SYNTHESIS OF DIETHYLENE TRIAMINE PENTAACETIC ACID (DTPA) CONJUGATED ANTIBODIES.

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SUMMARY

Antibodies labelled with radioisotopes via chelating agents are being used increasingly for in vivo localisation or radiotherapy of tumours. The most widely used chelating agent is DTPA. Here, we describe technical modifications of the standard DTPA cyclic anhydride conjugation procedure and determine the in vitro stability of the DTPA-antibody conjugates. The use of this derivative of DTPA combined with the use of appropriate buffers and small disposable gel filtration columns resulted in the development of a simpler and more rapid technique for the production of DTPA-conjugated antibodies.

Keywords: DTPA conjugation, antibodies.

INTRODUCTION

A procedure for covalently attaching DTPA to antibodies has been described (1), but several technical problems associated with the preparation of the conjugates have not been properly addressed. These involve the separation of free DTPA from the antibody conjugate and maintenance of freedom from contaminating metal ions in the synthesis and radiolabelling steps (2). Problems in separating DTPA from proteins arise from the multivalent negative charge on DTPA in neutral

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solutions which lead to strong ionic association with the protein. The radiolabelling solutions need to be kept scrupulously free from metal ion impurities because contamination results in low labelling efficiencies. This paper describes how these problems may be overcome, taking advantage of the unusual elution properties of free DTPA on gel chromatography, and using the weak chelating agent dithizone for extraction of cold metal ions from preparatory buffers.

EXPERIMENTAL.

All preparative gel filtration was carried out using 9 ml Sephadex G 25 columns (PD 10, Pharmacia).

Gel-chromatography of [^{14}C] DTPA and [^{65}Zn] DTPA complexes on Sephadex G 25.

a) DTPA was labelled with ^{65}Zn (1 μCi). 2.5M acetate pH6 (6 μl) and 0.01M DTPA (50 μl) were mixed in a clean 2 ml plastic tube. [^{65}Zn] zinc chloride (2 μl, 1 μCi) was added and the mixture was left for at least 15 mins at room temperature. It was then fractionated on PD 10 columns equilibrated and eluted with water, acetate buffers at pH 6, or 0.15M saline. Fractions (0.5 ml) were collected and counted in a Gamma Set 500 scintillation counter.

b) [^{14}C] DTPA (0.5 μCi, 50 μl) was fractionated on PD 10 columns equilibrated in water or 0.15M saline. Fractions (0.5 ml) were collected, scintillation fluid (10 ml) was added and the fractions counted in a Beckman beta counter.

Extraction of metal ions from buffers with dithizone.

a) Concentrated buffers were tested for the presence of polyvalent metal ions using dithizone (diphenylthiocarbazone - Sigma; (3)). Buffers (200 ml) in a 500 ml separating funnel were shaken with dithizone (4 mg) in redistilled chloroform (3 ml). If a positive test

resulted, ie, a pink coloration in the organic layer, redistilled chloroform (10 ml) and dithizone (8 mg) in chloroform (2 ml) were added. The mixture was shaken, and the layers allowed to separate. A blue organic layer indicated substantial freedom from polyvalent ions.

b) The chloroform layer was separated, and the aqueous layer re-exracted with chloroform (10 ml) until both the chloroform and aqueous layers were colourless.

c) The buffer was tested for metal ions as above. If a pink colour remained, step b) was repeated until the buffer was negative for polyvalent metal ions.

DTPA conjugation of antibodies using the cyclic anhydride method.

a) The cyclic anhydride was suspended in dry dimethyl sulphoxide at 10 mg/ml, and appropriate volumes of this mixture used to provide the required quantity of anhydride. DTPA cyclic anhydride (ca. 200 μg) was placed in a clean 2 ml plastic tube. Polyclonal sheep anti carcinoembryonic antigen (CEA) (10 mg) in bicarbonate (1 ml; 0.05M, pH 7) was added and the mixture agitated for 2 min at room temperature.

b) In order to determine the efficiency of conjugation of DTPA to the antibody, a 50 μl aliquot of the reaction mixture was removed, and 2.5M acetate buffer pH 6 (10 μl) was added, followed by [^{65}Zn] zinc chloride (1 μCi). Incubation proceeded for 15 mins at room temperature to allow chelation of the ^{65}Zn by antibody-bound and free DTPA (pK = -18.7).

c) The trace-labelled aliquot was fractionated on a PD 10 column. Each fraction was counted in a gamma scintillation counter. The efficiency of conjugation of DTPA to antibody was determined by summing the counts in the protein peak and the DTPA peak.

d) The original reaction mixture was fractionated on another PD 10 column, and the third and fourth 1 ml fractions were collected. A further 20 μl aliquot of the fourth fraction was removed, trace-labelled with 1 μCi of ^{65}Zn, and fractionated as before. The

protein eluted in a single peak in the seventh 0.5 ml fraction which was collected and radiolabelled as previously described (2).

