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Review

Drug residue analysis using immunoaffinity chromatography

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ABSTRACT

The background and applicability of immunoaffinity chromatographic separations and clean-up to drug residue analysis of agricultural commodities is discussed. The uses of antibody specificity for separation and concentration of drug residues are presented. Examples of immunoaffinity chromatography for the determination of residues of (1) nortestosterone and methyl testosterone in swine muscle, urine and bile; (2) chloramphenicol in swine tissue, eggs and milk; (3) clenbuterol in calf urine; (4) zeranol and β-zearalanolin in calf urine: (5) diethyl stilbesterol, dienestrol and hexestrol in calf urine are presented. Further, examples of the successful coupling of immunoaffinity separations with other chromatographic techniques such as gas chromatography and high-performance liquid chromatography are presented.

CONTENTS

1. INTRODUCTION

Immunoaffinity chromatography (IAC), in its various forms, is a rather specialized form of affinity chromatography [1,2] wherein the separatory ligand is either an immobilized antibody or antigen. For the purposes of these discussions on drug residue analysis, the antibody will be the separatory ligand. The selective separation occurs through the classical antibody–antigen reaction

$$Ab + Ag \rightleftharpoons AbAg$$
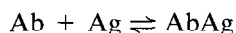
where Ab is the antibody and Ag is the antigen and the complex formed is represented by AbAg.

IAC for antigen isolation is completely dependent upon the antibody to separate the target compound. The antibody ligand is immobilized on a support and, as the target compound comes into contact with it, a complex is formed. The ligand–target compound complex is disassociated because of hydrophobic changes caused by the mobile phase and the target compound is eluted from the column. The disassociation of the complex takes place after other materials have passed or been washed through the column. The specificity of the antibody leaves a minimum of interfering materials to be eluted from the column. Thus, the eluate can be isolated relatively pure.

2. THE ANTIBODY

The literature is replete with references to the production and purification of antibodies. Interested readers should utilize the texts and papers referenced as a start in undertaking the process of preparing antibodies [3–5]. However, regardless of which type of antibody is used, monoclonal or polyclonal, the antibody should be considered as a reagent. The antibody reagent may have different stability and handling characteristics than the standard chemical reagent, but, if the singular attributes and liabilities of the antibody are understood, the antibody should pose no greater problem of handling and use than any other delicate reagent.

2.1. Polyclonal antibodies

These antibodies are raised by immunizing animals with a specific antigen. In general, molecules with molecular masses greater than 5000 usually can elicit an antibody reaction in the animal. Molecules that have low molecular masses, such as those of drugs, usually do not stimulate an immunogenic response. Such a molecule usually must be linked to a large molecule such as a protein (bovine serum albumin, ovalbumin, human serum albumin, thyroglobulin, hemocyanin) to become immunogenic. The serum of immunized animals contains several antibodies called polyclonal because the antibodies are products of several B-cell clones. It also may be considered that the polyclonal antibody is but a mixture of monoclonal antibodies that are extremely difficult to separate.

2.2. Monoclonal antibodies

Antibodies are raised in mice via immunization with an antigen complex followed by further injections 4–6 weeks later to boost the titer of antibodies. Within a few days after the booster treatment, the spleen is removed from the mouse and the mouse lymphocytes are fused in the presence of polyethylene glycol with cultured mouse myeloma cells that are deficient in the enzyme hypoxanthine–guanine rybosyltransferase (HAT). The fused cells or heterokaryons are cultured in a medium containing HAT which prevents the growth of the myeloma cell. After the death of the mouse lymphocytes, only heterokaryon cells that posses the combined traits of both the lymphocytes and the myeloma cells (hybridomas) survive. These hybridomas are screened for the production of the specific antibody. Cells, that produce the desired antibody, are cloned to produce a cell line that will produce antibody in either cell culture systems or through the growth of ascites tumors in mice.

3. PURIFICATION OF THE ANTIBODY

The classical first step in the purification of the antibody is precipitation with ammonium sulfate. Precipitation usually occurs at concentrations of 35–40% saturation; concentrations greater than this do not improve the antibody yield and, instead, will increase contamination with other proteins. Since the presence of ammonium ions can interfere with the further use of the antibody, ammonium ions are removed commonly by dialysis. Since ammonium sulfate precipitation rarely purifies the protein, other enrichment/concentration steps are necessary.

