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Note

High-performance fast affinity chromatographic purification of anti-benzodiazepine antibodies

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Affinity chromatography is one of the most useful methods available to isolate antibodies directed against a specific immunogen. This method relies on the unique biospecific interaction between an antibody and an antigen which has previously been covalently coupled to a chromatographic support. This is a powerful tool for obtaining a relatively pure population of antibodies directed against a single antigen. This procedure, however, is time-consuming as large volumes of eluting buffer must be passed through the column at slow flow-rates. Since the elutions are often carried out at reduced temperatures and since there is shrinking and swelling of the soft gel matrix with changing ionic conditions, there may be enhancement of non-specific interactions and a reduction in purity of recovered antibodies. Recently, a rigid high-performance liquid chromatography (HPLC) support for affinity chromatography has become

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available which may alleviate many of these problems. This report describes the rapid and efficient affinity purification of anti-benzodiazepine antibodies using high-performance fast affinity chromatography (HPFAC).

EXPERIMENTAL

Covalent coupling of benzodiazepine to protein

Compound I [Ro 7-1986, 1-(2-aminoethyl)-7-chloro-1,3-dihydro-5-(2-fluorophenyl)-2H-1,4-benzodiazepine-2-one dihydrochloride] was prepared by a modification [1] of the synthesis of Earley et al. [2] from 7-chloro-1,3-dihydro-5-(2-fluorophenyl)-2H-1,4-benzodiazepine-2-one. This compound was identical by thin-layer chromatography, melting point, infrared spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectroscopy to an authentic sample of I (Hoffman-La Roche, Nutley, NJ, U.S.A.). The synthesis of compound II [irazepine, 1-(2-isothiocyanatoethyl)-7-chloro-1,3-dihydro-5-(2-fluorophenyl)-2H-1,4-benzodiazepine-2-one] from compound I has been described previously [3]. Compound II (10 mg) was dissolved in 2.0 ml of dioxane which had been passed over basic alumina oxide to render it peroxide free. Triethylamine—dioxane (1:10, 0.16 ml) was added and the solution cooled to 5°C. Porcine thyroglobulin (Tg) (20 mg) was dissolved in 20 ml of distilled water and 8 ml of dioxane were added while stirring. The pH of the Tg solution was adjusted to 8.5 by the addition of $NaHCO_3$ and cooled to 5°C. The compound II solution was added to the Tg solution while stirring rapidly, reacted for 30 min at 5°C, then warmed to room temperature and reacted for 4 h. The mixture was dialyzed overnight against 500 ml of 50% dioxane in water pH 8.5, then dialyzed against 2 l of 0.05 *M* borate buffer, pH 9.0. The resulting conjugate was dialyzed against distilled water and concentrated by ultrafiltration on an Amicon PM10 filter. The epitope density was approximately 35 mol of II per mol of Tg.

Immunization and blood collection

Compound II conjugated to Tg was emulsified in complete Freund's adjuvant (1:1) and injected subcutaneously into the rear footpad and back of rabbits (75 μg in 0.2 ml). Booster injections were given monthly or bi-monthly using incomplete Freund's adjuvant (400 μg per 0.2 ml) and only subcutaneous back-injections were given.

Blood was obtained from the marginal ear vein several days after injection. Blood was allowed to clot overnight and serum was separated by centrifugation and stored at −80°C until used.

Chromatography

All chromatographic separations were carried out using a Beckman (Berkeley, CA, U.S.A.) Ultraffinity-EP column (50 × 4.6 mm) with a Beckman HPLC system which included a 110A pump, Model 163 UV detector (280 nm) and a 2-ml sample injection loop. Buffers were prepared in distilled, deionized water and samples were filtered through 0.45-μm filters (Millipore HA or HV; Bedford, MA, U.S.A.) before use.

Prior to derivatization, the column was washed with 20 ml of water followed

by 20 ml of derivatizing buffer (1 *M* potassium phosphate, pH 7.0). Recrystallized derivatizing ligand, compound I (12 mg), was dissolved in derivatizing buffer, filtered and circulated through the column at a flow-rate of 0.2 ml/min for 21 h at 22°C. The column was subsequently washed at a flow-rate of 1 ml/min with 30 ml of water, 120 ml of derivatizing buffer, 30 ml of loading buffer (50 m*M* Tris—HCl—120 m*M* sodium chloride, pH 7.1), 6 ml of 0.01 *M* hydrochloric acid, then 30 ml of loading buffer. The completeness of removal of non-derivatized (free) I from the chromatographic system was shown by demonstrating the inability of column fractions to inhibit the binding of [^{3}H]flunitrazepam to unfractionated anti-benzodiazepine antisera (see *Radioimmunoassay*).

