Optimization of the DTPA Mixed-Anhydride Reaction with Antibodies at Low Concentration

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Diethylenetriaminepentaacetic acid (DTPA) was conjugated with antibody to human serum albumin (Ab) at low concentration (300 μg/ml, 2.0 μM) via the DTPA carboxycarbonyl mixed-anhydride method. To study parameters determining the balance between the degree of conjugation and the antibody-binding activity of Ab, a known concentration of the anhydride prepared at isobutylchloroformate (IBC)-to-DTPA ratios of 1, 2.1, or 4.2 was reacted with Ab. The percentage yields of the anhydride were determined by spectrophotometric and gravimetric titration. By the former method the percentage yields, based on DTPA concentration, were 18, 24, and 220, respectively, when the IBC-to-DTPA ratios were 1, 2.1, and 4.2. The corresponding percentage yields were 17, 39, and 262 when determined by the latter method. When the anhydride was prepared at an IBC-to-DTPA ratio of 2.1, an optimum conjugation giving three indium atoms per Ab was obtained, with 64% retention of antibody-binding activity. For an IBC-to-DTPA ratio of 1, the antibody retained almost 100% binding activity but the number of indium atoms incorporated (0.2) was too small. For an IBC-to-DTPA ratio of 4.2, up to 22 indium atoms were incorporated but antibody-binding activity was completely destroyed.

J Nucl Med 24: 932–936, 1983

The mixed-anhydride method via the reaction of isobutylchloroformate with DTPA is a convenient two-step method to conjugate DTPA to antibody. This procedure seems to be ideal for indirect labeling of antibody with a metallic radionuclide because the In-111-DTPA complex is quite inert to ligand exchange to transferrin, with an exchange rate of about 1% per day (*1*)*. It was used successfully to conjugate DTPA to human serum albumin (*2,3*) and antibodies (*4,5*) in high concentration (5–20 mg/ml). The conjugation, however, is not reproducible under the stated conditions when a practical concentration (300 μg/ml, 2.0 μM) of antibody was used, mainly because the conjugation reaction cannot compete efficiently with the hydrolysis reaction.

Received Sept. 7, 1982; revision accepted Apr. 28, 1983.

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The reproducible conjugation is very important when antibody is conjugated because antibody-binding activity is affected by the attached chelating agent. It is necessary, therefore, to react antibody with a known concentration of the DTPA carboxycarbonyl mixed anhydride. Heretofore, the percentage conversion yield to the anhydride has not been determined, thus making difficult the comparison of various studies using the mixed-anhydride approach. This study provides two methods for the determination of the anhydride concentration, thus enabling us to correlate the number of the chelating groups incorporated with the anhydride concentration.

MATERIALS

Antibody to human serum albumin (Ab) was obtained commercially.† It was purified using an affinity column containing Sepharose 4B conjugated HSA. DTPA,

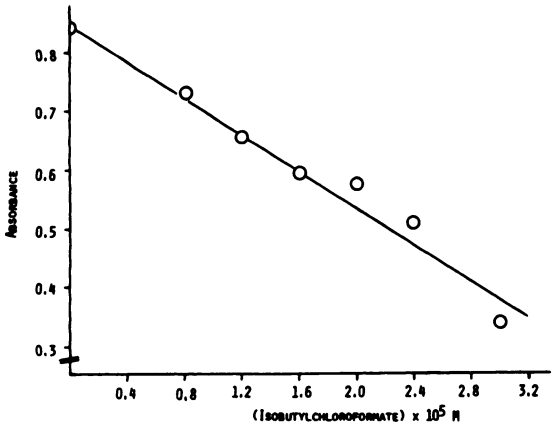

FIG. 1. Absorbance of N-(2,4,6-trinitrophenyl)-benzylamine plotted against concentration of isobutylchloroformate.

isobutylchloroformate, and triethylamine were also purchased.[‡] DTPA and isobutylchloroformate were used without further purification. Isobutylchloroformate was pure by NMR. Triethylamine was purified by the method of Swift (*6*). Acetonitrile was dried according to Muney and Coetzee (*7*). Human serum albumin (HSA fraction V, fatty-acid free) was purchased.[§] Monomeric HSA was isolated by a gel-filtration column containing Sephadex G-150. Commercial sources were used for human serum albumin (HSA fraction V, fatty-acid free)[§] and for CNBR-activated Sepharose 4B.

