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Preparation of Anti-theophylline Antiserum and Determination of Theophylline in Patient Blood by Radioimmunoassay Method

TAKASHI NISHIKAWA, MASAYUKI SAITO,[1a] and HIROAKI KUBO[1b]

School of Medicine, Kitasato University[1a] *and School of Pharmaceutical Sciences, Kitasato University*[1b]

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A radioimmunoassay method has been developed for the determination of theophylline in body fluids. The anti-theophylline antibodies were obtained from the rabbits which were immunized with theophylline-bovine serum albumin conjugate. Standard curve for theophylline determination was linear on logit-logarithm graph in the concentration range of 3 to 50 μg/(ml of blood plasma). The plasma samples were collected from theophylline-receiving patients, and analyzed by the method. The values correlated well to those determined by the high-performance liquid chromatography (correlation coefficient 0.981, regression line $y=0.96x-0.06$). This method was accurate, sensitive, simple and suitable for routine assay of large numbers of samples.

Keywords——theophylline; anti-theophylline antiserum; therapeutic drug monitoring; radioimmunoassay; bronchodilator; xanthines

Introduction

Theophylline is commonly used in the treatment of acute and chronic obstructive airway diseases. The therapeutic response is related to the plasma theophylline concentration, which should be maintained within relatively narrow range of 10 to 20 μg/ml.[2,3] At plasma levels above 20 μg/ml, serious side effects such as unpleasant gastrointestinal symptoms, vomiting, convulsion and agitation are observed.[2,4] Therefore it is desirable to monitor plasma theophylline concentration for the safe and effective use of this drug. For the plasma theophylline determination, several methods such as spectrophotometry,[5,6] gas chromatography[7,8] and high-performance liquid chromatography[9,10] are usually used. However these methods have disadvantages in sensitivity, accuracy, simplicity or convenience. We have developed the radioimmunoassay method because this method is expected to be readily adaptable to routine assay of large numbers of samples and to offer advantages in specificity, sensitivity, and elimination of troublesome pretreatment.

Materials and Methods

Reagents and Instruments——Theophylline-8-^{14}C (specific activity, 210 μCi/mg; radiochemical purity, over 98%) was obtained from Radio Chemical Center in England. Phosphate buffer solution (1/15 M Na_2-

1) Location: *a*) *1, Asamizodai, Sagamihara, Kanagawa*; *b*) *5-9-1, Shirogane, Minato-ku, Tokyo.*
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HPO_4–KH_2PO_4, pH 7.4) was used for dilution. Dextran-coated charcoal suspension[11] was prepared by adding 250 mg of dextran T70 (Pharmacia Fine Chemicals) and 2.5 g of Norit A Charcoal (American Norit Co.) to 100 ml of phosphate buffer. The suspension was used to separate antibody-bound from free-theophylline. High-performance liquid chromatography (Waters Associates, Model ALC/GPC 204, with a detector of ultraviolet absorbance at 280 nm, μBondapak C_{18} column) was used as the reference method for the plasma and salivary theophylline determination. The chromatographic conditions and the procedure were:[10] at room temperature, elution solvent of 0.01 M sodium acetate buffer (pH 4.00)/acetonitrile (9/1), flow rate of 1 ml/min; the body fluid was mixed with methanol for deproteinization and centrifuged, then an aliquot of the supernatant was injected into the high-performance liquid chromatograph. Radioactivity was measured on the liquid scintillation counter (Aloka, LSC-602). Scintillation cocktail solution of Bush *et al.*[12] was used.

Preparation of the Antiserum——1. Synthesis of Theophylline-7-propionic Acid: Theophylline (500 mg) was allowed to react with 3-bromopropionic acid (500 mg) at 60° for 16 hr in 30 ml of alkaline solution adjusted to pH 10.5 with 1 N NaOH. The reaction mixture was adjusted to pH 6.5 with 6 N HCl, extracted with ethyl acetate, and then the aqueous layer was adjusted to pH 1.5 with 6 N HCl, extracted again with ethyl acetate. The extract of the organic solvent was evaporated to dryness. The residue was dissolved in water/methanol (5/1 by volume) mixture. By recrystallization from the water/methanol mixture, white crystals of theophylline-7-propionic acid (450 mg) were obtained. Chemical purity was established by thin-layer chromatography [developing solvent of *n*-butanol/acetic acid/water (10/1/1 by volume), on silica gel impregnated with fluorescent indicator] and by elemental analysis [Found (%): C, 47.59; H, 4.82; N, 22.09. Calcd. (%): C, 47.62; H, 4.79; N, 22.21]. The results of ultraviolet spectrum (molar extinction coefficient 1.0×10^4 at 272 nm), mass spectrum (MS), *m/e* ($M^+ = 252$) and nuclear magnetic resonance (NMR) spectrum established the chemical structure of the recrystallized compound.