Efficiency of DTPA-anhydride conjugation after storage.

The efficiencies of conjugation of DTPA to antibodies were observed over several months after opening a vial of the cyclic anhydride, the compound having been stored at -20°C in a sealed container over dry silica gel. DTPA to antibody molar ratios in the reaction mixture were 5 or 10 to 1.

Immunoreactivity of the DTPA-conjugated antibody.

The conjugated antibodies were checked for reactivity with anti sheep IgG by immunoelectrophoresis, and for reactivity with CEA by double immunodiffusion in agarose gels.

Tests for covalent linkage of DTPA to antibody and stability of metal ion/DTPA bond.

a) Precipitation.

After radiolabelling with metal ions, the stability of the (ion)DTPA-protein bonds was determined by precipitation of the protein with saturated ammonium sulphate over a range of pH. Sheep IgG (50 µl at 10 mg/ml) to aid protein precipitation was added to ^{65}Zn labelled antibody (10 µl). Saturated ammonium sulphate, pH 5.3 (5 ml) was then added dropwise. Sheep serum (100 µl) was added to ^{65}Zn-labelled antibody (10 µl), followed by saturated ammonium sulphate pH 7 (5 ml). Precipitated protein was centrifuged at 2,500 g for 10 mins, the clear supernatant aspirated, and both the pellet and supernatant counted. This was repeated for ^{111}In-labelled antibody, and further repeated using saturated ammonium sulphate at pH 9.0.

The protein and supernatant fractions were obtained and counted.

b) Dialysis.

The long-term stability of ^{111}In-labelled antibody in various

buffered solutions was determined by dialysis. ^{111}In-labelled antibody was prepared and fractionated on a PD 10 column. The antibody was diluted in saline containing 2 mg/ml bovine serum albumin to an activity of 32,000 cpm/ml as recorded on a Packard gamma counter. The following solutions were prepared: 0.15M sodium chloride, 0.15M sodium acetate, pH 6, 0.15M EDTA, pH 4.7 and 0.15M tris, pH 10. Each of the solutions was supplemented with 2 mg/ml bovine serum albumin. Volumes (2 ml) of the radiolabelled antibody were pipetted into sealed dialysis tubing, and then placed in 10 ml plastic tubes containing one of the four solutions (6 ml) above. There were 3 tubes for each solution. After careful sealing, the tubes were fixed to a bench rotator and mixed for 24 hours. The contents of each dialysis bag was removed, placed in a plastic tube and counted on a Packard gamma counter.

c) Serum incubation.

In order to check the stability of the indium ion on chelate-linked antibody after incubation in human serum, radiolabelled antibody was fractionated on Sephacryl S 300. Immediately after preparation of ^{111}In-labelled antibody (specific activity > 13 mCi/mg), 5 µl (5 µCi) of the protein was mixed with PBS and fractionated on a Sephacryl S 300 column (27 x 3 cm) equilibrated with PBS using an LKB Superrac fraction collector. Fractions (2 ml volumes) were collected every 5 minutes and counted on a Packard gamma counter. The same radiolabelled preparation (10 µl) was added to fresh human serum (2 ml). Volumes (1 ml) of the serum were fractionated as above on Sephacryl S 300 after 5 min and 24 hours of incubation and counted in a Packard gamma counter.

RESULTS

Elution of DTPA complexes on Sephadex G 25.

Results of eluting ^{14}C-labelled DTPA in NaCl or water on PD 10 columns are shown in fig. 1. Results of the elution of ^{65}Zn complexes are summarised in table I.