Before antibody purification, the affinity column was equilibrated in loading buffer using a flow-rate of 1 ml/min. Unfractionated antisera (serum), which had previously been shown to have elevated titers of antibody activity to [^{3}H]flunitrazepam, was diluted in loading buffer to a final volume of 2 ml, filtered and applied to the column using the sample injector. When the UV absorption (280 nm) returned to baseline, 2 ml of 0.01 *M* hydrochloric acid (pH 2.0) was injected and fractions were collected until the UV absorption returned to baseline again. Acidic column fractions were neutralized with 1 *M* Tris—HCl (pH 7.4) to give a final concentration of 0.1 *M* Tris—HCl, and used immediately in radioimmunoassay (RIA). In some cases, the column fractions were dialyzed against 50 m*M* Tris—HCl (pH 7.4)—120 m*M* sodium chloride for 24—48 h. When necessary, samples were concentrated using a vacuum concentrator (Savant Instruments, Farmingdale, NY, U.S.A.).

Radioimmunoassay

Unfractionated antisera or neutralized column fractions were incubated in duplicate or triplicate with 1 n*M* [^{3}H]flunitrazepam (New England Nuclear, Boston, MA, U.S.A.; specific activity 79.6—92.3 Ci/mmol) in a final volume of 0.25 ml of loading buffer (50 m*M* Tris—HCl—120 m*M* sodium chloride, pH 7.4). Diazepam (10 μM) was used to define non-specific protein binding. Incubations were carried out for 0.5—18 h at 4°C and terminated by rapid filtration over glass fiber filters (GF/C, Whatman, Clifton, NJ, U.S.A.). The filters were washed three times with 4 ml of ice-cold loading buffer. Radioactivity remaining upon each filter was quantified by liquid scintillation spectroscopy.

Gel electrophoresis

Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out under reducing conditions [4]. The electrophoretic mobility of the heavy and light chains of purified antibody was compared with the mobility of the heavy and light chains of rabbit immunoglobulin (IgG, Cohn fraction II; Sigma, St. Louis, MO, U.S.A.).

RESULTS

When sera containing antibodies directed against a benzodiazepine hapten were applied to an HPFAC column previously derivatized with compound I the

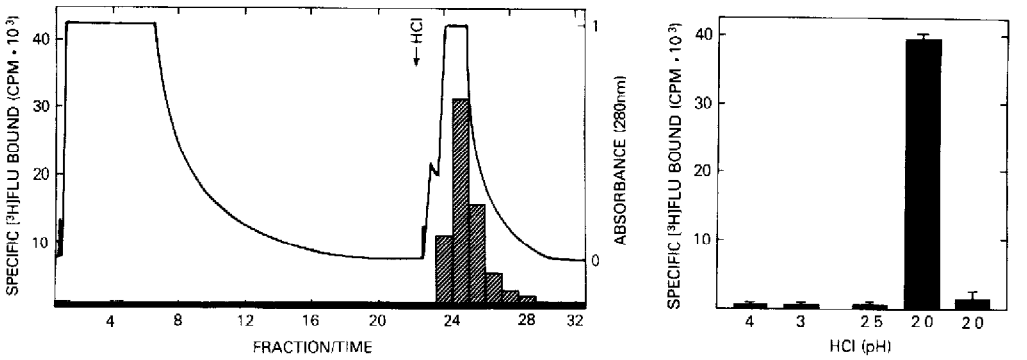

Fig. 1. High-performance fast affinity chromatographic purification of anti-benzodiazepine antibodies. Crude antisera (1 ml) was diluted to a volume of 2 ml, filtered and applied to an Ultraffinity EP column previously derivatized with I. Fractions (1 ml) were collected, neutralized and analyzed by radioimmunoassay as described in Experimental. Antibodies were eluted with 2 ml of 0.01 *M* hydrochloric acid. The radioimmunoassay was incubated for 0.5 h and terminated by filtration.

Fig. 2. pH-Dependence of antibody elution by hydrochloric acid. Crude antisera (0.5 ml) was applied to the affinity column The ability of dilute aqueous hydrochloric acid to elute the purified antibodies was examined by injecting 2-ml aliquots of increasing concentrations of acid onto the column. The second 2.0 represents a subsequent injection of pH 2.0 hydrochloric acid. The resulting eluents were neutralized and incubations were carried out in triplicate for 0.5 h. The results represent the mean ± S.E.M. of two separate chromatographic elutions.

anti-benzodiazepine activity was retained by the column and could be eluted with 0.01 *M* hydrochloric acid (Fig. 1). Following neutralization and dilution, certain fractions could be shown to specifically bind [^{3}H]flunitrazepam. For the experiment illustrated in Fig. 1, the time from injection of crude antisera to completion of the RIA with purified antibody was less than 2 h. Similar results were found using an RIA method which employed dextran-coated charcoal to separate bound from free ligand (results not shown). SDS gel electrophoresis of the eluted fraction revealed two distinct bands of 45 000 and 22 000 daltons corresponding to the heavy and light chains of rabbit IgG, respectively. In addition, the benzodiazepine binding activity could be absorbed to and eluted from a Protein A Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.), a characteristic of a large proportion (>90%) of rabbit immunoglobulins. Recovery of purified antibodies was estimated by rechromatographing the neutralized anti-benzodiazepine antibody and quantifying the amount of [^{3}H]flunitrazepam bound compared to the antibody solution that was chromatographed once. Using this method, the recovery was 60.3 ± 5.6% [mean ± standard error of the mean (S.E.M.); n = 3].

