaCl, and wiped with a filter paper, then added to 6 ml of scintillation fluid (6 g of DPO, 0.5 g of POPOP, 333 ml of Triton X-100, and 667 ml of toluene). The radioactivity was measured in a liquid scintillation counter. The binding capacity of the membrane was determined from the following equation:

% bound theophylline = ([8-^{3}H]theophylline remaining)/(total [8-^{3}H]theophylline added) × 100

Under the optimal conditions, the amount of antibody coupled to the membrane was calculated from the specific activity.

Preparation of Catalase-labelled 8-(3-Carboxypropyl)-1,3-dimethylxanthine——A solution of 8-(3-carboxypropyl)-1,3-dimethylxanthine (106.4 mg) in 1.5 ml of *N*,*N*-dimethylformamide was treated with *N*-hydroxy-

succinimide (46 mg) and DCC (82.6 mg).[4] The mixture was stirred at room temperature for 1 h, then a solution of catalase (115 mg) in 20 ml of 0.03 M phosphate buffer (pH 7.0) was added. The whole was incubated at 4°C for 4 h and then dialyzed against the same buffer. The dialyzate was directly used as the catalase-8-(3-carboxypropyl)-1,3-dimethylxanthine conjugate solution.

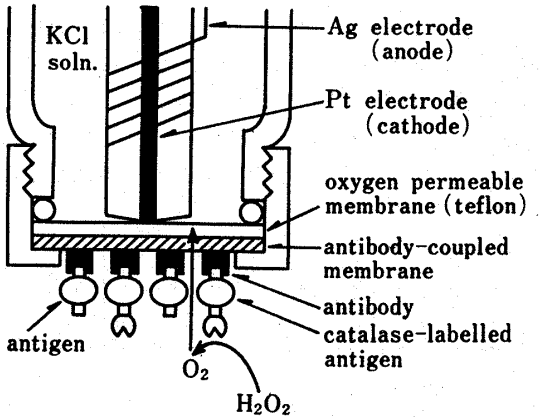

Fig. 2. Schematic Representation of the Enzyme Immunoelectrode and Its Mode of Action

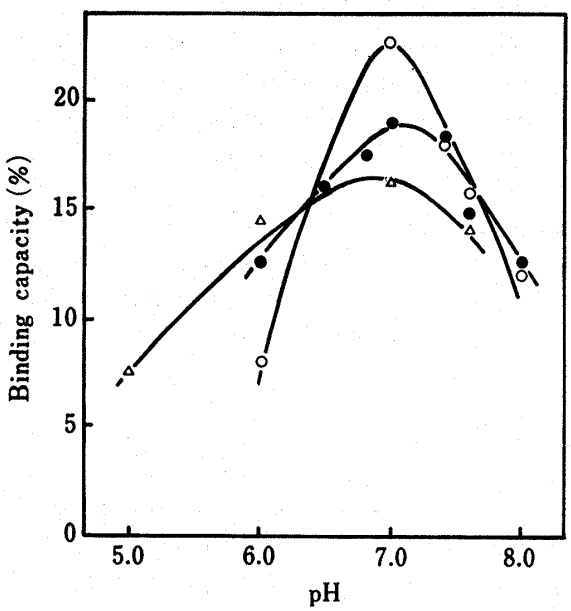

Fig. 3. pH-Dependence of the Binding in Various Media

A piece of antibody-coupled membrane was incubated with 100 μl of [8-^{3}H]theophylline and 900 μl of buffer for 24 h at 4°C
0.2 M Tris–HCl buffer (○),
0.1 M KH_2PO_4–0.05 M $Na_2B_4O_7$ buffer (●),
0.1 M Na_2HPO_4–0.2 M citric acid (△).

Immunoelectrode——The immunoelectrode is shown in Fig. 2. The electrode is composed of the antibody-coupled membrane and a handmade Clark-type oxygen electrode with an oxygen permeable Teflon membrane (Beckman P/N571649).

Assay Procedure——The Following components were placed in a test tube: 100 μl of sample solution, 800 μl of 0.1 M KH_2PO_4–0.05 M $Na_2B_4O_7$ buffer (pH 7.0), 100 μl of 10-fold diluted solution of catalase-labelled 8-(3-carboxypropyl)-1,3-dimethylxanthine, and finally a piece of antibody-coupled membrane. The reaction mixture was incubated at 4°C for 24 h, then the membrane was taken out, washed with 100 ml of 1 M NaCl and firmly fixed onto the Teflon membrane by means of a screw cap fitted with packing. The electrode was immersed in 10 ml of 0.03 M phosphate buffer (pH 7.0) and equilibrated against the reaction mixture, then 113 μl of 3% H_2O_2 was introduced. The output current was registered. From the initial current increase (di/dt), the concentration of theophylline was determined.

