Separation & Purification Reviews
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597294

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Online publication date: 09 August 2003

To cite this Article Clarke, William and Hage, David S.(2003) 'Clinical Applications of Affinity Chromatography ', Separation & Purification Reviews, 32: 1, 19 — 60
To link to this Article: DOI: 10.1081/SPM-120025025
URL: http://dx.doi.org/10.1081/SPM-120025025

SEPARATION AND PURIFICATION REVIEWS
Vol. 32, No. 1, pp. 19–60, 2003

Clinical Applications of Affinity Chromatography[#]

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INTRODUCTION

Liquid chromatographic methods like reversed-phase, normal-phase, size-exclusion, and ion-exchange methods are important in modern clinical laboratories. However, a related technique that is seeing growing use is affinity chromatography. *Affinity chromatography* can be defined as a liquid chromatographic technique that utilizes ''biological interactions'' for the separation and analysis of specific analytes in a sample (IUPAC). Examples of these interactions include the binding of an enzyme with an inhibitor, of hormones with receptors, or of an antibody with an antigen. Because of the

[#]Reprinted from *Separation Techniques in Clinical Chemistry*, Aboul-Enein, H. A., Ed.; Marcel Dekker, Inc.: New York, 2003; 321–359.

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DOI: 10.1081/SPM-120025025
1542-2119 (Print); 1542-2127 (Online)

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highly selective nature of these binding processes, affinity chromatography is quickly becoming the method of choice for separations in fields such as pharmaceutical science and biotechnology. Similar developments are beginning to occur in clinical laboratories, thus creating a need for workers in this area to be aware of this technique.

Terms and Definitions

The key component of affinity chromatography is the immobilized binding agent, known as the *affinity ligand*, which selectively interacts with the desired analyte. This is used by immobilizing the ligand onto a solid support and placing it within a column. Once the immobilized ligand has been prepared, it can then be used for isolation or quantitation of the analyte. The ligand in affinity chromatography is the key factor that determines the success of the method. Most of the ligands for affinity chromatography are of biological origin. However, ''affinity chromatography'' has also been used to describe columns that contain selective ligands of a nonbiological origin. Examples of these nonbiological ligands include boronates, immobilized metal ion complexes, and synthetic dyes.

The type of ligand present in the column is often used to break down affinity methods into various subcategories. For instance, *bioaffinity chromatography* and *biospecific adsorption* are terms used to specify whether the affinity ligand is really a biological compound. Other categories that are based on the type of ligand being used include lectin, immunoaffinity, dye ligand, and immobilized metal ion affinity chromatography (Hage, 1998a; Hermanson et al., 1992). Each of these methods will be examined in more detail later in this chapter.

Another factor used to distinguish between affinity methods is the type of support present within the column. In *low-performance* (or *column*) *affinity chromatography*, the support is usually a large-diameter non-rigid gel, such as agarose, dextran, or cellulose. In contrast, *high-performance affinity chromatography* (*HPAC*) uses small, rigid particles based on silica or synthetic polymers that are capable of withstanding the high flow rates and/or pressures that are characteristic of HPLC systems (Hage, 1998a; Larsson, 1987). Both low- and high-performance methods are used in the clinical laboratory. Low-performance affinity chromatography is generally used for sample extraction and pretreatment because it is relatively easy to set up and inexpensive. However, the better flow and pressure stability of high-performance supports make HPAC easier to incorporate into instrumental systems, giving it better speed and precision for the automated quantitation of analytes.

Principles and Separation Scheme

A typical approach for performing affinity chromatography is shown in Figure 1. In this scheme, the sample of interest is first injected onto the affinity column under conditions in which the analyte has strong binding to the immobilized ligand. This is usually performed at a pH and ionic strength that mimic the natural environment of the ligand and analyte. Because of the specificity of the analyte–ligand interaction, other components in the sample tend to have little or no interaction with the ligand and wash quickly from the column. After these nonretained solutes have been removed, an elution buffer is applied to dissociate the analyte–ligand complex. This commonly involves changing the pH or composition of the mobile phase to decrease the strength of the interaction, or adding a competing agent to the mobile phase to displace the analyte from the ligand. As the analyte elutes, it is either detected on-line or collected for further use. Later, the system is reequilibrated with the initial application buffer and the column is allowed to regenerate prior to the next sample injection. The result is a separation that is both selective and easy to perform. These features are what make affinity chromatography so appealing for solute purification or for the quantitation of sample components in a complex mixture.