METHODS

1. Determination of isobutylchloroformate concentration. Isobutylchloroformate in seven different concentrations ranging from 0.0 to 1.5 m*M* was reacted with benzylamine (3.0 m*M*) in dry acetonitrile at 0°C for 1.5 hr. One hundred microliters of the solution was then reacted with 2,4,6-trinitrobenzenesulfonate (1.0 m*M*) in 5 ml of 0.1 *M* borate buffer (pH 8.3) for 1.5 hr at 23°C (*8*). The absorbance of N-(2,4,6-trinitrophenyl) benzylamine was measured at λ_{max} 420 nm and was plotted against the initial concentration of isobutylchloroformate to give a straight line (Fig. 1). This linear relationship was used to determine the unknown concentration of isobutylchloroformate, which was stored for some period of time.

2. Preparation of DTPA anhydride. A modified method of Krejcarek and Tucker (*2*) was used. DTPA (3.93 g, 0.01 mole) and triethylamine (7.0 ml, 0.05 mole) (*6*) were dissolved in dry acetonitrile (*7*) by stirring at 60°C for 1 hr. The 100 ml of solution was then cooled to room temperature. A 5 ml aliquot was put in a vial with a rubber stopper and cooled to 0°C. Isobutylchloroformate (0.50 mmol, 1.05 mmol, or 2.10 mmol) was then added to the solution. The reaction mixture was shaken well and let stand in an ice bath for 1.5 hr. The samples were frozen at −80°C until used.

3. Determination of the DTPA anhydride concentration. (*a*). *Spectrophotometric method.* Aliquots of the anhydride solution from the reaction of IBC and DTPA (0.1 *M*) at molar ratios of 1, 2.1, or 4.2 were reacted with an excess molar concentration of benzylamine (3.0 m*M*) in dry acetonitrile at 0°C for 3 hr. One hundred microliters of the solution was then reacted with 5 ml of 1.0 m*M* 2,4,6-trinitrobenzenesulfonate in 0.1 *M* borate buffer at pH 8.3 for 1.5 hr at 23°C. The optical density of the product, N-(2,4,6-trinitrophenyl)-benzylamine, was measured at λ_{max} 420 nm. The concentration of the anhydride was then obtained by an extrapolation method (Fig. 1).

(*b*). *CO_2 method.* The anhydride (0.5 ml from the IBC:DTPA ratios of 1 and 2.1, or 0.1 ml from the ratio of 4.2) was reacted with 1 ml of 0.1 *N* $Ba(OH)_2$ solution. The solution was quickly diluted to 20 ml with freshly distilled water. The container was covered with Parafilm to prevent absorption of atmospheric CO_2 and immediately titrated with 0.01 *N* HCl until the phenolphthalein turned from pink to colorless. The solution was stirred gently with a magnetic stirrer. The solution containing 0.5 ml or 0.1 ml of DTPA·5Et_3N (0.1 *M*) and 1 ml of $Ba(OH)_2$ was titrated with HCl and used as a blank (*9*).

4. Conjugation of DTPA to antibody. The known concentration of the anhydride from the reaction of IBC and DTPA at three different ratios (Table 3) was reacted with 300 μg (2 nmol) of Ab in 0.1 *M* borate buffer at pH 8.3 for 20 hr at 0°C. The reaction volume was 1 ml. Half the solution was cooled on an ice bath and acidified with 0.3 ml of 0.1 *M* citrate buffer at pH 5.3. The solution was then reacted with 0.1 ml of 0.1 *M* $InCl_3$ for 30 min, along with a tracer amount of In-111 in 0.05 *M* HCl. The pH of the reaction solutions was 4.0. Unconsumed indium ion was then complexed with 0.15 ml of 0.1 *M* DTPA in order to prevent the formation of insoluble indium hydroxide. The solution was immediately neutralized by addition of a 0.65 ml of 0.1 *M* borate buffer at pH 9.3. Half of the solution was then put on a 0.9 × 60 cm column containing 54 cm of Sephadex G-50 at the bottom and 3 cm of Sepharose 4B conjugated HSA on the top. The column was eluted with 0.02 *M* phosphate buffer/0.5 *M* NaCl at pH 7.6 to separate inactive antibody and unconjugated DTPA from active antibody. The active antibody was then dissociated with 0.2 *M* glycine·HCl/0.5 *M* NaCl at pH 2.8. Active and inactive antibody were detected by a uv monitor and recorded on chart paper. The percentages of active and inactive antibody were then calculated from the peak intensities. The number of indium atoms incorporated into each antibody fraction was calculated based on the indium-111 activity associated with the fraction. The affinity column used for this study was washed before the experiment with 100 ml of 0.02 *M* phosphate buffer at pH 7.6 containing 300 mg of transferrin and 5 mmol of

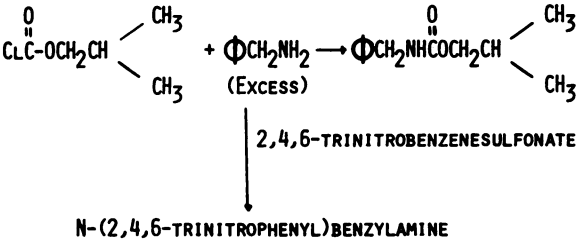

FIG. 2. Determination of isobutylchloroformate concentration.

