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SOLUBLE ANTIBODY AFFINITY CHROMATOGRAPHY TECHNIQUE INVESTIGATED WITH ULTRATRACE [^{125}I]THYROXINE

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SUMMARY

An amount of 10 pg of [^{125}I]thyroxine was subjected to DEAE agarose chromatography and then complexed with an antibody. Recycling of the [^{125}I]thyroxine now as a complex with the antibody through a second DEAE agarose column under the same conditions gave a change in the retention of the [^{125}I]thyroxine that potentially constitutes a specific shift away from co-eluting interferences from the first column. The [^{125}I]thyroxine was then dissociated from the antibody and subjected to a third DEAE agarose column for additional shifting of its chromatographic retention. Since the overall recovery of the [^{125}I]thyroxine is 36%, this soluble antibody affinity technique potentially is useful for sample clean-up in labeling analysis of ultratrace solutes such as thyroxine.

INTRODUCTION

The successful technique of affinity chromatography comes in two forms: solid phase and solution phase. In the solid technique, one of the specific binding substances is immobilized onto a solid particle or other chromatographic surface. A wide variety of such particles, surfaces and accompanying immobilization techniques are employed[1]. Contrasting with this, both binding substances are reacted together in a dissolved form in the solution phase or soluble affinity approach[2].

Like the solid affinity method, the solution technique has been used for both preparative and analytical purposes. When done preparatively, the solution procedure has been practiced mostly with one of the binding molecules conjugated to a soluble carrier material such as dextran[2,3], and also includes a special carrier designed to give subsequent precipitation[4].

In contrast, analytical applications of the soluble technique have relied more on the inherent shift in chromatographic retention of the molecular complex of interest, not involving any carrier. The Hummel-Dreyer method for determining an affinity constant provides an early example[5]. More recently, the interaction of single-stranded DNA and ribonuclease A was studied by a high-performance liquid chromatographic (HPLC) shift technique[6]. Also, antibodies have been used to shift

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the HPLC chromatographic retention of albumin, *S. mutans* polysaccharide, and bradykinin[7].

The solid phase affinity technique, largely due to its greater convenience, has been much more widely used than the solution approach for both analytical and preparative work, and this situation may not change. Nevertheless, several weaknesses that vary with the particular application for a given solid phase have sometimes limited this method. These weaknesses can include, with some overlap, unfavorable preparation, diffusion, porosity, capacity, stability, adsorption, steric,washing, leakage and elution properties of the affinity solid phase, as has been reviewed[1,8–11]. While these problems are sometimes minor and advances are continually being made, solution affinity techniques, in spite of other shortcomings, offer a contrasting approach.

In this paper we employ a form of soluble antibody affinity chromatography. The basic feature of this technique is that the solute of interest is chromatographed through the same type of column more than once, alternately in the absence of antibody, and then in the presence of antibody. During these steps, the other chromatographic conditions, aside from the presence or absence of antibody, are kept constant.

Although our method potentially may be used for both analytical and preparative purposes, we are particularly interested in using it for ultratrace (below the ng level) labeling analysis. Here, as a first stage, we apply it to the "purification" of [^{125}I]thyroxine as a model, ultratrace analyte, establishing conditions that both demonstrate the concept and give a satisfactory recovery of this analyte.

MATERIALS AND METHODS

Chemicals and reagents

Cation-exchange resin (AG 50 W-X2, 100–200 mesh, lithium form), anion-exchange resin (AG1-X2, 200–400 mesh, acetate form) and anion-exchange gel (DEAE agarose, 100–200 mesh) were all purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). [^{125}I]Thyroxine (*T_4) was from Clinical Assays (Cambridge, MA, U.S.A.). Rabbit thyroxine antiserum was obtained from Dr. P. R. Larsen at Brigham and Women's Hospital (Boston, MA, U.S.A.). Urea (high purity) was from Pierce (Rockford, IL, U.S.A.). Methanol (HPLC grade) was from Fisher Scientific (Fairlawn, NJ, U.S.A.). Phosphate buffer, unless indicated otherwise, was 0.015 *M* sodium phosphate, pH 7.0. All other regents were analytical grade and were used without further purification.

