[Chem. Pharm. Bull. 32(12)4951—4957(1984)]

Effect of Heterologous Combination on Competitive Nephelometric Immunoassay. I. Site-Heterologous Combination for Theophylline Immunoassay

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

School of Medicine, Kitasato University,[a] 1–15–1, Kitasato, Sagamihara, Kanagawa 228, Japan and School of Pharmaceutical Sciences, Kitasato University,[b] 5–9–1, Shirokane, Minato-ku, Tokyo 108, Japan

(Received March 2, 1984)

A competitive nephelometric immunoassay that is based on the inhibition of immunoprecipitation by a hapten was improved in specificity and in sensitivity for theophylline by the use of a heterologous combination of the assay reagents. Each of two theophylline derivatives, theophylline-7-propionic acid and theophylline-8-butyric acid, was conjugated to bovine serum albumin. These conjugates were injected into rabbits to raise anti-theophylline antiserum. Each derivative was also conjugated to human serum albumin. The competitive nephelometric immunoassay was performed by combined use of this human serum albumin conjugate with the antiserum. This immunoassay system allowed two homologous combinations and two heterologous combinations. Of these four combinations, the heterologous combination of the conjugate of (theophylline-7-propionyl)-(human serum albumin) with the antiserum against (theophylline-8-butyryl)-(bovine serum albumin) gave the most sensitive immunoassay for theophylline with the lowest interference by caffeine.

Keywords——precipitation inhibition; nephelometric immunoassay; heterologous combination immunoassay; theophylline; therapeutic drug monitoring

We have already reported a competitive nephelometric immunoassay for theophylline (1,3-dimethylxanthine).[1] Competitive nephelometric immunoassay is based on the inhibition of precipitation by a hapten. The majority of drugs can be haptens and thus can be determined by this assay method. Commercial kits utilizing this assay principle are now supplied to clinical laboratories for therapeutic monitoring of various drugs because this assay method has many practical advantages.[2–5]

We previously reported the characteristics of the antiserum against theophylline.[6] As described in that report, the antiserum was produced by using as an immunogen the conjugate of theophylline-7-propionic acid with bovine serum albumin. However, the antiserum strongly cross-reacted with caffeine (1,3,7-trimethylxanthine, or 7-methyltheophylline), probably because the immunogen was a 7-alkylated theophylline conjugate and caffeine is 7-methyltheophylline. Therefore caffeine interfered with our immunoassay of theophylline. This antiserum was not suitable for routine therapeutic monitoring of theophylline in the blood of patients because caffeine is present in some drinks and is sometimes present in human blood at relatively high concentrations. So, the interference by caffeine should be removed.

In label immunoassays such as radioimmunoassay and enzyme immunoassay, specificity and sensitivity could be improved when the hapten derivatives used for preparations of immunogens and labeled compounds differed slightly.[7,8] Homologous assays, in which identical derivatives were used for preparations of immunogens and labeled compounds, were sometimes less specific and less sensitive than heterologous assays.

Precipitation inhibition immunoassay, which includes competitive nephelometric immunoassay, is not a label immunoassay but allows homologous and heterologous combinations.

All of our previous competitive nephelometric immunoassays employed homologous combinations for drugs such as theophylline,[1] phenobarbital and phenytoin,[9] and carbamazepine.[10] We expected that competitive nephelometric immunoassay could also be improved by the employment of a suitable heterologous combination and that the interference of caffeine with our theophylline assay could be removed. Therefore we synthesized another theophylline derivative, theophylline-8-butyric acid. Since we had two theophylline derivatives, we could carry out heterologous combination immunoassay and examine the effects of the heterologous combination.

Materials and Methods

Details of the materials and methods were described in our previous reports.[1,6,9,10] Bovine serum albumin (BSA), human serum albumin (HSA), and human γ-globulin were purchased from Miles Laboratories Inc.

Preparations of the Antisera and Polyhaptenic Molecules——Theophylline-7-propionic acid was conjugated to BSA, and the conjugate (T-7-BSA) was used as an immunogen to raise anti-theophylline antiserum. This theophylline derivative was also conjugated to HSA, and the conjugate (T-7-HSA) was used as a polyhaptenic molecule for the precipitation inhibition immunoassay.