The elution of anti-benzodiazepine antibodies from the HPFAC column by hydrochloric acid was pH-dependent (Fig. 2). At pH values of 2.5 or greater, no detectable antibody activity could be eluted from the column. Using 0.01 *M* hydrochloric acid (pH 2.0), antibodies were recovered in the eluent and a subsequent injection of 2 ml of 0.01 *M* hydrochloric acid did not result in the further elution of significant quantities of antibodies from the column. In addition to hydrochloric acid, several solutions were examined as potential

TABLE I

RELATIVE POTENCIES OF BENZODIAZEPINES AND NON-BENZODIAZEPINES FOR INHIBITING THE SPECIFIC BINDING OF [^{3}H]FLUNITRAZEPAM TO CRUDE ANTI-BENZODIAZEPINE ANTISERA AND HPFAC-PURIFIED ANTI-BENZODIAZEPINE ANTIBODIES

Crude antisera and purified antibodies were diluted so that the total binding was approximately 6000 cpm per tube. Incubations were carried out in duplicate for 18 h. Values represent the mean ± S.E.M. of the IC_{50} values (concentration inhibiting 50% of specific binding) of two separate experiments. The chemical names of the following compounds are: 4'-chlorodiazepam (Ro 5-4864), ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5*a*]-[1,4]benzodiazepine-3-carboxylate (III, Ro 15-1788), ethyl-β-carboline carboxylate (β-CCE) and gamma-aminobutyric acid (GABA).

Drug	IC_{50} (n*M*)	
	Crude antisera	Affinity-purified antibodies
Compound II	0.3 ± 0.02	0.4 ± 0.10
Flunitrazepam	2.8 ± 0.2	2.5 ± 0.4
Compound I	6.5 ± 0.4	7.5 ± 1.8
4'-Chlorodiazepam	9.0 ± 0.7	7.0 ± 0.7
Diazepam	9.0 ± 0.7	12.5 ± 5.3
Clonazepam	27.5 ± 1.8	35.0 ± 10.6
Flurazepam	>100	>100
Compound III	>100	>100
β-CCE	>100	>100
Chlorpromazine	>100	>100
Propranolol	>100	>100
GABA	>1 m*M*	>1 m*M*

eluting buffers. Deoxycholate (0.1%), magnesium chloride (3.5 *M*), and potassium dihydrogen phosphate (1 *M*, pH 2.4) all resulted in significantly less recovery compared to 0.01 *M* hydrochloric acid (results not shown). In addition, these eluting buffers required dialysis prior to RIA. Elution of anti-benzodiazepine antibodies was also effected by diazepam (100 μ*M*); however, extensive dialysis was necessary to remove the diazepam.

Using the unfractionated antisera or purified antibodies, II was the most potent inhibitor of [^{3}H] flunitrazepam binding (Table I). Several other benzodiazepines were also potent inhibitors of [^{3}H] flunitrazepam binding including flunitrazepam, I, 4'-chlorodiazepam, diazepam and clonazepam. Using these compounds, there was a significant correlation (Pearsons product moment) between the ability of drugs to inhibit [^{3}H] flunitrazepam binding to the unfractionated antisera and the purified antibodies ($r = 0.98, P < 0.001$).

DISCUSSION

In the present study, an HPLC anti-benzodiazepine affinity column was prepared by derivatizing a rigid commercially available chromatographic support containing free epoxide groups with I, a benzodiazepine possessing a primary amino group. Antibodies directed against a related benzodiazepine, II, bound to the column and were eluted rapidly with various treatments including acids, salts or diazepam (Figs. 1 and 2). Several lines of evidence con-

firm that the material eluted from the column which bound [^{3}H] flunitrazepam was an antibody directed against benzodiazepines. First, the anti-[^{3}H] flunitrazepam activity could be adsorbed to and eluted from protein A Sepharose (results not shown). Second, the HPFAC-purified material displayed the same mobility by SDS gel electrophoresis as purified rabbit IgG. Third, normal (non-immune) rabbit serum did not contain components that were retained by the column and eluted with 0.01 *M* hydrochloric acid. Fourth, II was the most potent inhibitor of [^{3}H] flunitrazepam binding to the purified material. Fifth, other compounds structurally similar to II were also potent inhibitors of [^{3}H] flunitrazepam binding to the purified antisera whereas compounds structurally less similar to II were less potent inhibitors of binding. There was not a significant difference between the abilities of many structurally related agents to inhibit [^{3}H] flunitrazepam binding to the crude antisera compared to the purified antibodies suggesting that the purification/elution process did not alter the antibody characteristics.

In summary, this study demonstrates that antibodies to benzodiazepines can be rapidly and efficiently purified using HPFAC. In addition to using derivatizing ligands containing primary amines, compounds containing sulfhydryl or hydroxyl groups may also be used. This procedure should greatly facilitate the purification of antibodies for many uses including immunocytochemical localization and purification of molecules. In addition, affinity columns of this type may prove useful in the purification of receptors.

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