Dextran-coated charcoal was prepared by dissolving 0.125 mg of dextran (Sigma clinical grade) in 100 ml of phosphate buffer, adding 1.25 mg of charcoal, and stirring for 20 min. The suspension was stored at 4°C and was mixed for 10 min before each use.

*Purification of *T_4

Procedures described previously for purifying thyroxine[12,13] were combined and modified to give the procedure reported here.

The pH of 1 ml of *T_4 was adjusted to 12.0 (pH paper) with 1 *N* NaOH. This sample was applied to a 50 × 5 mm anion-exchange column (AG1-X2) which had been equilibrated with 0.5 *N* NaOH. The column was washed twice with 1 ml of 0.5

N NaOH, three times with 1 ml of 0.25 *M* ammonium acetate (pH 9.0), once with 1 ml of 0.1 *M* ammonium acetate (pH 7.0), twice with 1 ml 0.05 *M* ammonium acetate (pH 5.0) and three times with 1 ml of 2.5 *M* acetic acid. The $*T_4$ was eluted with 2 ml of acetic acid–methanol–water (6:2:2, v/v), and applied to a 50 × 5 mm cation-exchange column (AG50-X2) which had been equilibrated with acetic acid–methanol–water (6:2:2, v/v). This column was washed once with 1 ml of acetic acid–methanol–water (6:2:2), three times with 1 ml of 0.2 *M* ammonium acetate (pH 7.0) containing 25% isopropanol (v/v) and twice with 1 ml of water. The $*T_4$ was eluted with 1 ml of methanol–ammonium hydroxide–water (72:3:25, v/v). The eluted fraction was evaporated to dryness under nitrogen at 40°C. The residue was dissolved in methanol–water (50:50, v/v) and stored at −20°C. An aliquot of the final dilution was counted and the concentration of the solution was determined from the specific activity of the starting material (1000–1500 cpm/pg) and the efficiency (63.5%) of the counter. All $*T_4$ was purified before use unless indicated otherwise.

Purification of thyroxine antibody

A volume of 5 ml saturated ammonium sulfate (pH 7.8) was added dropwise to 10 ml of rabbit thyroxine antiserum with constant stirring. The resulting suspension was stirred for an additional 2–3 h and then centrifuged for 30 min at room temperature at 3000 rpm. The precipitate was dissolved in 10 ml of 0.85% saline, and the precipitation and centrifugation steps were repeated two more times. The precipitate was dissolved in 5 ml phosphate buffer, followed by 36 h of dialysis against 3 × 1 l of butter. Insoluble material was removed by centrifugation, and the supernatant was applied to a 20 × 1.6 cm DEAE cellulose column which had been pre-equilibrated with phosphate buffer. Fractions (5 ml) were collected at 2 ml/min and monitored at 280 nm. The first peak (100 ml), containing the IgG fraction, was pooled, aliquoted, and stored at −20°C until use. The titer of the antibody (Ab) was determined with 5500 cpm of $*T_4$ as described[14]. This antibody was used undiluted unless indicated otherwise.

Polyethylene glycol (PEG)-focused thyroxine antibody

For some experiments, as indidated, the antibody was concentrated by PEG precipitation. For this, 1 ml of purified antibody was treated with 1 ml of 20% PEG. This mixture was vortexed for 30 sec, then centrifuged for 15 min at 2500 rpm, 25°C. The supernatant was discarded and the precipitate was washed once with 0.3 ml distilled water, and centrifuged again. The supernatant was discarded and the precipitate was dissolved in 0.3 ml of phosphate buffer.

Effect of urea on $*T_4$ binding by antibody

The stability of the $*T_4$–antibody complex ($*T_4$–Ab) in the presence of urea was investigated. To 1 ml of phosphate buffer containing varying concentrations of urea (0, 0.6, 1.5, 2.0, 3.0, and 6.0 *M*) were added 0.2 ml of antibody and 0.01 ml of nonpurified $*T_4$ (40,000 cpm). This mixture was vortexed and then incubated for 30 min at room temperature. A volume of 0.5 ml of dextran-coated charcoal was added. The suspension was mixed, incubated for 10 min at room temperature, and centrifuged for 15 min at 3000 rpm. The supernatant was decanted and both the charcoal and supernatant fractions were counted to determine the percent of $*T_4$ still bound to antibody.