Theophylline-8-butyric acid was prepared by the reported method[11] with a slight modification. This theophylline derivative was conjugated to BSA and to HSA, and these conjugates (T-8-BSA and T-8-HSA) were used as an immunogen and a polyhaptenic molecule, respectively. These conjugations were performed by the mixed anhydride method[12] under the same conditions to couple equal numbers of molecules of the two derivatives onto albumin. The average incorporations of the theophylline derivatives were similar (18 to 22 mol/mol) as estimated by ultraviolet spectrophotometry. The conjugate of theophylline-8-butyric acid with human γ-globulin (T-8-G) was prepared by the use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Three male rabbits were immunized with each of these immunogens. The differences among the results given by the antisera from the three rabbits were small, and representative results are described below.

Radioimmunoassay Procedure——Theophylline-8-^{14}C (210 μCi/mg) was used as a labeled compound. Xanthine derivative solution (50 μl), theophylline-8-^{14}C solution (100 μl), and 20- or 25-fold diluted antiserum (100 μl) were mixed. The dextran-coated charcoal method was used to separate antibody-bound from antibody-unbound theophylline-8-^{14}C.

Competitive Nephelometric Immunoassay Procedure——The anti-theophylline antiserum was diluted 100- or 150-fold with a phosphate buffer (1/15 M Na_2HPO_4–KH_2PO_4, pH 7.4) which contained polyethylene glycol 6000 (25 g/l). The solution was allowed to stand for 40 min and filtered through a membrane (pore size 0.4 μm). The theophylline derivative solution in the phosphate buffer (50 μl), the polyhaptenic molecule solution in the phosphate buffer containing polyethylene glycol (100 μl), and the filtered antiserum solution (1.00 ml) were then added to a glass assay tube. A laser nephelometer (Hyland Laboratories Inc.) was used to measure the scattered light from the immunoprecipitate particles in the assay tube.

Results

Characterization of Antibodies by Double Immunodiffusion

The antisera were analyzed by double immunodiffusion on agar gel plates. T-8-G was used because γ-globulin has a quite different structure from that of serum albumin. These two antisera produced precipitation lines with BSA, indicating that the two antisera contained the antibodies against BSA (the hapten-carrier of the immunogen). Though HSA has a quite similar amino acid sequence to that of BSA, neither of the antisera produced any lines with HSA. Therefore HSA could be used as a hapten-carrier of the polyhaptenic molecule.

As Fig. 1 shows, the antiserum against T-7-BSA produced a line with T-7-HSA, and also with T-8-HSA and with T-8-G. These three lines fused completely with each other, showing a pattern of complete identity. In this antiserum, antibodies that would react with both theophylline-7-alkylated and theophylline-8-alkylated derivatives should be present.

As Fig. 2 shows, the antiserum against T-8-BSA also produced a precipitation line with each of these three conjugates. The line with T-8-HSA completely fused with the line with T-8-G, but the line with T-7-HSA incompletely fused with the lines with T-8-HSA and T-8-G,

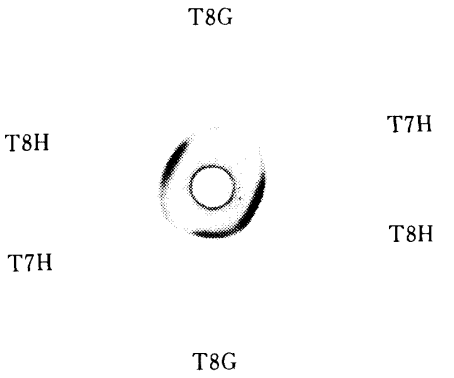

Fig. 1. Double Immunodiffusion Analysis of the Anti-(T-7-BSA) Antiserum

The center well was filled with the antiserum. The other wells were filled with T8G=(theophylline-8-butyryl)-(human γ-globulin), T7H=(theophylline-7-propionyl)-HSA, and T8H=(theophylline-8-butyryl)-HSA.