2. Conjugation of Theophylline-7-propionic Acid with Bovine Serum Albumin: Theophylline-7-propionic acid (75 mg) was allowed to react with 450 mg of bovine serum albumin (BSA) to synthesize the conjugate (T-BSA) by the mixed anhydride method.[13] About twenty theophylline moieties per BSA were incorporated, which was determined by ultraviolet spectrophotometry. This conjugate (T-BSA) was used as immunogen. Theophylline-human serum albumin conjugate (T-HSA) and theophylline-rabbit serum albumin conjugate (T-RSA) were synthesized by the same method as T-BSA. These conjugates were used for the double immunodiffusion analysis described later.

3. Immunization of Rabbits with T-BSA: The solution of T-BSA (0.5 to 4 mg/ml of buffer solution) was emulsified with an equal volume of complete Freund's adjuvant. Each male rabbit was immunized with 1.0 ml of the emulsion, being injected subcutaneously in several sites at the back and at the foot pad A booster injection of 0.1 to 1 mg of T-BSA in the emulsion was given every two or three weeks.

Results

Antiserum Characterization

1. Double Immunodiffusion Analysis——Fig. 1 shows the precipitation pattern obtained by double immunodiffusion method on agar gel plate. The antiserum produced precipitation lines against BSA, T–BSA, T–HSA and T–RSA. The line of BSA made a partial fusion with the line of T–BSA. The line of BSA made a crossing with the line of T–RSA. The line of T–HSA made a fusion with the line of T–RSA. The line of T–BSA made a partial fusion with the line of T–HSA. The antiserum could not produce precipitation line against either HSA or RSA. These results indicated that the rabbit serum had both anti-theophylline antibodies and anti-BSA antibodies.

2. Affinity for Theophylline——The affinity constant of anti-theophylline antibodies was estimated by the Scatchard plot method.[14] After varying amounts of ^{14}C-theophylline were incubated with constant amounts of the antiserum at 4° for 18 hr, dextran-coated charcoal suspension[11] was added to separate antibody-bound from free ^{14}C-theophylline. The Scatchard plot was thus obtained at 1: 20 and 1: 40 antiserum dilutions in the immunoreaction mixture. As Fig. 2 shows, straight lines were obtained. The affinity constant of the

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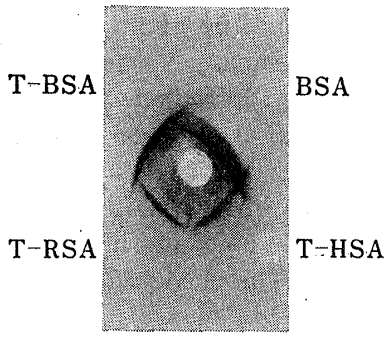


Fig. 1. Precipitation Pattern obtained by Double Immunodiffusion Method on Agar Gel Plate

The center well had been filled with the antiserum. Each well had been filled with as follows: upper right with BSA, upper left with T-BSA, down left with T-HSA and down right with T-RSA. The precipitation lines were stained with amide-black 10B (5 g/l).

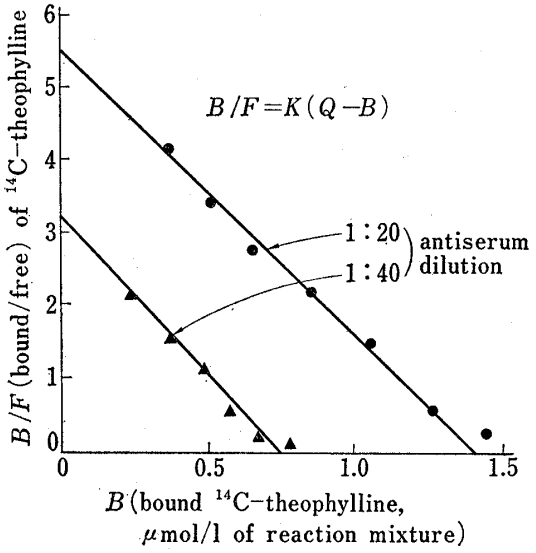


Fig. 2. Scatchard Plot obtained after Incubation of Varying Amounts of ^{14}C-Theophylline with Constant Amounts of the Antiserum

The antibody-bound ^{14}C to free ^{14}C ratio (B/F) was plotted against antibody-bound (B). Straight lines were obtained with constant antiserum dilutions of 1: 20 (—●—) and 1: 40 (—▲—). The intercept on B axis is Q which is the concentration of total antibody-binding sites, the intercept on B/F axis is KQ, when K is affinity constant.