Ion-exchange chromatography using DEAE cellulose resin is one of the basic modalities for the purification of antibody. Antibodies are basic serum proteins with isoelectric points between 6 and 8. At a pH of 8, the antibodies carry a negative charge while the DEAE cellulose has a strong positive charge. Thus, there is a binding usually at low ionic strengths. The antibodies are eluted with the increasing strength of competing anions usually provided by a gradient elution system. Because the antibodies are very basic serum proteins, they elute from the column first. Similarly, antibodies can be eluted in the order of their isoelectric point by lowering the pH of the eluent.

If greater purification of the antibody is necessary, gel permeation chromatography can be used. Gel permeation chromatography is more of an adjunct to other purification systems than a primary method. Affinity chromatography can be used as a purification technique for antibodies by immobilizing the antigen on a solid matrix such as agarose and binding the antibody from solution. Elution of the antibody from the solid phase matrix can be accomplished with a relatively small volume of eluent. A cautionary word is required. It is extremely important to be careful and not use conditions that will denature the antibody.

The reverse of the system described for the purification of antibody is fundamentally IAC. Antibodies are immobilized upon a solid matrix allowing the specificity of the antibody–antigen complex to separate the target compound. Interferences are then washed away and the relatively pure target compound is eluted in small volumes from the column [6].

4. THE ANTIBODY–ANTIGEN REACTION

The antibody–antigen reaction is best described as a bimolecular reaction
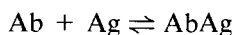
$$Ab + Ag \rightleftharpoons AbAg$$

with

$$K_a = [AbAg]/[Ab][Ag]$$

where [AbAg] is the equilibrium concentration of the bound ligand complex; [Ab] is the equilibrium concentration of the free ligand and [Ag] is the equilibrium concentration of the antigen. By substitut-

ing B for [AgAb], p for the total concentration of ligand, both bound and free, and q as the total concentration of binding sites the equation becomes

$$K_a = B / [(p - B) \cdot (q - B)]$$

At equilibrium, $p - B$ becomes the concentration of the free ligand. Thus, it follows that

B(bound ligand) / F(free ligand) $\equiv$ R(response variable)

If one substitutes then, $R \equiv K_{a1} - K_{a2}$ which is the classical relationship developed by Scatchard [7]. However, this equation holds for only one species of antibody binding site. If there were a second species of antibody, the equation becomes

$$R \equiv K_{a1} (q_1 - B_1) = K_{a2} (q_2 - B_2)$$

In chromatographic assays the response variable R equals the distribution coefficient K_d, which is the ratio of matrix-bound analyte to free analyte. If this relationship is substituted into the basic equation for the retention of an analyte on chromatographic columns, where $V_e = V_0 + V_0 K_d$, the relationship becomes

$$V_e = V_0 + [V_0(K_a q - K_a B)]$$

From this basic equation, Van Ginkel [8] calculated the potential efficiencies of immunoaffinity columns and showed the exquisite sensitivity possible. At very low concentrations, $10^8 M$, IAC would not have the problems associated with other chromatographic interactions. Van Ginkel, demonstrated rather conclusively, the IAC would be especially effective for drug residue analysis where nanogram and picogram quantities need to be isolated and measured.

Another considereation is the affinity of the antibody. Since the immunochemical reaction is rather specific, it is not critical to have high capacities for the retention of analyte. In general, the high capacity of other chromatographic systems allows retention of more interfering compounds, with loss of the advantages of high retentive capacity. The key to the balance between the capacity necessary for the retention of analyte and the ability to reuse the column lies with the use of eluting solvents.

5. THE COLUMN

As with any column chromatographic system, the heart and soul of the separation is the column. Immunoaffinity columns that can be used with a high-performance liquid chromatographic (HPLC) system must be able to withstand the rigors of the system. Phillips [9] outlined the basic requirements for HPIAC columns. He recommended stainless steel columns, usually ranging in length from 5 to 25 cm with an internal diameter of 4.6 mm. After the packing has been prepared to contain the antibody, the column can be packed either as a slurry or dry. The pump-slurry technique uses buffers with a low salt content, such as Tris or 0.01 M phosphate buffer to minimize friction and denaturation of the immobilized antibody (ligand). If the solid support consists of glass beads, the packing can be freeze dried after antibody attachment and packed dry.