Purification by recycling

Three 200 × 5 mm glass columns (QS-GE, Isolab, Akron, OH, U.S.A.) were packed to a height of 100 mm with DEAE agarose that had been equilibrated with phosphate buffer (pH 8.0) containing 3 *M* urea and 5% (v/v) methanol. $*T_4$ (15,000 cpm, 10 pg) in 0.2 ml of this buffer was applied to one column (0.5 cycle). The sample was washed onto the column with 0.4 ml of buffer, followed by elution at 0.5 ml/min. Fractions (1 ml) were collected, and those containing radioactivity were pooled, giving a 10-ml volume. The pH was adjusted to 4.5 (pH paper) with 1.0 *M* acetic acid, and the $*T_4$ was enriched by cation-exchange chromatography as described for the purification of $*T_4$. The appropriate fraction was evaporated to dryness under nitrogen at 40°C, and the residue was dissolved in 0.2 ml of antibody. This mixture was incubated at ambient temperature for 0.5 h, and then applied and eluted on a second DEAE agarose column (1.0 cycle) as before. The $*T_4$–Ab peak was collected and the $*T_4$ was released from the Ab by raising the pH to 12 (pH paper) with 1 *N* NaOH. The $*T_4$ was then enriched (and further purified) by anion-exchange chromatography using the same procedure as described for the purification of $*T_4$. The eluted fraction of $*T_4$ was evaporated to dryness under nitrogen at 40°C, and the residue was redissolved in 1 ml of methanol–water (1:1) and evaporated again to remove any traces of acetic acid. The residue was dissolved in 0.2 ml of elution buffer, applied to the third DEAE agarose column (1.5 cycle) and eluted and collected as on the 0.5-cycle DEAE agarose column.

RESULTS AND DISCUSSION

The concept for our recycling technique is presented in Fig. 1. After the solute of interest is first separated from most of the interferences on an initial chromatography column, the collected peak for this solute and residual interferences is combined with a specific antibody. The resulting antibody–solute complex is then applied to a second column of the same type and conditions. Here it is intended that the solute is antibody-shifted away from the residual interferences. Standard techniques

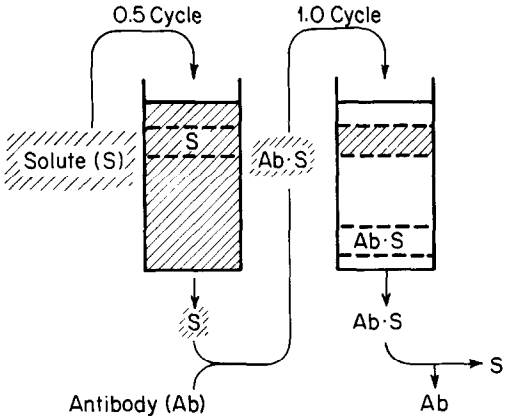

Fig. 1. Concept of the recycling technique. The solute of interest is subjected to the same type of column and conditions twice, first in the absence of antibody, and then as a specific complex with antibody. Background interferences potentially removed by this technique are represented by the shading.

then are used to remove the solute from the soluble antibody, including the possibility, as done here, to cycle further through a third such column in which the solute shifts back to its initial retention position.

Potentially this overall affinity separation technique may be quite powerful. This is because solution transport of the dissolved antibody–solute complex through a chromatographic column having different retention characteristics for this complex than the interferences should tend to strip these interferences away from this complex. Although, in theory, a comparable affinity step is present in solid-phase affinity chromatography, in practice the complex adsorption and desorption characteristics of the affinity solid phase tend to limit its removal of interferences[15,16], even with ligand elution[17,18]. Thus, solid-phase affinity techniques seem to provide less purification than expected considering their theoretical selectivity.

The data to be presented here do not bear on this selectivity aspect of the method. Instead, the purpose of this first paper is to demonstrate some conditions and an acceptable recovery for a model, ultratrace solute.

Strong conditions

We chose to employ "strong conditions" for our initial model procedure, *i.e.* conditions that minimize non-covalent solute interactions, to enhance the stripping of interferences from the antibody–solute complex. Although the strength of such conditions is limited by the need to maintain adequate antibody–solute binding, this latter binding, depending on the particular system, can resist several types of moderately strong conditions. Thus, antibody–solute complexes have tolerated mild pH extremes[19], low concentrations of non-ionic detergents[20] or organic solvents[21], and the presence of moderate concentrations of denaturants such as urea[22]. We selected the latter based on its common use.