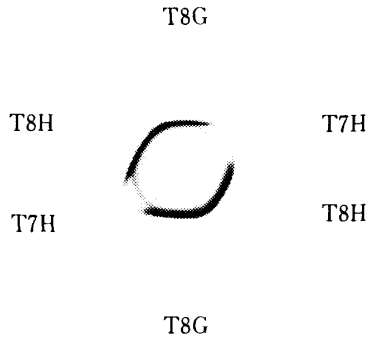

Fig. 2. Double Immunodiffusion Analysis of the Anti-(T-8-BSA) Antiserum

The center well was filled with the antiserum. The other wells were filled in a manner similar to that in Fig. 1.

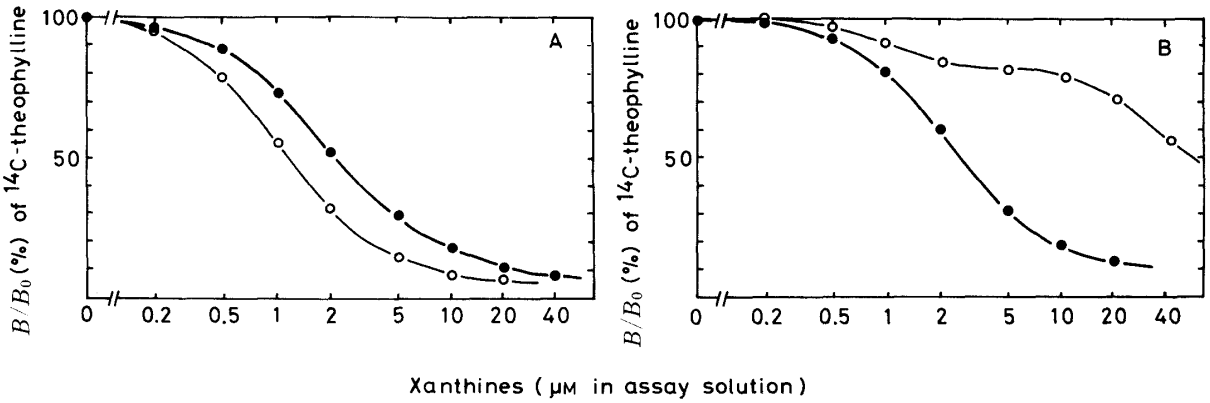

Fig. 3. Standard Curves for Radioimmunoassay of Theophylline and Caffeine

A: anti-(T-7-BSA) antiserum was used after 20-fold dilution. B: anti-(T-8-BSA) antiserum was used after 25-fold dilution.
●, theophylline; ○, caffeine.
B/B_0 = (^{14}C cpm at each dose)/(^{14}C cpm at zero dose) in the antibody-bound fraction.

showing a pattern of partial identity. Therefore, this antiserum should contain the antibodies that reacted with the two theophylline derivatives as well as the antibodies that reacted with only theophylline-8-alkylated derivative.

Characterization of Antibodies by Radioimmunoassay Methods

Figure 3A shows the standard dose-response curves obtained by use of the antiserum against T-7-BSA. The binding of ^{14}C-theophylline was inhibited by caffeine more extensively than by theophylline. Therefore the immunoassay using this antiserum was more sensitive to caffeine than to theophylline. The relative cross-reactivity (caffeine/theophylline, molar ratio) was estimated to be 1.83 by the method of 50% inhibition dose ratio. This antiserum was an anti-caffeine antiserum rather than an anti-theophylline antiserum. However, the immunoassay using the antiserum against T-8-BSA was more sensitive to theophylline than to caffeine (Fig. 3B). The relative cross-reactivity was as little as 0.05, so this antiserum was essentially an anti-theophylline antiserum. Other authors have reported similar cross-reactivity of antisera that were raised against theophylline-8-alkylated derivative.[11,13]