The scheme shown in Figure 1 is known as the *on/off* or *direct detection mode* of affinity chromatography and has been the basis of numerous clinical applications. There are several reasons for the popularity of this format. For example, when this is performed by an HPLC system, the precision is generally in the range of 1 to 5%, and the run times are often as low as 5 or 6 min per sample (Hage, 1998a,b; Larsson, 1987). The greater speed of these systems compared to many other ligand-based techniques (e.g., traditional immunoassays) is largely due to the better mass transfer properties and increased analyte–ligand binding rates that are produced by the supports in affinity columns. The increased precision of this approach is due to the reproducible sample volumes, flow rates, and column residence times that are possible with modern HPLC equipment. Another factor that leads to good precision is the reduced batch-to-batch variation, which is created by using the same ligand for the analysis of multiple samples and standards. In many studies it has been reported that several hundred injections can be performed on the same affinity column under properly selected elution and regeneration conditions (de Frutos and Regnier, 1993; Hage, 1998a,b).

A limitation of the direct detection format in affinity chromatography is that it requires the presence of enough analyte to allow the measurement of this compound as it elutes from the affinity column. In HPLC systems this is usually performed by on-line UV/Vis absorbance or fluorescence detectors.

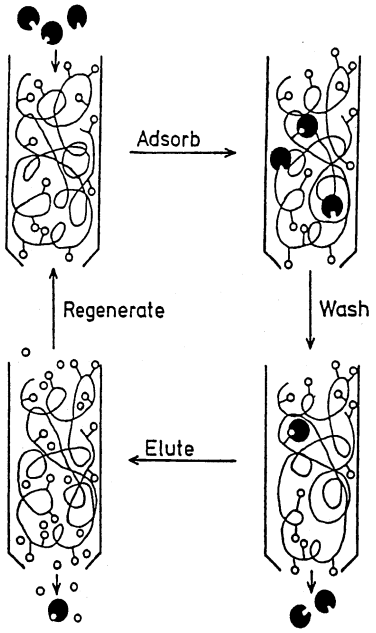

Figure 1. General operating scheme for affinity chromatography. [Reproduced with permission from Hage (1998a).]

This requirement tends to make the direct detection mode most useful when dealing with intermediate-to-high concentration solutes. However, it is possible to employ direct detection with trace sample components if the affinity column is combined with precolumn derivatization and/or more sensitive detection schemes, like an off-line immunoassay or a suitable postcolumn reactor (Hage, 1998b). Another potential limitation of the direct detection mode is that samples and standards are analyzed sequentially, which limits its effectiveness in situations where high throughput is needed. This makes this format most valuable in situations where low-to-moderate numbers of samples are being processed and/or fast turnaround times per sample are desired. However, sequential analysis has the advantage of making affinity chromatography more convenient to troubleshoot than batch-mode techniques, and it is easier to determine whether the assay is operating at satisfactory levels before patient samples are tested.

In the next few sections there will be numerous examples given in which the direct detection mode is used for clinical testing with affinity ligands. This will include the use of boronates, lectins, protein A and protein G, and other miscellaneous ligands for such methods. But there are

other ways in which affinity chromatography can also be used by clinical laboratories. Examples will be seen later when the topics of affinity extraction, postcolumn affinity detection, chiral separations, and studies of biomolecular interactions are discussed.