DTPA in order to block nonspecific binding sites of the column for proteins, and also to eliminate metallic ions in the column. The column was then eluted with the glycine buffer and neutralized before the samples were eluted. Upon blocking the nonspecific binding sites, we were able to reproduce the same peak intensity for 2.0 μM Ab within 5% error limit.

RESULTS

1. Determination of isobutylchloroformate concentration. Isobutylchloroformate is thermally unstable and also is inactivated by hydrolysis. Therefore it is necessary to measure the percent activity of isobutylchloroformate in order to obtain quantitative data on the conjugation studies. We used an indirect spectrophotometric method to determine the percent activity of isobutylchloroformate.

The absorbance of N-(2,4,6-trinitrophenyl)benzylamine was inversely proportional to the concentration of isobutylchloroformate up to the molar ratio of benzylamine-to-isobutylchloroformate of two. This linear relationship (Fig. 1) was used to determine the concentration of isobutylchloroformate that was stored for a prolonged time. TLC (silica gel) of the product solution from the reaction of the chloroformate with benzylamine showed only one product spot when it was developed with chloroform ($R_f = 0.53$) or 5%:95% methanol:chloroform ($R_f = 0.87$). This indicates that one mole of ϕCH_2NH_2 was consumed.

2. Determination of the DTPA anhydride concentration. The results from the spectrophotometric and CO_2 methods are very similar except for the anhydride from the reaction at the molar ratio of 2.1. They are shown in Tables 1 and 2. The CO_2 method is preferable because of its simplicity. The percentage yields of the anhydride at the reaction time of 1.5 hr are 17, 39, and 262 when IBC-to-DTPA ratios are 1, 2.1, and 4.2, respectively. The percentage yield of 260 is equivalent to 2.6 anhydride per DTPA. The yield diminishes with duration of reaction time. We kept the reaction time at 1.5 hr in order to ensure the complete disappearance of IBC. The absence of IBC in the reaction solutions from the molar ratio of 1 and 2.1 was confirmed by the absence of its benzylamine derivative upon the reaction with benzylamine. The solution from the molar ratio of 4.2, however, showed a small spot on TLC corresponding to the benzylamine derivative of isobutylchloroformate.

TABLE 1. PERCENTAGE YIELD OF THE DTPA ANHYDRIDE DETERMINED BY THE SPECTROPHOTOMETRIC METHOD

Isobutylchloroformate: DTPA ratio	Percentage yield of anhydride*
1.0	18.3 (10–28)
2.1	23.9 (18–32)
4.2	220 (200–270)

* The percentage yield was calculated based on the concentration of DTPA and is an average of four to ten values, with range in parentheses.

3. Conjugation of DTPA to Ab. Among the anhydrides we prepared, the one from the reaction of isobutylchloroformate and DTPA at 1:1 molar ratio is the least efficient conjugating agent. The reaction of this anhydride with Ab (300 μg/ml, 2 μM) at molar ratio of 870 gave rise to only 0.2 indium atoms per antibody, although the antibody binding activity remained intact. We did not increase the ratio further because the reaction solution for the molar ratio of 870 contained 10% acetonitrile, and further increase of its concentration might denature the Ab. The anhydride from IBC-to-DTPA ratio of 4.2 was the most efficient in the conjugation of DTPA, but it caused complete deactivation of the Ab-binding activity. The anhydride that gave the optimum conjugation was that from the molar ratio of 2.1. When the anhydride and Ab at the molar ratio of 1170 were reacted, DTPA conjugated per active Ab determined by the indium complexation method was 3. This conjugation reduced the binding activity to 63.6% from 87.4%. Our control experiments showed that 10% acetonitrile did not deactivate Ab. Ab was quite stable and was not deactivated by the reaction conditions, such

TABLE 2. PERCENTAGE OF THE DTPA ANHYDRIDE DETERMINED BY THE CO_2 METHOD

Isobutylchloroformate: DTPA ratio	Percentage yield of anhydride*
1.0	17.4 (12–25)
2.1	39.1 (29–56)
4.2	262 (240–285)

* The percentage yield was calculated based on the concentration of DTPA and is an average of four to eight values, with range in parentheses.