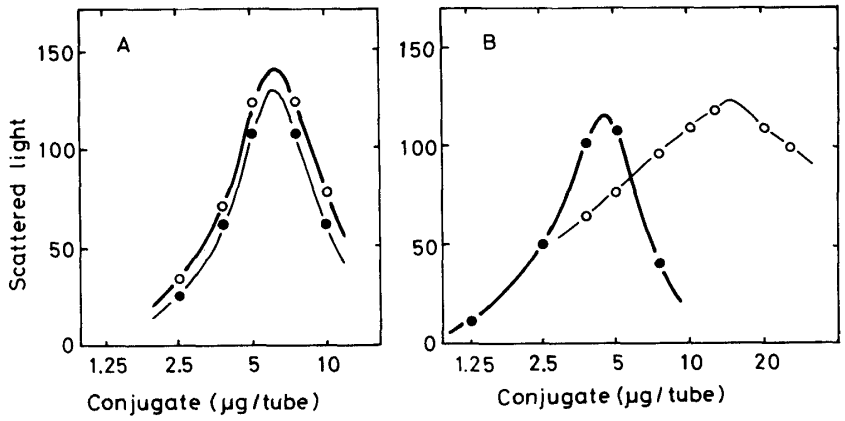

Fig. 4. Precipitation Curves of the Two Antisera

One ml of the diluted antiserum was allowed to react for 15 min with 100 μl of the conjugate solution. A: the reaction of the 100-fold diluted anti-(T-7-BSA) antiserum with the T-7-HSA (○) or T-8-HSA (●) conjugate. B: the reaction of the 150-fold diluted anti-(T-8-BSA) antiserum with the T-7-HSA (○) or T-8-HSA (●) conjugate.

Competitive Nephelometric Immunoassay

Various amounts of the polyhaptenic molecules were allowed to react with a constant amount of the antiserum in a glass assay tube, and the immunoprecipitates formed were quantitated at the steady state of the reaction (15 min) by nephelometry. Typical precipitation curves were obtained (Fig. 4). When the antiserum against T-7-BSA was used, the equivalent amount of T-8-HSA was similar to that of T-7-HSA. The maximum scattered light intensities were similar to each other. As the double immunodiffusion result showed, the same antibodies were involved in the immunoprecipitations of the two conjugates. When the antiserum against T-8-BSA was used, the equivalent amount of T-7-HSA was much greater than that of T-8-HSA. However the maximum scattered light intensities were similar to each other. As the double immunodiffusion result showed, some antibodies reacted with only one of the two conjugates, T-8-HSA.

The equivalent amount of the polyhaptenic molecule was allowed to react with the antiserum in the presence of 0.6 μM theophylline. When the antiserum against T-7-BSA was used, theophylline inhibited the precipitation of T-7-HSA by 40% and that of T-8-HSA by 30% (Fig. 5). When the antiserum against T-8-BSA was used, the inhibitions were greater. The precipitation of T-7-HSA was inhibited by 90%, and that of T-8-HSA was inhibited by 80% (Fig. 6).

Standard dose–response curves for theophylline and for caffeine were obtained with the four combinations of the assay reagents (Figs. 7 and 8). The cross-reactivities of (caffeine/theophylline) were estimated by the 50% inhibition dose method (Table I).

As Fig. 7 and Table I show, when the antiserum against T-7-BSA was used, caffeine inhibited the precipitations of the two polyhaptenic molecules more extensively than theophylline did. Therefore this antiserum should contain antibodies with a higher affinity for caffeine than for theophylline. This result coincided with that of the radioimmunoassay. The heterologous combination of the anti-(T-7-BSA) antiserum with the T-8-HSA conjugate (Fig. 7B) gave a more sensitive immunoassay for caffeine than did the homologous combination of the anti-(T-7-BSA) antiserum with the T-7-HSA conjugate (Fig. 7A). The heterologous assay had 1.4 times higher sensitivity and 1.4 times higher cross-reactivity for caffeine. Therefore the heterologous assay using this antiserum was more suitable for the determination of caffeine than the homologous assay.