BORONATE AFFINITY CHROMATOGRAPHY

Affinity methods using boronic acid or boronates as ligands are one group of techniques that have been particularly successful in the clinical laboratory. This set of methods is known collectively as *boronate affinity chromatography*. At a pH above 8, most boronate derivatives form covalent bonds with compounds that contain *cis*-diol groups in their structure. Since sugars like glucose possess such groups, boronates are valuable for resolving glycoproteins from nonglycoproteins (Mayer and Freedman, 1983). The first use of a boronate affinity column in clinical testing was for the determination of glycated hemoglobin as a means for the assessment of long-term diabetes management. This was reported by Mallia et al. (1981), where absorbance measurements at 414 nm were used to quantitate the retained and nonretained hemoglobin fractions in hemolysate samples. Similar low-performance methods have been reported or evaluated by other groups (Fluckiger et al., 1984; Gould et al., 1982; Johnson and Baker, 1988; Klenk et al., 1982) and have been adapted for use in HPAC (Gould et al., 1982; Hjerten and Li, 1990; Kitagawa and Treat Clemens, 1991; Singhal and DeSilva, 1992). This approach has also been used as a point-of-care test for glycated hemoglobin (Stevenson, 1999).

In addition to hemoglobin, boronate columns can be used to look at other types of glycoproteins (Li et al., 2000; Singhal and DeSilva, 1992). For example, by monitoring absorbance at 280 nm instead of 410–415 nm, the same technique used for glycated hemoglobin can be modified to determine the relative amount of all glycated proteins in a sample (Singhal and DeSilva, 1992). Boronate chromatography can also be combined with mass spectrometry to determine the extent of glycation of protein samples (Silver et al., 1991). Alternatively, a particular type of glycoprotein can be examined by combining a boronic acid column with a detection method that is specific for the protein of interest. Examples include a boronic acid column followed by an immunoassay for the detection of glycated albumin in serum and urine (Silver et al., 1991) or glycated apolipoprotein B in serum (Pantehini et al., 1994), as well as the combination of boronate chromatography with latex immunoagglutination for the determination of glycated apolipoprotein A-I (Shishino et al., 2000). Additional studies have been performed using boronate affinity chromatography to isolate RNA (Singh and Willson, 1999).

Although hemoglobin A_{1c} is the major form of glycated hemoglobin, there are several minor species that coelute with this from boronate columns. In one study, electrospray ionization mass spectrometry was used off-line to analyze the glycated hemoglobin fraction collected from a boronate column (see Figure 2) (Peterson et al., 1998). This hemoglobin was characterized with respect to the extent of glycation and whether this modification occurred at the α- or β-chain of hemoglobin. With this approach it was possible to demonstrate that both α- and β-chains showed increased glycation as the glycated hemoglobin levels increased, but that the extent of modification of these two chains did not increase to the same degree.

LECTIN AFFINITY CHROMATOGRAPHY

Lectins are another class of ligands that can be used for the detection of clinical analytes by affinity chromatography. The result is a method known as *lectin affinity chromatography*. Lectins are non-immune system proteins that are able to recognize and bind certain carbohydrate residues (Liener et al., 1986). Two lectins often used in affinity chromatography are concanavalin A, which binds to α-D-mannose and α-D-glucose residues, and wheat germ agglutinin, which binds to D-*N*-acetylglucosamines. Other lectins that can be used are jakalin and lectins found in peas, peanuts, and soybeans. These ligands are commonly used in the isolation of polysaccharides, glycoproteins, and glycolipids (Hage, 1998a; Hermanson et al., 1992).

One clinical application of lectin affinity chromatography has been in the study of prostate cancer and prostate tumor markers. Serial lectin affinity chromatography with concanavalin A and wheat germ agglutinin was used to characterize prostatic acid phosphatase (PAP) with respect to an altered asparagine-linked sugar chain structure in patients with prostatic carcinoma (Yoshida et al., 1997). A similar approach using a combination of concanavalin A and phytohemagglutinin has been employed to study prostate specific antigen (PSA) (Sumi et al., 1999). These studies isolated their respective analytes from samples and divided the eluent into fractions based on their relative strength of lectin binding. The amount of PAP or PSA was quantitated by measuring the enzyme activity in the collected fractions. The results showed significant differences in both markers between patients with benign prostatic hyperplasia and those diagnosed with prostate carcinoma (see Figure 3). These data suggest that asparagine-linked sugar chains for both PAP and PSA were significantly altered during oncogenesis and may be used to discriminate between benign prostate hyperplasia and prostate cancer.