Results and Discussion

Properties of Antibody-coupled Membrane

By reaction with [8-^{3}H]theophylline, the amount of antibody coupled to the membrane was estimated to be about 1.6×10^{10} mol/cm^2.

Although antibody-coupled membrane can be prepared by direct reaction of antibody with *O*-alkylated nylon net,[7] such a membrane had a smaller binding capacity (data not shown) than that coupled through a spacer molecule and a cross-linking reagent as described in this experiment. Several reports have shown that the use of nylon net for the immobilization of enzyme[8] or antibody[9] is advantageous, and the newly developed membrane had excellent permeability to dissolved oxygen and was stable to handling.

Assay Conditions

The assay procedure using the enzyme immunosensor involves two steps, *i.e.*, the competitive immunochemical reaction of antibody-coupled membrane with catalase-labelled and nonlabelled theophylline, and the electrochemical determination of enzyme activity of the membrane-bound catalase.

To determine whether this antibody-coupled membrane possesses immunochemical reactivity, binding capacities were determined at various values of pH, incubation time, and incubation temperature using [8-^{3}H]theophylline as a tracer. Fig. 3 shows the immunochemical reaction of antibody-coupled membrane with [8-^{3}H]theophylline at various pH values for 24 h at 4°C. In each buffer solution, pH 7.0 was the optimum for the immunochemical reaction. Since the buffer capacity of 0.2 M Tris–HCl buffer is weak at pH 7.0, subsequent experiments were carried out in 0.1 M KH_2PO_4– 0.05 M $Na_2B_4O_7$ buffer at this pH.

The effects of incubation time and incubation temperature on the reaction are presented in Fig. 4. The reaction increased with incubation time in the range from 10 min to 24 h and

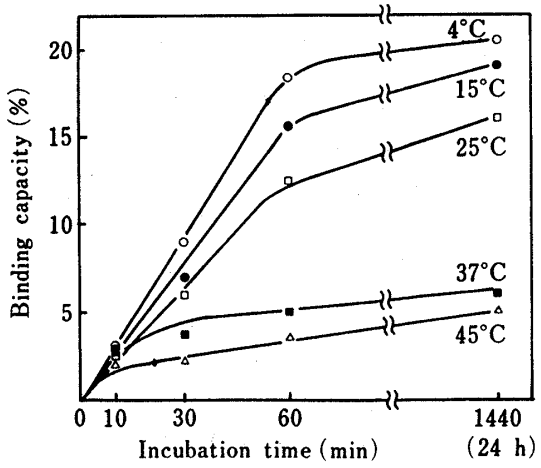

Fig. 4. Effect of Incubation Temperature on the Binding of Theophylline

A piece of antibody-coupled membrane was incubated with 100 μl of [8-^{3}H]theophylline and 900 μl of 0.1 M KH_2PO_4–0.05 M $Na_2B_4O_7$ buffer (pH 7.0).

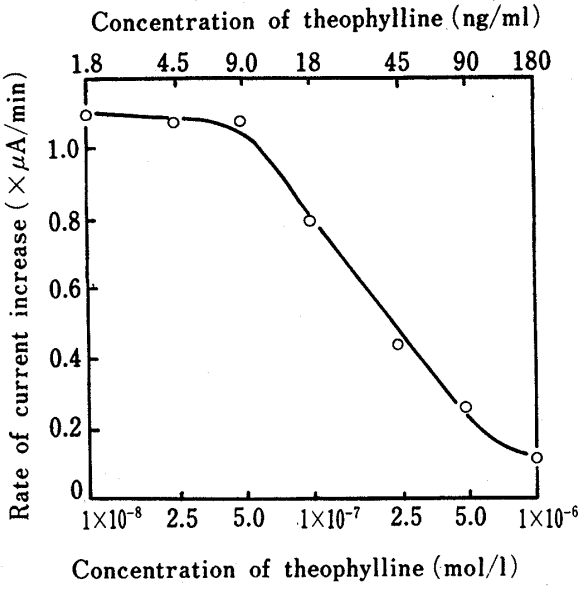

Fig. 5. Calibration Curve for Theophylline

A piece of antibody-coupled membrane was incubated in a mixture of theophylline standard solution (100 μl), 800 μl of 0.1 M KH_2PO_4–0.05 M $Na_2B_4O_7$ buffer (pH 7.0), and 10-fold diluted catalase-labelled 8-(3-carboxypropyl)-1,3-dimethylxanthine solution (100 μl) for 24 h at 4°C.