As Fig. 8 and Table I show, when the antiserum against T-8-BSA was used, theophylline

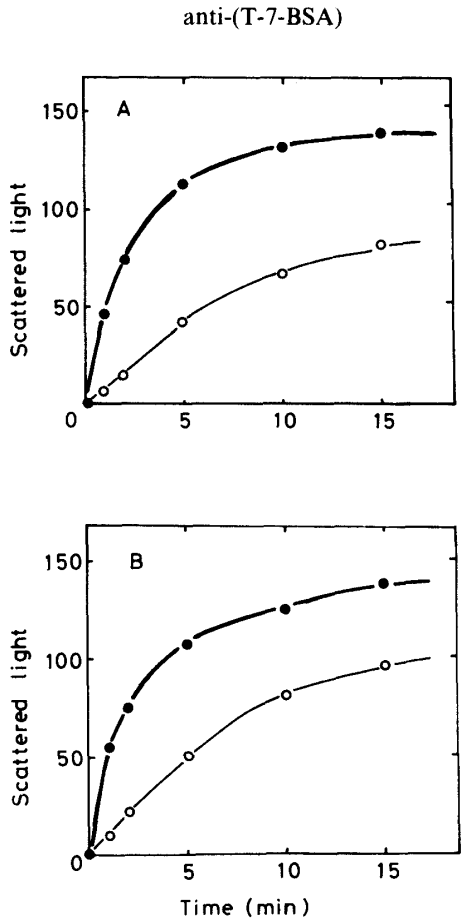

Fig. 5. Time Course of the Immunoprecipitation of the Anti-(T-7-BSA) Antiserum at the Theophylline Concentration of 0 μM or 0.6 μM in the Assay Mixture

A: the reaction with the T-7-HSA conjugate. B: the reaction with the T-8-HSA conjugate.
●, 0 μM theophylline; ○, 0.6 μM theophylline.

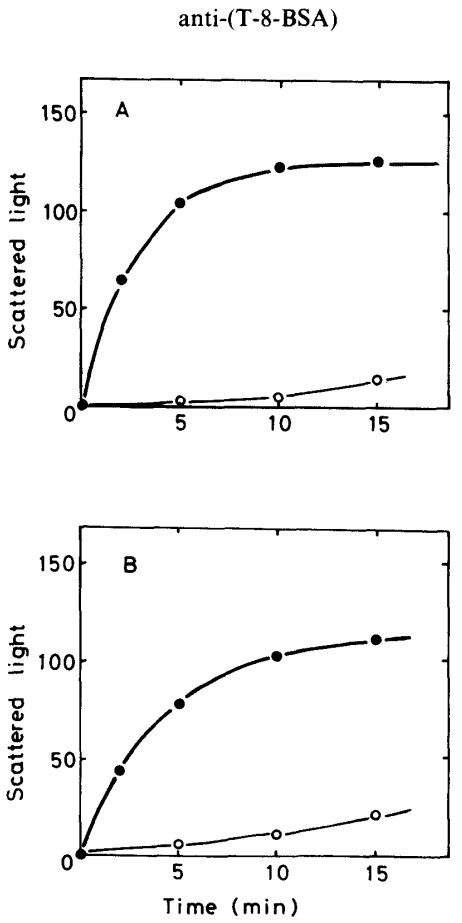

Fig. 6. Time Course of the Immunoprecipitation of the Antiserum against T-8-BSA at the Theophylline Concentration of 0 μM or 0.6 μM in the Assay Mixture

A: the reaction with the T-7-HSA conjugate. B: the reaction with the T-8-HSA conjugate.
●, 0 μM theophylline; ○, 0.6 μM theophylline.

inhibited the precipitations of the two polyhaptenic molecules more extensively than caffeine did. Therefore this antiserum should contain the antibodies that had a higher affinity for theophylline than for caffeine. This result coincided with that of the radioimmunoassay. The heterologous combination of the anti-(T-8-BSA) antiserum with the T-7-HSA conjugate (Fig. 8A) gave a more sensitive and more specific immunoassay for theophylline than did the homologous combination of the anti-(T-8-BSA) antiserum with the T-8-HSA conjugate (Fig. 8B). The heterologous assay had 1.6 times higher sensitivity for theophylline and 5.3 times lower cross-reactivity for caffeine.

As these curves show, both the heterologous combinations influenced not only the sensitivity and the specificity, but also the steepness of the curve. The heterologous assay gave steeper curves, which yielded more precise assay.

