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Effect of Heterologous Combination on Competitive Nephelometric Immunoassay. II. Bridge-Heterologous Combination for Ethosuximide Immunoassay

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We developed a competitive nephelometric immunoassay for the determination of ethosuximide. The method was based on the inhibition of immunoprecipitation by a hapten. Each of two ethosuximide derivatives, ethosuximide acetic acid and ethosuximide butyric acid, was conjugated to bovine serum albumin. These conjugates were injected into rabbits to raise anti-ethosuximide antisera. Each derivative was also conjugated to human serum albumin. These two conjugates were allowed to react with the two antisera in combination to form precipitates. Ethosuximide and its derivatives quantitatively inhibited these four precipitation reactions. The results indicated that the anti-(ethosuximide butyrate) antiserum recognized the bridge moiety better than the anti-(ethosuximide acetate) antiserum. Of the four combinations, the heterologous combination of the anti-(ethosuximide butyrate) antiserum with the (ethosuximide acetyl)-human serum albumin conjugate gave the most sensitive immunoassay for ethosuximide.

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

We have developed competitive nephelometric immunoassays of theophylline[1,2] phenobarbital and phenytoin[3] and carbamazepine.[4] The method is based on the inhibition of immunoprecipitation by a hapten. The precipitates in an assay solution are determined by measurement of the scattered light from the precipitates with a nephelometer.

Ethosuximide (2-ethyl-2-methylsuccinimide, molecular weight 141, ES) is a relatively small compound as an antigenic determinant. We thought that the competitive nephelometric immunoassay for ES might be insensitive because the antibody might have a weak affinity for a small compound such as ES. However, the sensitivity of a competitive nephelometric immunoassay could be increased by employment of a site-heterologous combination of the assay reagents, as we described in our previous report.[2] Therefore, we expected that the sensitivity for ES could also be increased by employment of a heterologous combination. We prepared two ES derivatives, of which one had an acetate group and the other had a butyrate group at the same position of ES, so we could make a bridge-heterologous combination.

Ethosuximide has been used as an antiepileptic drug. Blood plasma level monitoring of this drug can play an important role in the clinical management of the patients, and the therapeutic plasma level range is 40 to 100 μg/ml (285 to 710 μmol/l).[5]

Materials and Methods

Details of the materials and methods were described in our previous reports.[1–4]

Preparations of the Antisera and Polyhaptenic Molecules——*N*-Carboxymethylethosuximide (ethosuximide acetic acid, ES·C_2) and *N*-carboxypropylethosuximide (ethosuximide butyric acid, ES·C_4) were conjugated to bovine

serum albumin (BSA) by the mixed anhydride method.[6] Each of these conjugates (ES·C_2-BSA and ES·C_4-BSA) was injected into two rabbits to raise an anti-ethosuximide antiserum. The difference between the results given by the antisera from the two rabbits was small, so representative results are described below. ES·C_2 and ES·C_4 were also conjugated to human serum albumin (HSA). These conjugates (ES·C_2-HSA and ES·C_4-HSA) were used as polyhaptenic molecules for the nephelometric immunoassay.

Assay Procedure——The antiserum was diluted with a phosphate buffer (pH 7.4) in which polyethylene glycol had been dissolved at 25 g/l. The solution was allowed to stand for 40 min and filtered through a membrane (pore size 0.4 μm). The ethosuximide or other inhibitor solution (50 μl), the filtered antiserum solution (1.00 ml) and the polyhaptenic molecule solution (100 μl) were added in that order to a glass assay tube. The subsequent reaction was monitored with a laser nephelometer (Hyland Laboratories Inc.).