Attachment of the antibody to the solid support requires that the combining sites of the antibody be properly oriented to the mobile phase. The antibody should be linked by the Fc or tail portion of the antibody to a binding compound or linker molecule. To accomplish this task, reagents such as carbonyl diimidizole can be used. This reagent reacts with primary amine groups of the ligand, usually at a pH of 9, which in turn is attached to the glass beads. Aminopropyl or alkylamines can be used to react with primary amino groups; this requires either diazotization or succinimide ester modification before attachment can take place. Compounds containing carboxyl groups will react with primary amine groups on the ligand at a pH of 8 to 9. Thiol groups will react with carbonyl groups of the ligand through a carbodiimide linkage at pH 9. The use of these materials for linking the antibody (ligand) to the glass bead could result in the randomized binding of the different segments of the antibody Y rather than the Fc portion. This would result in the loss of ligand binding capacity. A completely randomized binding reaction might lead to a loss of 66% of the binding capacity.

Orginally obtained from the cell wall of *Staphylococcus aureus* and now available in recombinant form, Protein A binds antibodies through the Fc portion of the molecule. When immobilized on the glass beads, by any of the afore-mentioned systems, two of the five subunits of the Protein A will have Fc units available for attachment of antibody. The immobilized Protein A with ligand attached is now in the proper orientation. Once the antibody is attached, the other reactive sites on the glass beads are reacted with another nonreactive protein (a protein that will not react with the antigen or target compound).

Protein G, orginally derived from a streptococcus and now available as a commercial recombinant product, appears to be an excellent material for immobilizing antibodies on glass supports. It is attached to the glass in essentially the same manner as Protein A and appears to have binding qualities for many IgG antibodies that is superior to that of Protein A [9].

6. ELUTION OF TARGET COMPOUND FROM AN IMMUNOAFFINITY COLUMN

To elute the antigen from the antibody–antigen complex, the conditions on the column must be changed. The K_a must be reduced. Reduction can be accomplished by elution with a linear pH gradient or a linear chaotropic ion gradient using ions such as Cl^-, ClO_4^-, SCN^-, or CCl_3COO^-. Changing the polarity by using solvents such as methanol and ethanol or using denaturing agents such as detergents, urea or guanidine can accomplish the breaking the antibody–antigen bond, but, this could damage the antibody and limit the useful life of a column. Chaotropic agent have the tendency to reduce hydrophobic interactions and can cause some denaturation. Changing the columns temperature can also be used. As the temperature rises, antibodies have a tendency to denature. Even at room temperature, antibodies have a tendency to slowly denature. The lower the temperature the longer the life of the column and the better the peak resolution.

7. FLOW-RATES

The flow-rates govern the speed of the antibody–antigen reaction. The binding reaction is less efficient with faster flow-rates. Flow-rates between 0.4 and 4.0 ml/min are common. Optimal flow-rates are functions of the solvent systems used during the isolation of the analyte and the gradient system used for the elution. In general, it is best to determine the optimum flow-rate for each separation [9].

8. OPERATIONAL PRESSURE OF THE HIGH-PERFORM-ANCE IMMUNOAFFINITY CHROMATOGRAPHIC (HPIAC) COLUMN

A continuing source of concern is the operating pressure of an immunoaffinity column. Although the flows will be low in general, excessively high pressures ($>3.4 \cdot 10^6$ Pa) should not be used because the pressure will generate shear-type forces that could cause the destruction of the antibody–matrix bond and lower the efficiency of the column. In addition, it is possible to add impurities from the column material to the separated analyte. In general, pressures should be approximately $0.34 \cdot 10^6$ Pa to prevent loss of immobilized antibody.