First we determined the dilution ten times of our purified antibody that maintained a plateau binding (74%) of 40,000 to 60,000 total cpm of non-purified $*T_4$. This involved 0.5 h of incubation at room temperature followed by a charcoal separation step. We then carried out the same procedure in the presence of 3 *M* and 6 *M* urea, and observed no significant change in $*T_4$ binding by our antibody. The concentration of 3 *M* was selected for our system. Also, we chose to use the undiluted, purified antibody for our subsequent work.

Choice of column and conditions

At the outset, a DEAE type of column was selected, because it was anticipated that conditions could be established on such a column giving a different retention for $*T_4$ and $*T_4$–Ab. It is well-established that antibodies can be eluted rapidly from DEAE columns[23], while it is expected that $*T_4$, because of its phenolic ionization (pK_a = 6.7; ref. 24) could be retained on such a column. The hydrophobic and other characteristics of $*T_4$ might contribute to its retention as well.

Preliminary work with commercial DEAE versions of Sephadex, cellulose and agarose (10 × 0.5 cm columns) eluted with 3 *M* urea in 0.015 *M* sodium phosphate buffer, pH 7, gave recoveries of 0, 19 and 63%, respectively, for 20,000 cpm (13 pg) of $*T_4$. We also observed, under the same chromatographic conditions, that peak tailing was more severe on the DEAE-cellulose as opposed to the latter two columns, at least when PEG-focused antibody was analyzed, involving absorbance detection

at 280 nm. Further, the recovery in terms of cpm in the antibody peak when preformed *T_4–Ab was chromatographed was 73, 97 and 94%, respectively, on these three DEAE columns. Thus, we chose the DEAE agarose packing for our subsequent work due to its better overall recovery and peak shape for our solutes.

The chromatographic conditions for the DEAE-agarose column were then refined in two respects. First of all, the recovery of *T_4 was raised from 63 to 82% by including 5% methanol in the mobile phase. This result suggested that the prior losses on this column included a hydrophobic effect. Secondly, the pH was adjusted to 8.0 to increase the ionic retention of *T_4 relative to that of *T_4–Ab.

We also tried, unsuccessfully, to reduce the tailing of the *T_4 peak. At pH 7, a higher buffer concentration (0.03 *M* sodium phosphate) increased rather than decreased the tailing, along with the retention time, apparently due to an enhanced hydrophobic interaction of the *T_4 with the DEAE agarose. The presence, instead, of 0.1% Triton X-100 in the mobile phase had a similar effect, that we speculated, in retrospect, to involve a dynamic hydrophobic retention[25]. Although the band shape for *T_4 was improved at pH 6, this condition was not acceptable, because the *T_4 peak eluted more rapidly, overlapping about 50% with the *T_4–Ab peak. Thus, we maintained our initial pH 8.0 conditions.

Accessory steps

Our method as practiced here included two accessory steps to overcome sample dilution, isolate the solute free from the antibody, and give further purification of the *T_4. The first one was a cation-exchange enrichment of *T_4 after the first DEAE column. The second one, used to release *T_4 from *T_4–Ab after the second DEAE column, involved sodium hydroxide addition followed by anion-exchange chromatography. Both of these steps were first optimized with appropriate model samples, giving recoveries of *T_4 (10–30 pg) above 90% in each case.

1.5-Recycle procedure

With the individual steps now individually optimized, we then conducted a recycle antibody-shift procedure starting with an ultratrace amount (10 pg, 15,000 cpm) of *T_4 that was taken successively through all of the steps. A 1.5-cycle procedure (three DEAE columns having the same conditions) was selected. This is summarized in Fig. 2, showing the sequence of steps, the absolute recovery for each step (ranging from 82 to 94%), and the overall, acceptable recovery of 36%. The corresponding chromatograms for the three DEAE columns are shown in Fig. 3. Although the peaks are broad due to the type of packing employed, the basic concept of this new technique is demonstrated, including a good recovery for an ultratrace solute.