Results

Characterization of Antibodies by Double Immunodiffusion

The antisera were analyzed by double immunodiffusion on agar gel plates. These two antisera produced precipitation lines with BSA, though the lines were very faint (Figs. 1 and 2). Therefore these antisera contained antibodies to BSA (the hapten-carrier of the immunogen). Neither antisera produced any line with HSA. Therefore HSA could be used as a hapten-carrier of the polyhaptenic molecule. As Fig. 1 shows, the anti-(ES·C_2-BSA) antiserum produced lines with all the conjugates, ES·C_2-BSA, ES·C_2-HSA, ES·C_4-BSA and ES·C_4-HSA. The line of ES·C_4-HSA fused incompletely with the line of ES·C_2-HSA. Therefore this antiserum should contain antibodies that reacted with the two HSA conjugates as well as antibodies that reacted with only ES·C_2-HSA. The latter antibodies could recognize the difference between the acetyl and butyryl bridges. The result with the anti-(ES·C_4-BSA) antiserum is shown in Fig. 2. The line of ES·C_2-HSA fused incompletely with the line of ES·C_4-HSA. Therefore this antiserum should contain antibodies that reacted with the two HSA conjugates as well as antibodies that reacted with only ES·C_4-HSA. The latter antibodies could also recognize the difference between the acetyl and butyryl bridges.

Precipitation Curves

The anti-(ES·C_2-BSA) antiserum and the anti-(ES·C_4-BSA) antiserum were diluted 100- and 300-fold, respectively. The antiserum solutions were mixed with the ES·C_2-HSA or ES·C_4-HSA solutions in the manner described before. All of the four precipitation reactions at the equivalent dose reached equilibrium within 20 to 40 min. The nephelometric readings at 5 min were plotted and the precipitation curves were obtained (Fig. 3). The nephelometric readings at more than 5 min gave a less steep standard curve and a less sensitive immunoassay for ethosuximide.

Standard Curves

An equivalent amount of the polyhaptenic molecule was allowed to react with the antiserum in the presence of ES, ES·C_2 or ES·C_4. These three compounds inhibited the four precipitations (Fig. 4).

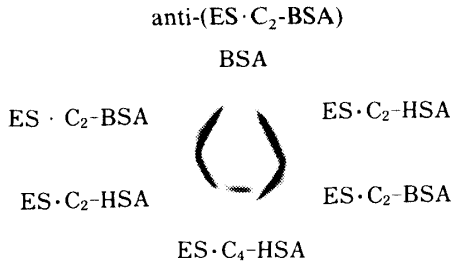

Fig. 1. Double Immunodiffusion Analysis of the Antiserum against ES·C_2-BSA

The center well was filled with the antiserum.

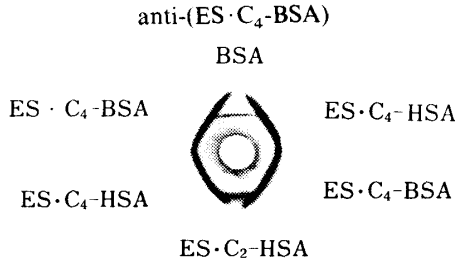

Fig. 2. Double Immunodiffusion Analysis of the Antiserum against ES·C_4-BSA

The center well was filled with the antiserum.

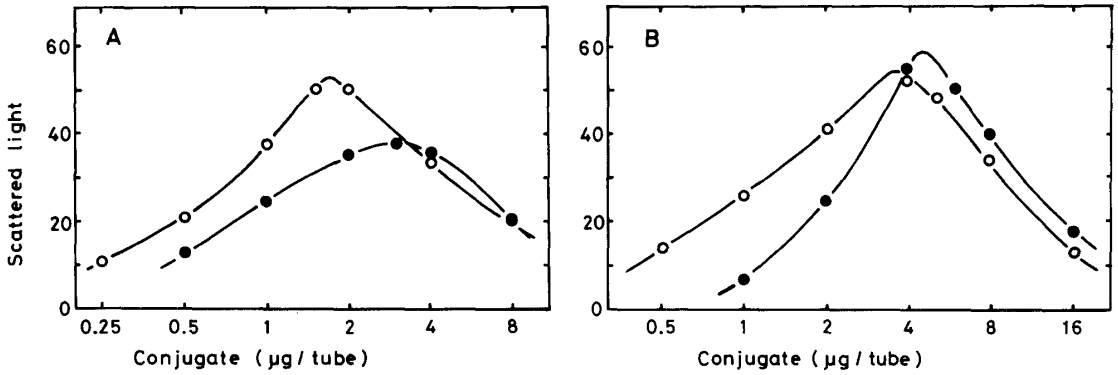

Fig. 3. Precipitation Curves

One ml of the diluted antiserum was allowed to react with 100 μl of the polyhaptenic molecule solution.