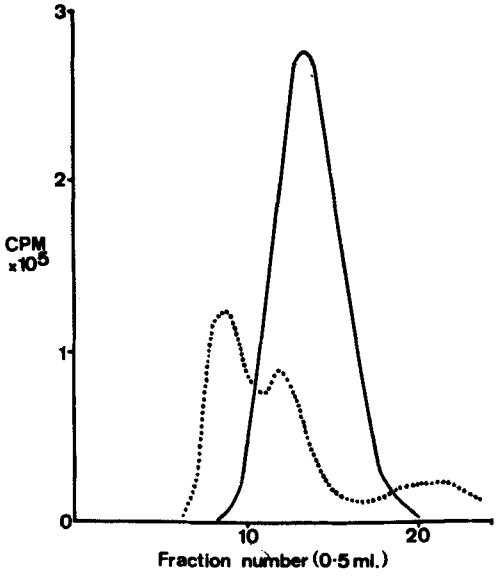

Figure 1. Elution profiles of [^{14}C] DTPA on PD 10 columns eluted with water (••••••) and 0.15M saline (——).

Table I. Elution of DTPA complexes on Sephadex G25 columns (PD 10).

No. moles of DTPA	eluting buffer	Main elution peak (0.5 ml fraction no.)
5 x 10^{-7}	0.15M saline	12
5 x 10^{-10}	"	12
0 (^{65}Zn only)	"	28
5 x 10^{-7}	water	8
"	0.25M acetate	12 - 13
"	0.01M acetate	10
"	0.001M acetate	8

The main observation from the elution data was that DTPA and its complexes did not elute the same way in all buffers. The elution volume of the DTPA depended on the concentration of the buffer (identical results with 0.25M acetate and 0.15M saline - the elution peak being in the 12th 0.5 ml fraction). In triple distilled water, DTPA and its complexes eluted in the fractions expected for radiolabelled proteins (ie, in 0.5 ml fractions 6 to 8).

Buffer extraction with dithizone.

All buffers were effectively freed from contaminating metal ions using the solvent extraction method with dithizone, as judged by visual assessment of colour changes in the organic layer. The method reduced metal ion contamination to below 1 x 10^{-10}M (4).

Conjugation of polyclonal sheep anti CEA with DTPA cyclic anhydride and antibody purification.

Trace labelling of the DTPA/antibody mixture with ^{65}Zn followed by fractionation on a PD 10 column in 0.15M acetate indicated a very efficient separation of DTPA-antibody conjugate from free DTPA (fig.2). The antibody conjugate eluted mainly in 0.5 ml fractions 7 to 8, while free DTPA eluted in fractions 9 - 18. Ratios of the counts in the protein and free DTPA elution peaks indicated a conjugation efficiency of approximately 65% for fresh anhydride and antibody concentration of 10 mg/ml (3.5 DTPA per antibody). No residual radioactivity was found on the column.

Having shown that the mixture could be fractionated under appropriate conditions of ionic strength, it was eluted on a PD 10 column. Protein eluted in 0.5 ml fraction 8. Trace-labelling of protein (100 μg) with ^{65}Zn (20 μCi), followed by re-elution on PD 10 showed that 100% of the radioactivity was associated with the protein fraction. (See fig.2, lower profile).

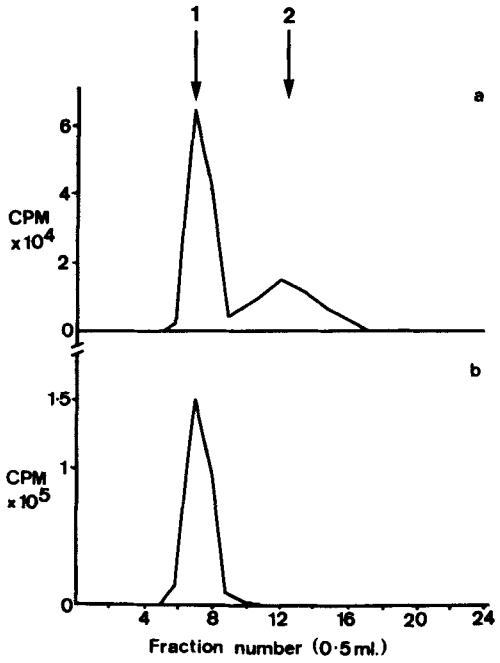

Figure 2. a) An anti CEA / DTPA cyclic anhydride reaction mixture trace labelled with ^{65}Zn and fractionated on a PD 10 column equilibrated with 0.25M acetate, pH 6. Peak 1: DTPA-protein. Peak 2: free DTPA. b) DTPA-anti CEA trace labelled after purification on a PD 10 column equilibrated with 0.25M acetate, pH 6.