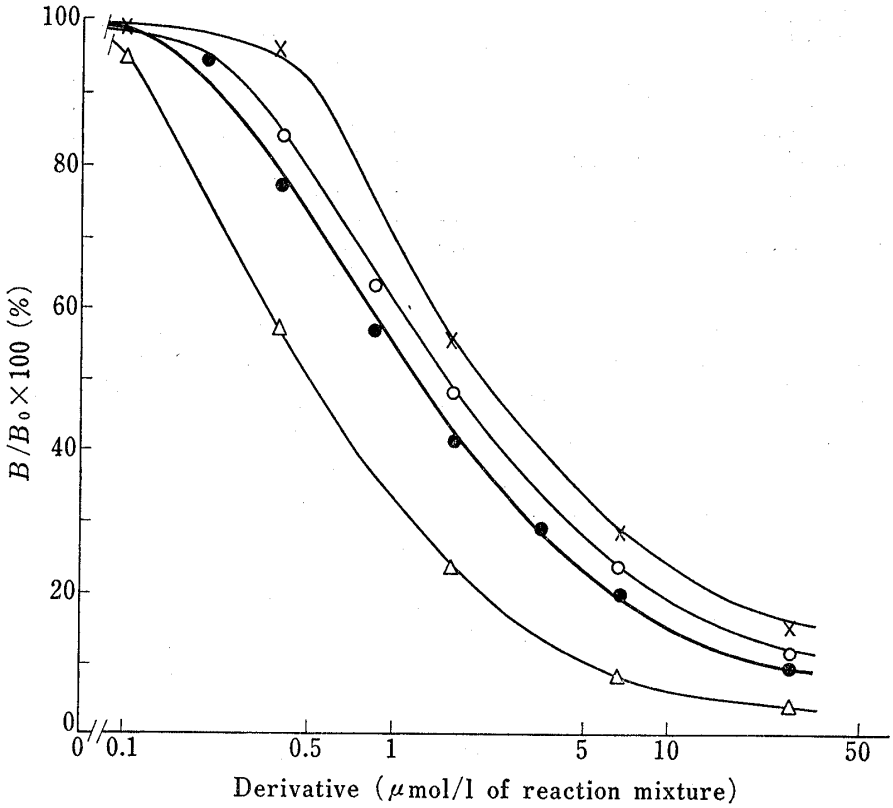


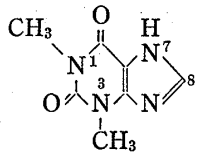
Fig. 3. Inhibition of Antibody-^{14}C-theophylline Binding by Xanthine Derivatives

The ratio of bound ^{14}C to zero-dose bound ^{14}C (B/B_0) was plotted against log-transformed concentration of xanthine derivative.
theophylline (—●—).
dyphylline (—×—).
7-(2-hydroxyethyl)theophylline (—○—).
theophylline-7-propionic acid (—△—).

antibodies and the concentration of total antibody-binding sites in antiserum were estimated to be 4.0×10^6 1/mol, 0.028 mmol/l of antiserum (at 1: 20 antiserum dilution), 4.3×10^6 1/mol, 0.029 mmol/l of antiserum (at 1: 40 antiserum dilution), respectively. The antisera of all the other rabbits gave similar values to those described above. The affinity constant was high enough for the determination of theophylline in patient plasma. Normal rabbit serum could hardly bind ^{14}C-theophylline, because less than 1% of the total radioactivity was found in the supernatant after the treatment of dextran-coated charcoal at the each serum dilution.

3. Cross-reactivity——In order to examine the cross-reactivity of the antibodies, some compounds were incubated with the antiserum in the presence of ^{14}C-theophylline and the inhibition of the antibody-^{14}C-theophylline binding was measured. Some inhibition curves and the cross-reactivities were shown in Fig. 3 and Table I. The theophylline derivatives

Table I. Relative Cross-Reactivity of the Antibodies with Xanthine Derivative



Theophylline (1,3-Dimethylxanthine)