A: the reaction of the anti-(ES·C_2-BSA) antiserum with the ES·C_2-HSA conjugate (○) and the ES·C_4-HSA conjugate (●).

B: the reaction of the anti-(ES·C_4-BSA) antiserum with the ES·C_2-HSA conjugate (○) and the ES·C_4-HSA conjugate (●).

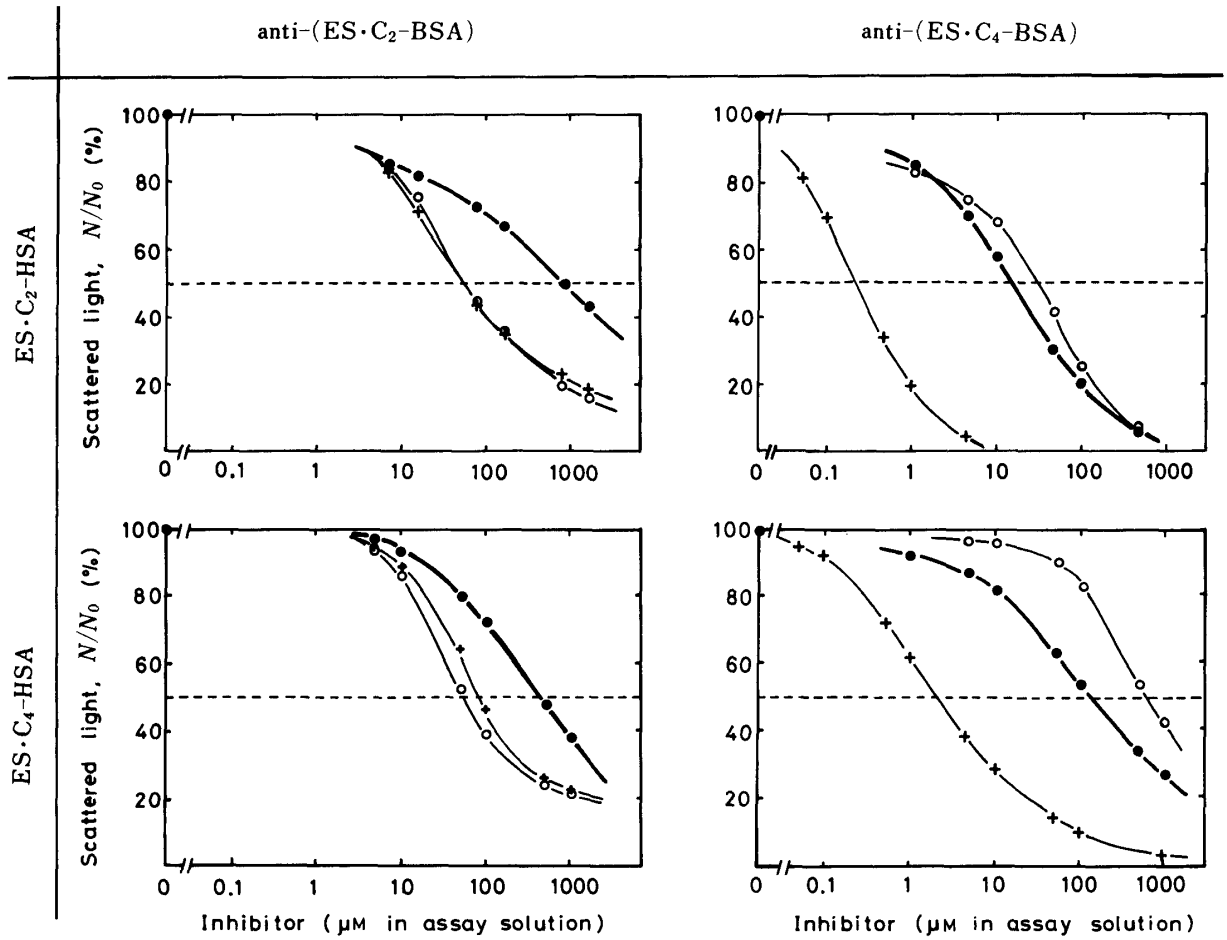

Fig. 4. Standard Curves

ES, ES·C_2, and ES·C_4 were used as inhibitors of the precipitations. N/N_0 = (scattered light at each dose)/(scattered light at zero dose).