Effect of storage time on conjugation efficiencies.

The effect of storage time on conjugation efficiency with the cyclic anhydride is shown in table II. The labelling efficiency decreased markedly after opening of the vial and storage over silica gel at -20°C.

Table II. Effect of storage time on antibody conjugation efficiencies with DTPA cyclic anhydride.

Storage time	Antibody:DTPA anhydride ratio	Efficiency (%)
0	1:5	48 - 65
> 1 month	1:5	26
> 2 months	1:10	4
> 4 months	1:10	3

Characterisation of DTPA-conjugated antibody and antigen binding.

Immunoelectrophoresis of the conjugated antibody showed that it had a higher electrophoretic mobility than the unconjugated antibody (not shown). Double immunodiffusion against CEA showed that the antibody was able to form a precipitate with the antigen at appropriate dilutions. This was comparable with the unconjugated antibody.

Results of tests for in vitro stability of metal ion labelling.

a) Precipitation.

The results of ammonium sulphate precipitation of ^{65}Zn and ^{111}In-labelled antibodies are shown in table III. Under conditions of neutral pH and a large excess of carrier protein, over 80% of the ^{111}In and ^{65}Zn was precipitated.

Table III. Precipitation of metal ion labelled antibodies with saturated ammonium sulphate under various conditions.

Precipitation medium	Radioisotope	% precipitated
Saturated ammonium sulphate, pH 5.3 + 50 µl IgG (10 mg/ml).	^{65}Zn	20.2
Saturated ammonium sulphate, pH 9.0 + 50 µl IgG (10 mg/ml).	^{65}Zn	4.4
Saturated ammonium sulphate, pH 7.0 + 100 µl NSS*.	^{65}Zn	80.0
"	^{111}In	92.0

Note: * NSS = normal sheep serum.

b) Dialysis.

The results of dialysing ^{111}In-labelled antibody against various buffers are shown in fig.3. A high proportion of the total

radioactivity added was retained in both the saline and acetate experiments; reduced activity was recovered from dialysis tubing in the EDTA experiments. In tris buffer, pH 10, most of the radioactivity was eluted from the dialysis membrane, to the level expected for equilibration of free indium. The triplicate experiments were in good agreement, (S.E.M. < 2%).

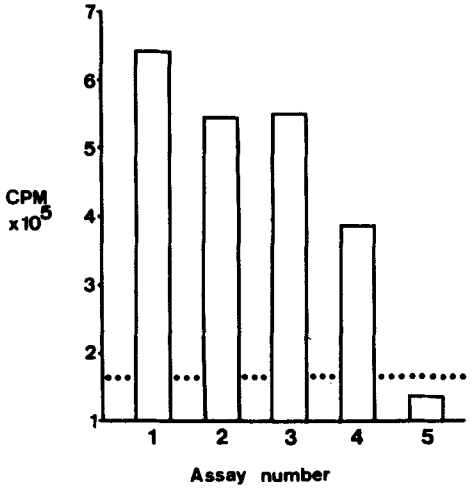

Figure 3. Dialysis of ^{111}In-labelled anti CEA against various buffers. 1) Total radioactivity added to dialysis membranes. 2) 0.15M saline. 3) 0.15M acetate, pH 6. 4) 0.15M EDTA, pH 4.7. 5) 0.15M tris, pH 10. The dotted line represents the equilibrium level for freely diffusible radioisotope.

c) Serum incubation.

^{111}In-labelled antibody either freshly prepared or incubated for 4 mins or 24 hours at 37°C in human serum showed identical elution profiles when fractionated on Sephacryl S 300 (see fig.4). Two peaks of radioactivity were eluted, the first corresponding to IgG and the

second, smaller peak to free DTPA. The IgG peak contained approximately 88% of the radioactivity added to the column.

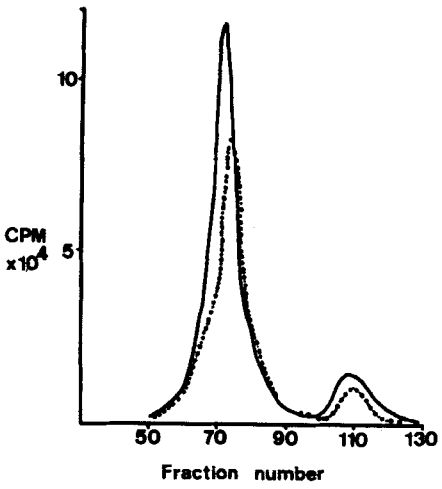

Figure 4. Elution profiles of ^{111}In-labelled anti CEA on Sephacryl S 300 prior to serum incubation (———), and after 24 hours in human serum at 37°C (•••••••).