Xanthine derivative	Position different from theophylline	Relative cross-reactivity
Theophylline	—	1.00
Theophylline-7-propionic acid	7-CH_2CH_2COOH	2.3
Caffeine	7-CH_3	1.9
7-(2-Hydroxyethyl)-theophylline	7-CH_2CH_2OH	0.87
Dyphylline	7-$CH_2CHOHCH_2OH$	0.53
8-Chlorotheophylline	8-Cl	0.002
Theobromine	1-H, 7-CH_3	0.001
3-Isobutyl-1-methylxanthine	3-$CH_2CH(CH_3)_2$	$<$0.001
Xanthine	1-H, 3-H	$<$0.001

Cross-reactivity was expressed as follows: molar ratio of theophylline dose to derivative dose, each required to inhibit 50% of antibody-^{14}C-theophylline binding.

which differ from theophylline at position 7 on xanthine ring, such as dyphylline and 7-(2-hydroxyethyl)theophylline, competed with ^{14}C-theophylline for the binding sites. It indicated that they had high affinity for the antibodies and that they could be also determined by the radioimmunoassay method. In contrast, the other derivatives which differ at position 1, 3, or 8 had extremely low affinity. This characteristic in cross-reactivity of the antibodies was understandable in that the conjugate of BSA with theophylline moiety at position 7 was used as immunogen.

Establishment of Theophylline Radioimmunoassay Method

1. Radioimmunoassay Procedure——Standard solutions or unknowns were diluted five-fold with the buffer solution. Then 50 μl of the diluted solution, 100 μl of ^{14}C-theophylline solution containing about 13000 dpm, and 100 μl of about 1: 16 diluted antiserum were pipetted into each assay tube and mixed. After incubation overnight at 4°, 200 μl of dextran-coated charcoal suspension[11] was added, the mixture was allowed to stand for 10 minutes at room temperature, and centrifuged at 3500 rpm for 10 minutes. The radioactivity in 250 μl of the supernatant was measured on the liquid scintillation counter.

Standard solutions were prepared by adding known amounts of theophylline to the pooled heparinized plasma. The radioactivity in antibody-bound fraction, *i.e.* not adsorbed on dextran-coated charcoal surface, increased rapidly as the immunoreaction proceeded, and then the immunoreaction reached equilibrium state. It took about 3 hr at room temperature

or about 7 hr at 4° to reach equilibrium state. Therefore the incubation time should be more than 3 hr or 7 hr at each temperature for the precise determination of theophylline. From the practical point of view, the immunoreaction mixture was incubated overnight at 4° in the subsequent experiments.

2. Standard Curve——Fig. 4 shows the typical standard curve. A linear response curve was obtained when logit (B/B_0), *i.e.* (bound in cpm/zero-dose bound in cpm), was plotted against logarithm theophylline concentration in the range of 3 to 50 μg/ml of plasma. Theophylline concentration in unknown sample was determined from the line on the logit–logarithm graph.

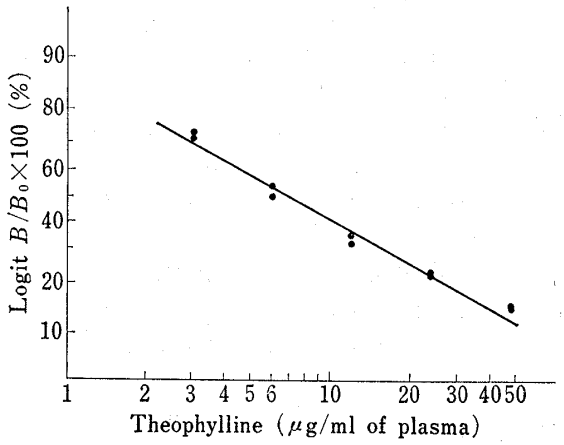


Fig. 4. Standard Curve for Theophylline Determination

Logit-transformed B/B_0, *i.e.* log $(B/B_0)/(1-B/B_0)$, was plotted against log-transformed theophylline concentration in pooled plasma. Duplicate determinations were made for each point.

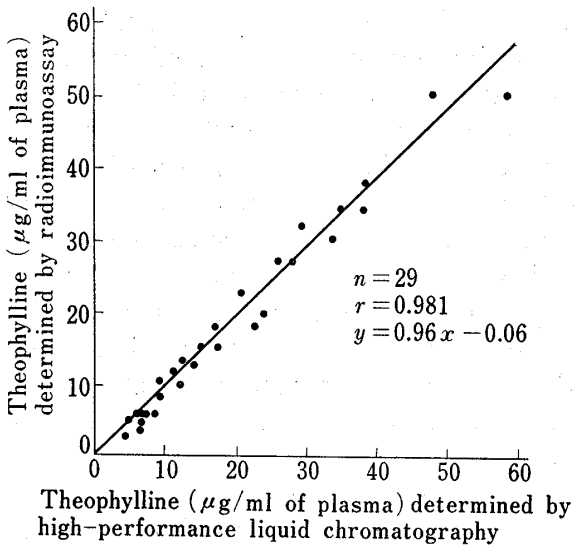


Fig. 5. Comparison of the Radiommunoassay with the High-performance Liquid Chromatography