Strengths and weaknesses

The general strengths of this method, relative to those of most forms of solid-phase antibody affinity chromatography, are that: (1) the antibody is used directly; (2) some of the complexities of the affinity solid phase are avoided; (3) various chromatographic modes (*e.g.* ion exchange, size exclusion, hydrophobic) probably can be used; (4) the solute is recovered from a soluble antibody (although sometimes this will be a weakness); (5) constant elution conditions are employed; and (6) the antibody, when it is not destroyed (as it is here) potentially can be repurified and replenished for repeated use.

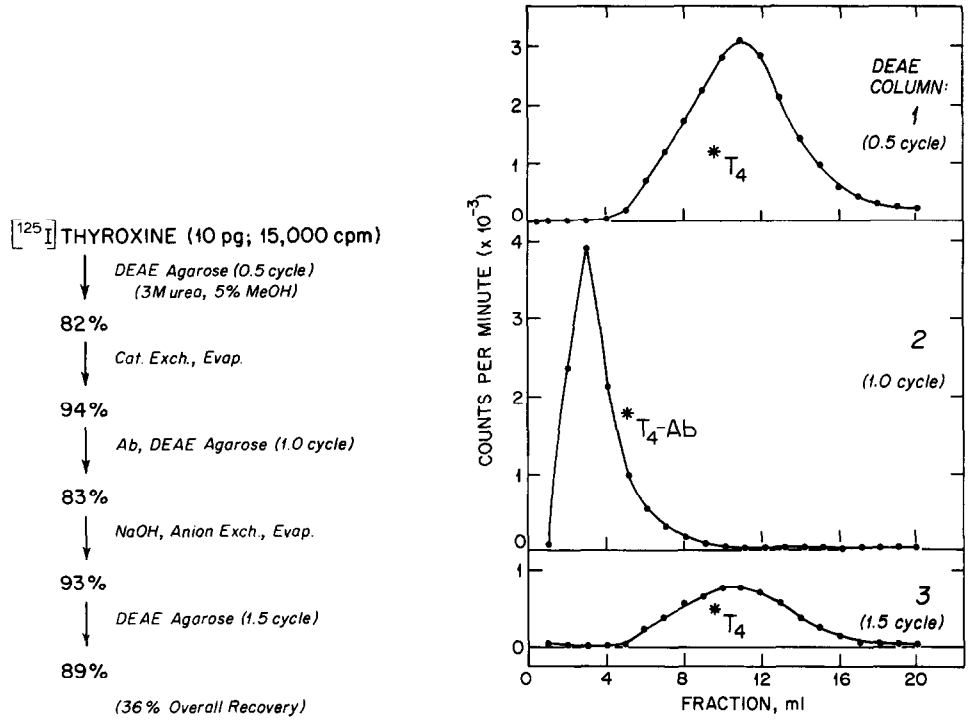

Fig. 2. Scheme giving the major steps, recoveries from each of these steps, and overall recovery of $*T_4$ throughout our 1.5-cycle procedure. These data were obtained the second time that this experiment was done; similar results were obtained the first time, where the overall recovery was 28%.

Fig. 3. Chromatograms from DEAE columns (1, 0.5 cycle; 2, 1.0 cycle; 3, 1.5 cycle) for recycle antibody-shift chromatography of [^{125}I]thyroxine ($*T_4$) involving the use of a thyroxine-specific antibody (Ab). See text and Fig. 2 for details.

The corresponding weaknesses for our method are that: (1) solid-phase affinity chromatography is well-established and currently more convenient; (2) rapidly-dissociating antibody–solute complexes may be more difficult to control by the soluble technique; and (3) chromatographic columns and conditions for different types of solutes by the soluble approach need to be established.

Future

In future work, we plan to compare the degree of sample clean-up provided by our technique with that of solid-phase affinity chromatography. We intend to conduct this work in conjunction with release tag labeling analysis[12].

Like solid-phase affinity chromatography, our technique of liquid affinity chromatography can be expected to benefit from the use of HPLC techniques and also monoclonal antibodies. The resultant sharper chromatographic bands from each will provide the usual chromatographic advantages, and the higher speed of HPLC should allow more rapidly-dissociating antibody–solute complexes to be done.

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