DISCUSSION

Experiments showed that the elution of DTPA on Sephadex G 25 could be varied by adjusting the concentration of the eluting buffer. The elution was considerably retarded by buffers of a concentration above 0.1M. This unusual characteristic was taken advantage of in the preparation of DTPA-conjugated antibodies. By using a buffer of high concentration, it was possible to carry out the purification procedure on disposable columns, using small buffer volumes and reducing the chance of contamination with cold ions.

When the cyclic anhydride of DTPA was used to conjugate antibodies with DTPA, a higher efficiency of reaction was achieved when compared

with the mixed anhydride method reported by Krejcarek and Tucker (5). This was due to the lower susceptibility of the cyclic anhydride to hydrolysis. The use of the cyclic anhydride method and small disposable columns for protein purification resulted in the development of a simple and rapid technique for the production of DTPA-conjugated antibodies. It was possible to achieve the labelling and purification in three hours, whereas the total time required for the mixed anhydride method was in excess of three weeks, as the mixed anhydride must be synthesised in the laboratory. The new method was also more reproducible.

The cyclic anhydride was more stable towards hydrolysis than the mixed anhydride. However, the evidence here suggested that the normal conditions for storage (-20°C over silica gel) were not adequate to prevent hydrolysis. Phosphorus pentoxide is a more powerful dessicant and more suitable for maintaining the cyclic anhydride in an anhydrous state.

The higher electrophoretic mobility of the DTPA-conjugated antibody was to be expected, since the addition of several DTPA molecules onto the protein would increase the total negative charge on the protein.

The ability of the conjugated antibody to precipitate CEA at the same dilutions as the unconjugated antibody showed that binding affinity of the modified antibody was unaffected by the conjugation procedure (see also (7)), since an antibody contains in excess of 50 lysine residues, and only one is required for linkage of each DTPA molecule. It was therefore unlikely that conjugation would damage antigen binding sites.

Ammonium sulphate precipitation of the radiolabelled antibodies gave varying results. The presence of carrier proteins was necessary to aid the precipitation; both low and high pH reduced the proportion of radioisotope on the protein. This may have been due to either failure of the protein to precipitate under these conditions rather than dissociation of the isotope from the chelate.

A better method for determining the stability of ^{111}In in DTPA-conjugated protein was dialysis. Allowing for failure to recover all radioactivity from the dialysis bags, neither acetate buffer nor saline reduced the amount of radioactivity bound to protein. EDTA, a stong chelating agent similar to DTPA, was able to reduce it by competition for ^{111}In. However, the kinetics of the exchange were slow, and the EDTA was in considerable excess over the DTPA. Tris buffer, pH 10, was most effective in dissociating indium from the antibody. The reason for this is unknown, but the result demonstrates that the integrity of (In)DTPA cannot be guaranteed in all pH conditions. Dissociation of the complex under physiological conditions may explain the accumulation of ^{111}In or ^{90}Y in the livers of animals and patients after administration of labelled antibody (6,7).

Fractionation of freshly prepared ^{111}In-labelled antibody on Sephacryl S 300 showed that the protein preparation contained a small proportion of unbound DTPA (approximately 12%). However, this was considered an adequate degree of purity. After both 4 mins and 24 hours of incubation in human serum at 37°C, the radiolabelled antibody possessed an identical elution profile to the fresh sample, indicating that the [^{111}]DTPA complex was stable against transferrin and other plasma proteins under physiological conditions.

In our hands, the antibodies conjugated to DTPA via the cyclic anhydride were superior in all respects to those conjugated via the mixed anhydride (5), in terms of freedom from unbound DTPA, radiolabelling efficiency with ^{111}In and other ions, and half-life in serum.

ACKNOWLEDGEMENTS.

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Addendum.

Purification of acetate buffer by dithizone is most effectively achieved by making a concentrated solution of sodium acetate and purifying it with dithizone prior to adjustment of the pH with pure hydrochloric acid (produced by diffusion of HCl into triple distilled water in a closed container). The resulting acetate buffer contains a small quantity of chloride ions which are of no concern in subsequent procedures.