9. SPECIFIC *VERSUS* MULTISPECIFIC ANTIBODY

Whenever the analytical problem requires the analysis of a single specific analyte and antibodies can be raised against that analyte, the analytical system is rather simple. If there are several members to a drug family, as is the case with the sulfonamide drugs, it would be logical to raise an antibody that will recognize the basic molecular structure of the drug family and use that antibody. Questions arise from this approach, namely, (a) will the multispecific antibody have sufficient affinity for all the members of the family? (b) Will the members of the family elute from the column as discrete peaks or will they come off as combined peak(s)? (c) Would a polyclonal antibody be sufficient to meet the analytical needs? (d) Would a mixture of monoclonal antibodies be required to obtain the proper separation/elution? (e) Where would metabolic products appear in this analytical scheme? (f) In multi-residue or multi-analyte analysis, would it be best to use the IAC column as a method of relatively specific separation/concentration and use more conventional HPLC procedures for separation and HPLC–mass spectrometry (MS) systems for identification?

All these questions are not totally definable at this point in time. Individual analytical situations will dictate the necessary approaches. Van Ginkel [8] discussed this problem with specific emphasis on the β-agonists and hormones and pointed out that most laboratories need multi-residue methods and that combinations of analytical separations are often necessary for completeness.

10. AVAILABILITY OF MATERIALS

With rare exception, most analytical laboratories are not equipped to produce antibodies. Nor are analytical personnel trained or desirous of producing them. IAC, in its various forms, is therefore dependent upon the commercial availability of such reagents. All too often, antibodies are proprietary products used for some other analytical purpose and are unavailable to the analytical scientist. When commercialization of antibody production for analytical purposes becomes commonplace, IAC will then realize its potential.

11. APPLICATIONS OF IAC AND HPIAC FOR RESIDUE ANALYSIS

11.1. Immunoaffinity clean up

By far the most common use of IAC for drug residues analyses has been in the area of sample clean-up. Clean-up of samples is as important to an analytical determination as any other aspect of the analytical system. Separation/concentration/purification of trace quantities of analyte (μg, ng to pg/kg) from a complex matrix is the backbone of every analytical determination. For this purpose, immunaffinity chromatography is well-suited.

A multi-immunoaffinity chromatographic column (MIAC) was used by Van Ginkel *et al.* [10] for the analysis of picogram quantities of nortestosterone and methyl testosterone in muscle. After digestion and extraction of the muscle tissue and defatting, the extract was passed through antibody-coupled tresyl-activated Sepharose column. The bound anabolic steroids were eluted with a small volume, 2 ml, of 50% ethanol. The MIAC column was regenerated with subsequent washings of 90% methanol, 0.1 *M* sodium acetate and phosphate buffer. Final determination was by gas chromatography (GC)–MS. Recoveries were 80% at levels of 0.5 μg/kg. Two aspects were especially noteworthy, namely, the ability to reuse the MIAC clean-up column at least 25 times without loss of capacity and the ability to detect and measure low levels, 0.5 μg/kg.

In a similar vein, Van Ginkel and co-workers [11–13] used a clean-up system using IAC coupled with HPLC with UV detection for residues of nortestosterone and its major metabolite in bovine urine and bile and for trenbolone and its metabo-

lite. Residues at levels of 0.1 μg/kg or less were detectable. Van Ginkel and co-workers noted that when an antibody is available, IAC surpasses many other techniques for sample clean-up.

Van de Water and Haagsma [14] used monoclonal antibody-mediated clean-up in the detection of chloramphenicol in swine muscle, milk and eggs. The skimmed milk and centrifuged egg homogenates were filtered and applied directly to the immunoaffinity column. The column was prepared by coupling the antibody to chloramphenicol to a carbonyldiimizole-activated support. No matrix interferences were noted and the recoveries, when the capacity of the column was not exceeded, was essentially 100% at spiking levels of 1 μg/kg. When a glycine–NaCl buffer was used as the eluting solvent, the immunoaffinity columns lost no binding capacity; methanol use caused a 85% loss in capacity after only ·6 cycles. Van de Water and Haagsma [15,16] used a similar clean-up in a system for the detection of chloramphenicol in swine muscle tissue. The tissue was extracted with water rather than solvent and passed through a immunoaffinity column prepared by coupling antibody to CNBr-activated Sepharose. After washing, the antibody-bound chloramphenicol was eluted with methanol. After evaporation of the methanol, the residue was dissolved in mobile phase solvent and assayed by HPLC. Although the overall recoveries were 70%, the were no losses attributed to the immunoaffinity clean-up. Co-extracted meat components did not influence the immunoaffinity clean-up.