Discussion

We determined caffeine concentration in blood plasma from five volunteers by high-performance liquid chromatography.[1] After the subjects had taken one or two cups of coffee, the caffeine concentrations peaked within 0.5—2.5 h. All the peak concentrations were below

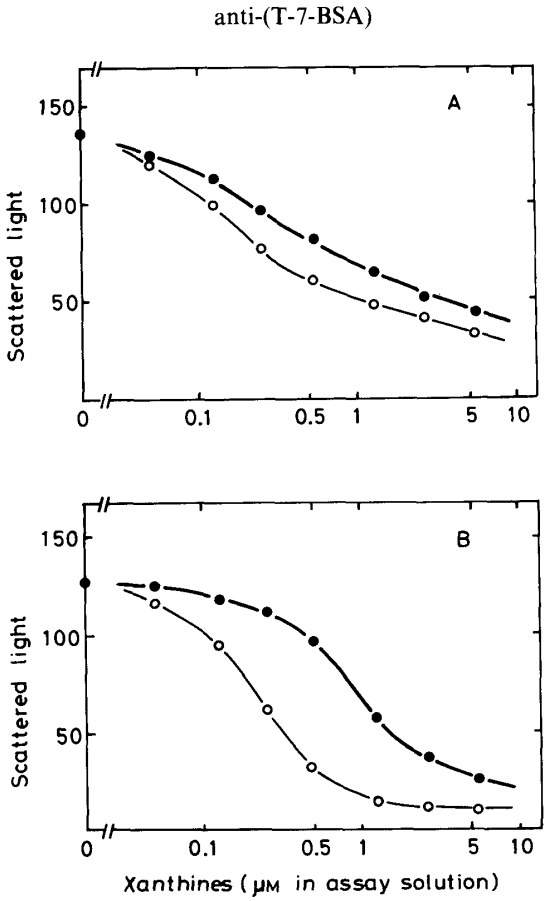

Fig. 7. Standard Dose–Response Curves for Competitive Nephelometric Immunoassay of Theophylline and Caffeine, Obtained with the Anti-(T-7-BSA) Antiserum

A: the reaction with the T-7-HSA conjugate. B: the reaction with the T-8-HSA conjugate.
●, theophylline; ○, caffeine.
The reaction time was 15 min.

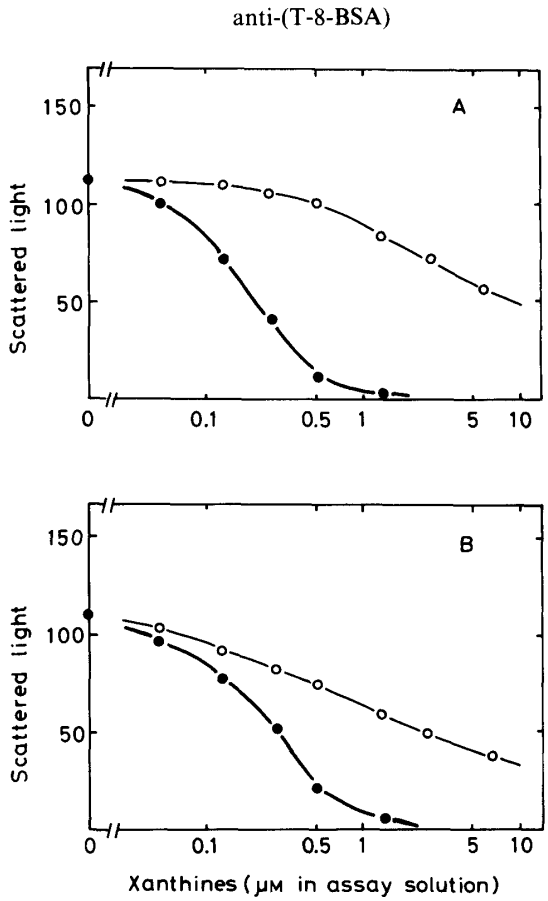

Fig. 8. Standard Dose–Response Curves for Competitive Nephelometric Immunoassay of Theophylline and Caffeine, Obtained with the Anti-(T-8-BSA) Antiserum

A: the reaction with the T-7-HSA conjugate. B: the reaction with the T-8-HSA conjugate.
●, theophylline; ○, caffeine.
The reaction time was 15 min.