(●), ES; (○), ES·C_2; (+), ES·C_4.

Left: the precipitation of the anti-(ES·C_2-BSA) antiserum with ES·C_2-HSA (above) and with ES·C_4-HSA (below).

Right: the precipitation of the anti-(ES·C_4-BSA) antiserum with ES·C_2-HSA (above) and with ES·C_4-HSA (below).

When the antiserum against ES·C_2-BSA was used (left in Fig. 4), ES·C_2 and ES·C_4 inhibited the precipitations more extensively than ES. The pattern obtained with the homologous combination of anti-(ES·C_2-BSA) and ES·C_2-HSA was similar to that obtained with the heterologous combination of anti-(ES·C_2-BSA) and ES·C_4-HSA. The effect of heterologous combination was not significant. Neither the homologous nor the heterologous immunoassay with the use of this antiserum was sensitive enough to determine the therapeutic plasma range of ethosuximide.

When the antiserum against ES·C_4-BSA was used (right in Fig. 4), ES·C_4 inhibited the precipitations most extensively, followed by ES and ES·C_2 in that order. The effect of the heterologous combination of this antiserum was significant. The sensitivity for ES was increased about 10-fold by employment of the heterologous combination as estimated by the method of 50% inhibition dose ratio. The therapeutic plasma range of ethosuximide could be determined by this heterologous combination immunoassay, though the accuracy was still relatively low. When plasma containing ethosuximide at 70 μg/ml (in the therapeutic range) was diluted 5-fold and the diluted solution was analyzed by this heterologous combination immunoassay, the inhibition was 40% and the coefficient of variation was 9.6%.

Discussion

Sensitivity for ethosuximide was increased by the employment of a suitable heterologous combination of the assay reagents. Of the four combinations tested, the anti-(ES·C_4-BSA) antiserum and the ES·C_2-HSA conjugate combination gave the highest sensitivity. The reason was thought to be as follows. The anti-(ES·C_4-BSA) antibody would have a high affinity for ES·C_4-HSA because the bridge moiety is the same, and a low affinity for ES·C_2-HSA because the bridge moiety is different. Therefore ES could not effectively inhibit the former high-affinity binding of the homologous combination. In contrast, ES could effectively inhibit the latter weak binding of the heterologous combination, which increased the sensitivity for ES.

The precipitation inhibition immunoassay of ethosuximide is distinct from those of other drugs in that the precipitation between the antibody and the conjugate occurred well but the precipitation was not so effectively inhibited by the drug even in the heterologous combination. Therefore the sensitivity for ethosuximide was low. However, ES·C_4 extensively inhibited the precipitation of the anti-(ES·C_4-BSA) antiserum, and the sensitivity for ES·C_4 was as high as those for the other drugs. The results suggested that ES and ES·C_2 were not large enough but ES·C_4 was large enough as an antigenic determinant. It is likely that the bridge moiety contributes greatly to the antigenic determinant when a hapten is as small as ethosuximide.

We previously reported the effect of a site-heterologous combination.[4] One of the site-heterologous assays was more sensitive for theophylline, while the other site-heterologous assay was more sensitive for caffeine. The site-heterology influenced not only the sensitivity but also the specificity and the steepness of the standard curve. The present report describes the effect of a bridge-heterologous combination. The bridge-heterologous assay was also more sensitive. Our results indicated that bridge-heterology has a smaller effect on the specificity and the steepness of the curve. However, it is sometimes difficult to synthesize two hapten derivatives with site-heterology. In contrast, it is usually easy to synthesize two hapten derivatives with bridge-heterology and to develop a bridge-heterologous assay system. We think that our results have general implications for hapten immunoassays which allow heterologous combination. Examples of such assays are radioimmunoassay, enzyme immunoassay,[7,8] fluoroimmunoassay and precipitation inhibition immunoassay.

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