Van de Water and Haagsma [15] compared antibody-mediated clean-up (AMC) to determine residues of chloramphenicol in swine tissue and milk and compared the results with an ELISA, solid phase extraction (SPE) and a qualitative card test. Correlation was excellent between the methods. The chromatograms using the AMC were very clean with no interfering peaks.

Haasnoot et al. [17] used similar immunoaffinity clean-up approaches for determining clenbuterol, a β-agonist drug, in urine of calves. Similarly, H. Ong et al. [18] used immunoaffinity clean-up coupled with reversed-phase HPLC for the determination of albuterol, a β-adrenergic agonist in human plasma. Plasma levels of 0.79–1.56 ng/ml could be detected.

Bagnati et al. [19] utilized immunoaffinity separation with GC–MS of the pentafluorobenzyl ethers of zeranol and β-zearalanolin in calf urine. Levels as low as 0.17 μg zeranol and 0.24 μg zearalanolin/g were detected. Recoveries from spiked samples using the immunoaffinity columns were 84 and 64%, respectively. Urine samples containing incurred residues were treated with β-glucuronidase and arylsulfatase to hydrolyze the phase 2 metabolites (sugar or amino acid conjugates of the parent compound or some other metabolic product). Elution from the immunoaffinity column was accomplished with acetone–water (95:5, v/v). These columns were used for at least 30 analytical cycles without appreciable decline. The authors noted that similar affinity columns lasted for up to 100 cycles.

Bagnati et al. [20] used a very similar analytical scheme for the analysis of diethylstilbesterol, dienestrol and hexestrol residues using immunoaffinity chromatographic clean-up followed by derivatization with pentafluorobenzyl bromide and detection using gas chromatography coupled with negative ion chemical ionization mass spectrometry. The antibody used was a polyclonal raised in rabbits. Average recoveries from buffer of the afore-mentioned compounds and their isomers ranged from 42 to 92%; from urine, the recoveries ranged from 52 to 96%; from plasma, recoveries ranged from 28 to 83%. Levels as low as 0.011 μg cis isomer of diethylstilbesterol/kg and 0.021 μg trans isomer/kg were detectable. The affinity columns used were able to extract all expected compounds through the polymorphism of the polyclonal and the presence of cis and trans compounds in the immunizing complex. Noteworthy was the fact that natural estrogens were not extracted. This points to the selectivity of the immunoaffinity clean-up system.

11.2. High-performance immunoaffinity analysis

The use of HPIAC for the direct analysis of residues is far less common, in fact, there is a dearth of reports on the direct use of of HPIAC for residue analysis. Although Phillips [9] indicated that HPIAC "can be applied to the isolation of any material to which an antibody can be produced" there are some very practical reasons that direct use has not burgeoned. Elution from the immunoaffinity column may not yield sharp peaks because the desorption is slower than desirable. Thus, the resulting peak is braodened with the expected quantitation problems [21]. This phenomenon was reported by

Haagsma and Van de Water [22] in their use of this technique for the analysis of chloramphenicol residues in milk and meat. To remove the problems of peak braodening and disruption of baseline stability, Haagsma and Van de Water coupled the immunoaffinity column directly to a C_8 RP-HPLC column. The eluate from the immunoaffinity column was concentrated upon the HPLC column. The target compound, chloramphenicol was chromatographed with no reported matrix interferences at residue levels. This combined system was rugged since repeated uses, 150 samples over a 3-month period, did not result in a loss of analytical performance.

12. CONCLUSIONS

Although there are limited numbers of applications of HPIAC in drug residue analysis to report, it is apparent that immunoaffinity systems for selective clean-up, separation and concentration of target compounds in residue analysis has significant value to the food scientist, pharmacologist and regulatory official. The coupling of a immunoaffinity columns with standard HPLC columns offers the analytical scientist the best of both techniques. The commercial availability of antibodies to drugs, both monoclonal and polyclonal, will be the limiting factor in the application of this technique.

13. ACKNOWLEDGEMENT

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