Table I. Cross-Reactivities (Caffeine/Theophylline) Obtained with the Different Combinations of Assay Reagents

	Anti-(T-7-BSA)			Anti-(T-8-BSA)		
	50% inhibition dose		Cross-reactivity of caffeine to theophylline	50% inhibition dose		Cross-reactivity of caffeine to theophylline
	Caffeine (μM)	Theophylline (μM)		Caffeine (μM)	Theophylline (μM)	
T-7-HSA	0.34	1.0	2.9	6.0	0.18	0.03
T-8-HSA	0.25	1.0	4.0	1.75	0.28	0.16

1.5 μg/ml plasma. Other authors also reported that caffeine concentration in serum was usually much lower than the therapeutic theophylline concentration of 10—20 μg/ml for asthma.[13] Therefore if the homologous combination of the anti-(T-8-BSA) antiserum with the T-8-HSA conjugate is used for therapeutic monitoring of theophylline, the interference by caffeine would be clinically negligible, because the therapeutic concentration of theophylline is

usually much higher than caffeine concentration and the cross-reactivity with caffeine is sufficiently low.

The heterology type used in the present study was bridge-heterology as well as site-heterology. For derivatization, position 7 and position 8 were used, while for bridging, the propionyl group and the butyryl group were used. Because the difference between propionyl group and butyryl group is small, site-heterology should have a greater effect than the bridge-heterology in the assay improvement. The present combination was considered to be essentially site-heterologous.

As we have described, the heterologous combination gave higher sensitivity than the homologous combination. The reason is not clear, but may be as follows. Because the antigenic determinant included the bridge moiety as well as theophylline moiety, the antibodies against theophylline-8-derivative would have a strong affinity for T-8-HSA. Therefore theophylline could not effectively inhibit this strong binding. However, these antibodies had a weak affinity for T-7-HSA, so theophylline could effectively inhibit this weak binding. Therefore this heterologous combination gave higher sensitivity for theophylline. However it is not clear why the cross-reactivity and the steepness of the curve changed.

In recent years, monoclonal antibodies produced by hybrid cells have been used in some immunoassays in order to get high specificity. However this technique is rather sophisticated and it is sometimes difficult to select a suitable antibody-producing hybrid cell. In contrast, a heterologous combination assay system is easily developed and may be successfully applied to increase sensitivity and precision as well as specificity. Of course a heterologous combination using monoclonal antibodies might give a better assay performance.

References

1) T. Nishikawa, H. Kubo and M. Saito, *Clin. Chim. Acta*, **91**, 59 (1979).
2) P. R. Finley, J. A. Dye, R. J. Williams and D. A. Lichti, *Clin. Chem.*, **27**, 405 (1981).
3) M. Samoszuk, E. Kaplan and H. I. Finkle, *Therapeutic Drug Monitoring*, **5**, 113 (1983).
4) J. W. Wu, S. Hoskin, S. M. Riebe, J. E. Gifford and S. P. O'Neill, *Clin. Chem.*, **28**, 659 (1982).
5) J. W. Wu, C. Bunyagidj, S. Hoskin, S. M. Riebe, J. Aucker, K. White and S. P. O'Neill, *Clin. Chem.*, **29**, 1540 (1983).
6) T. Nishikawa, M. Saito and H. Kubo, *Chem. Pharm. Bull.*, **27**, 893 (1979).
7) B. K. Van Weemen and A. H. W. M. Schuurs, *Immunochemistry*, **12**, 667 (1975).
8) H. Hosoda, N. Kawamura and T. Nambara, *Chem. Pharm. Bull.*, **29**, 1969 (1981).
9) T. Nishikawa, H. Kubo and M. Saito, *J. Immunol. Methods*, **29**, 85 (1979).
10) T. Nishikawa and M. Saito, *J. Pharm. Dyn.*, **4**, 77 (1981).
11) C. E. Cook, M. E. Twine, M. Meyers, E. Amerson, J. A. Kepler and G. F. Taylor, *Res. Commun. Chem. Path. Pharm.*, **13**, 497 (1976).
12) B. F. Erlenger, F. Borek, S. M. Beiser and S. Lieberman, *J. Biol. Chem.*, **228**, 713 (1957).
13) T. M. Li, J. L. Benovic, R. T. Buckler and J. F. Burd, *Clin. Chem.*, **27**, 22 (1981).