TABLE 3. EFFECT OF CONJUGATION OF DTPA ON ANTIBODY-BINDING ACTIVITY

IBC: DTPA ratio	Anhydride:[a] Ab ratio	Active Ab		Nonactive Ab	
		%	#In/Ab	%	#In/Ab
—	—	87.4[b]	—	12.6[b]	—
1.0	522	86.0 (84.1–87.9)	—	14.0 (12.1–15.9)	1.6 (1.0–2.3)
1.0	870	83.2 (81.9–84.5)	0.2 (0.1–0.3)	16.8 (15.5–18.1)	1.2 (1.2–1.3)
2.1	195	81.4 (79.9–84.0)	0.5 (0.4–0.6)	18.6 (16.0–20.1)	1.3 (0.9–1.6)
2.1	1173	63.6 (60.1–68.8)	3.1 (3.0–3.2)	36.4 (31.2–39.9)	3.3 (2.4–4.2)
2.1	1955	50.4 (49.8–51.0)	3.6 (3.0–4.1)	49.6 (49.0–50.2)	4.6 (4.3–4.9)
4.2	1310	—	—	100	9.2 (8.6–9.7)
4.2	7860	—	—	100	19.3 (18.0–21.6)
4.2	13100	—	—	100	22.1 (15.0–22.5)

[a] Anhydride concentration was measured by the CO_2 method. Concentration of Ab was 300 μg/ml (2.0 μM).

[b] Percentage of active or nonactive antibody in the stock antibody solution. Data are average numbers of triplicate experiments, with ranges in parentheses.

as pH 8.3 and incubation at room temperature for 20 hr.

DISCUSSION

Antibody-binding activity is affected greatly by attached foreign molecules. It is very important, therefore, to know the concentration of the reactive anhydride in order to optimize the balance between the degree of the DTPA conjugation and the antibody-binding activity of the antibody. The advantage of the DTPA mixed anhydride is that its conjugation reaction takes place fast even at a low antibody concentration because the anhydride is very reactive. The problem with the DTPA anhydride is that it is thermally unstable and reacts with water too rapidly to be isolated for identification. Nevertheless, this anhydride method has been used by a number of investigators without detailed investigation of the anhydride concentration or the effects of the IBC-to-DTPA ratio on the antibody-binding activity (*4*,*5*).

They reported successful conjugations of DTPA to antibodies when high antibody concentrations (5 mg/ml) were used and the anhydride was prepared at an IBC-to-DTPA ratio of 1. The conjugation was more difficult, however, when a practical antibody concentration (300 μg/ml) was used, mainly because the conjugation reaction could not compete effectively with the hydrolysis reaction (*10*). This necessitated a more detailed investigation of this method.

We chose two indirect methods, the spectrophotometric method and the CO_2 method, to determine the concentration of the anhydride. Both methods rendered the determination of the concentration within 25% error limit. The CO_2 method, however, is preferable because of its simplicity. The percentage yield obtained from the spectrophotometric method is somewhat smaller than those from the CO_2 method, probably because of hydrolysis side reactions with a minute amount of water in acetonitrile even though the solvent was dried.

The reaction of the antibody with the known concentrations of the anhydride enabled us to obtain reproducible conjugations, as shown in Table 3. Among three DTPA anhydrides, the one from the IBC-to-DTPA ratio of 2.1 gave rise to an optimum conjugation, producing three indium atoms incorporated per antibody, with 64% retention of antibody-binding activity. The anhydride from the IBC-to-DTPA ratio of 4.2 gave the highest conjugation but caused an extensive deactivation of the antibody. This deactivation might have been caused by inter- and intramolecular cross-linking of Ab, because the reaction with an IBC-to-DTPA ratio of 4.2 produced DTPA mixed anhydride containing an average 2.6 anhydride per DTPA (Table 2). Another contributing factor for the deactivation seems to be the reaction of Ab with a small amount of IBC that was left unreacted after anhydride formation. The anhydride from the IBC-to-DTPA ratio of 1 was the least efficient conjugating agent.

This study provides two methods for the determination of the DTPA anhydride, thus the number of the incorporated DTPA molecules can be related to the anhydride concentrations. With this information, investigators can be assured of a reproducible bifunctional chelate for the radiolabeling of antibodies.

FOOTNOTES

* Volkert WA, Sohn M, Paik C, et al: unpublished results. We obtained a slower exchange rate than that reported previously in *J Radionanal Chem* 57:553-564, 1980. The discrepancy was probably caused by fluctuation of pH and aged serum used in the previous experiment.

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§ Sigma Chemical Company, St. Louis, MO 63178.

ACKNOWLEDGMENT

We thank Ronald Simon for his technical assistance. This work was supported in part by Grant No. CA 28462 awarded by the National Cancer Institute, DHEW. We also thank New England Nuclear for supplying the In-111 used in this project.

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