decreased with incubation temperature in the range from 4 to 45°C. At a low incubation temperature, the effect of incubation time is more marked. The maximum binding was obtained at 4°C for 24 h. The binding capacity after incubation for 1 h is about 90% of that at 24 h. For practical applications, an incubation time of 1 h is reasonable to determine the drug concentration in clinical samples. Thus, the standard assay conditions were defined as pH 7.0, incubation temperature 4°C, and incubation time 24 h.

Further, various conditions, including concentration of substrate, temperature, and pH, were tested for determination of the enzyme activity of membrane-bound catalase. The results were the same as those for catalase-labelled insulin in the previous report.[2] The standard assay conditions were defined as pH 7.0, temperature 30°C, and 10 mM H_2O_2 concentration.

Sensitivity of Assay

Under the conditions described above, the sensitivity of the assay was determined (Fig. 5). In the range from 5×10^{-8} to 5×10^{-7} M, the response was proportional to the logarithm of theophylline concentration. About 9.0 ng/ml could be detected with this electrode. In this experiment, the sample and antibody-coupled membrane were preincubated in a smaller volume (100 μl) than that of the previous report (2 ml),[2] so high sensitivity could be obtained.

Specificity

The specificity of the assay was tested with several analogs of xanthine and the concentrations required for 50% inhibition (IC_{50}) were determined (Table I). Caffeine, 7-(2,3-dihydroxypropyl)-theophylline, theobromine, xanthine, hypoxanthine, and uric acid are structurally related to theophylline, and 3-methylxanthine and 1,3-dimethyluric acid are metabolites of theophylline. Among these analogs, caffeine ($IC_{50}=3\times10^{-6}$ M) was the most effective inhibitor. The IC_{50} values were approximately 3×10^{-5} and 1×10^{-4} M for 7-(2,3-dihydroxypropyl)-theophylline and theobromine, respectively. The other xanthine derivatives were even less efficient competitors.

TABLE I. Specificity of the Enzyme Immunoelectrode

Compound	% cross-reactivity
Theophylline	100 (2×10^{-7} M)
Caffeine	6.6 (3×10^{-6} M)
7-(2, 3-Dihydroxypropyl)-theophylline	0.7 (3×10^{-5} M)
Theobromine	0.3 (1×10^{-4} M)
Xanthine	<0.01
Hypoxanthine	<0.01
3-Methylxanthine	<0.01
Uric acid	<0.01
1, 3-Dimethyluric acid	<0.01

Cross-reactivities were expressed as the concentrations required for 50% inhibition of the binding of theophylline (2×10^{-7} M).

Theophylline, which is used in the treatment of bronchospasm associated with both asthma and chronic obstructive lung disease, has also recently been suggested for the prevention of apnea and bradycardia in infants. The therapeutic response is related to the plasma theophylline concentration, which should be maintained within the relatively narrow range of 10 to 20 μg/ml.[10] At plasma levels above 20 μg/ml, serious side effects such as unpleasant gastrointestinal symptoms, vomiting, convulsion, and agitation are observed. Moreover, the low blood volume of infants and possible requirement for multiple monitoring in other patients mean that there is a need for sensitive analytical procedures.

For plasma theophylline determination, several methods such as spectrophotometry,[11] gas-liquid chromatography,[12] high performance liquid chromatography,[13] radioimmunoassay,[3,14] and enzyme immunoassay[15] are usually used. The classic spectrophotometric method developed by Schack and Waxler[11] requires 3 ml of plasma and is unsuitable for repeated measurements in small infants. Gas-liquid chromatography[12] and high performance liquid chromatography[13] require specialized instruments which are expensive and limited in availability. The principal advantage of immunological techniques (*i.e.* radioimmunoassay[3,14] and enzyme immunoassay[15]) are ease of sample handling, rapidity of analysis, sensitivity, specificity, and no pretreatment of the sample, but there are several disadvantage, including the use of radioisotope and the need for expensive equipment.

The enzyme immunoelectrode described here eliminates both the need for expensive equipment and the use of a radioisotope, and requires only a small amount of sample. Since the sensitivity of this method is equal to that of radioimmunoassay or enzyme immunoassay,

this enzyme immunoelectrode should be useful as a drug sensor for clinical use.

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