3. Accuracy and Comparison of the Radioimmunoassay with the High-performance Liquid Chromatography——Within-run precision of the radioimmunoassay was estimated by analyzing the plasma samples containing theophylline at 10 to 30 μg/ml. The data showed that the precision was excellent with a coefficient of variation of 2 to 7%. The recovery tests were performed on the plasma containing uric acid at 590 μmol/l of plasma and the plasma containing bilirubin at 85 μmol/l of plasma. The results indicated that neither uric acid nor bilirubin interfered with the theophylline determination.

Theophylline concentrations in plasma samples from normal men with normal diets were lower than 3 μg/ml when they were determined by the radioimmunoassay method and also by the high-performance liquid chromatography. The 29 samples of plasma were collected from patients who had been intravenously infused with theophylline, and the plasma drug concentrations were determined by the radioimmunoassay method and by the high-performance liquid chromatography. The values were compared each other. As Fig. 5 shows, there was a good correlation between the values determined by these two methods. Correlation coefficient was 0.981 and regression line was expressed as $y=0.96x-0.06$.

For the determination of theophylline in saliva and in cerebrospinal fluid by the radioimmunoassay method, standard solutions were prepared by dissolving known amounts of theophylline in these body fluids and also in the buffer solution in a similar manner to the plasma standard solutions. All of these standard solutions gave the same standard curves. The result indicated that it was possible to determine theophylline in saliva and in cerebro-

spinal fluid by the radioimmunoassay method as well as in plasma, and indicated the absence of interfering substance in these body fluids. The nine samples of mixed saliva were collected from patients who had been intravenously infused with theophylline, and the salivary drug concentrations were determined by the two methods described above. There was also a good correlation between the salivary drug concentrations determined by the two methods. The salivary concentration range was about 3 to 15 μg/ml, correlation coefficient was 0.900 and regression line was expressed as $y=0.93x-0.10$.

Discussion

Generally immunoassay method has advantages and disadvantages over chromatographic method for therapeutic drug monitoring. The advantages are: possibility of serial adaptation to many samples, higher sensitivity, easiness of technique. The disadvantages are: unapparentness of interference when interference incidently occurs; difficulty to remove interference. Radioimmunoassay has disadvantage in that particular care must be taken in handling radioactive compound. However radioimmunoassay has advantages over non-radioisotopic immunoassay such as enzyme immunoassay in: enzymatic, biological or other physicochemical activity, with which drug is labeled, is sometimes affected whereas radioactivity is rarely affected; non-radioactivity-labeled drug has sometimes different affinity for the antibody from unlabeled drug in sample whereas radioisotopic effect is rarely seen. The radioimmunoassay method established in this work for therapeutic theophylline monitoring was sensitive, accurate and not interfered, and was suitable for routine assay of large numbers of samples.

Some theophylline derivatives, such as dyphylline and 7-(2-hydroxyethyl)theophylline, were used also in treatment of obstructive airway disease and these two drugs had high affinity for the antibodies as Fig. 3 and Table I shows. Therefore this method could be applied for the determination of these derivatives, though relationships between the blood concentration of these drugs and the clinical responses were not clearly known.

In blood of theophylline-receiving human, both free (not protein-bound) form and protein-bound form of theophylline are present.[15] Total theophylline concentration was determined by the radioimmunoassay method because the values correlated to those determined by the high-performance liquid chromatography which employed the procedure for total theophylline determination. In addition, the other result indicated the absence of interfering substance in normal plasma in which the drug-binding proteins were present. These results suggested that the antibodies had higher affinity for theophylline than the drug-binding proteins in human plasma, therefore total theophylline concentration was determined. Koysooko *et al.*[15] reported that there was an excellent linear relationship between theophylline concentrations in plasma and in saliva, and that the theophylline concentrations in saliva were about 48% lower than those in plasma and were equal to the concentration of free form theophylline in plasma. We also found that there was a linear relationship, however the concentration of theophylline in saliva were 60 to 90% of those in the paired plasma. Because we analyzed only nine paired (saliva/plasma) samples, a further investigation is now undertaken to define whether theophylline determination in saliva is useful for the safe and effective therapy.

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