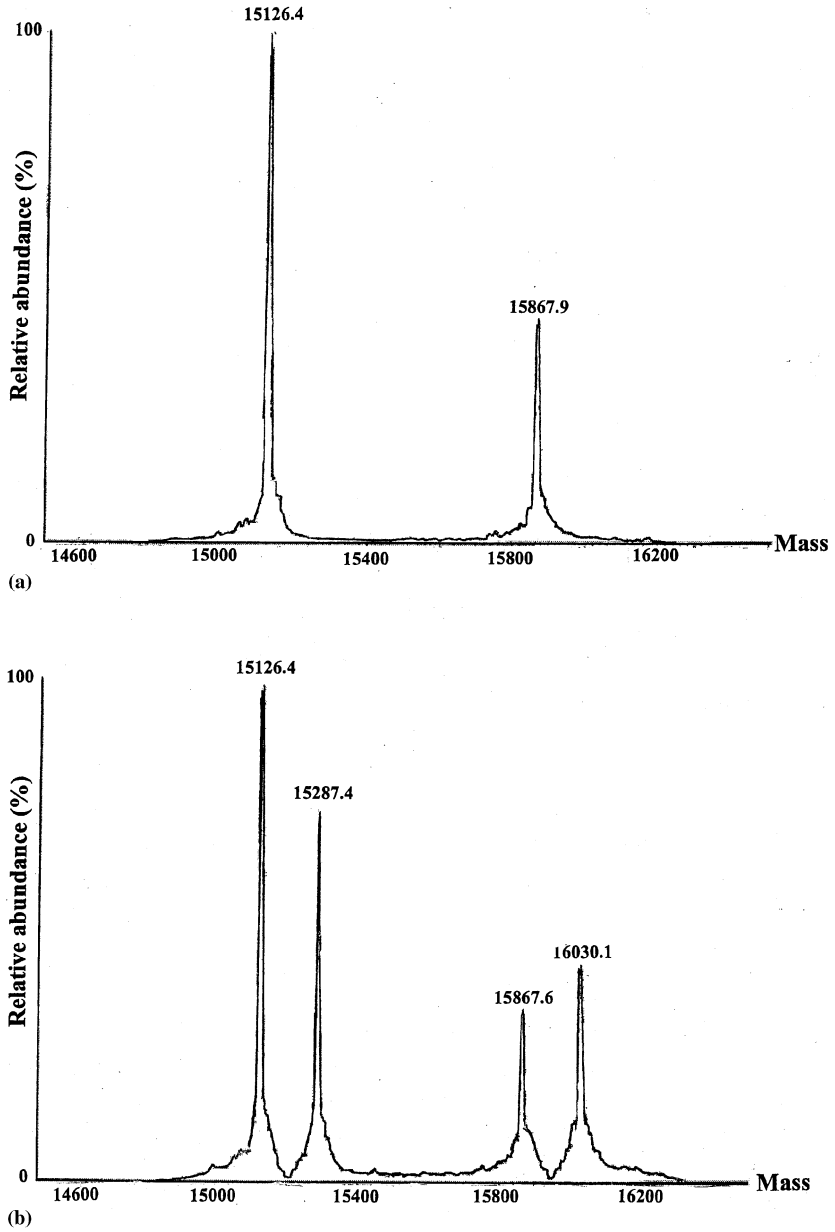

Figure 2. Deconvoluted electrospray mass spectra for (a) normal hemoglobin and (b) glycated hemoglobin collected from a boronate affinity column. [Reproduced with permission from Peterson et al. (1998).]

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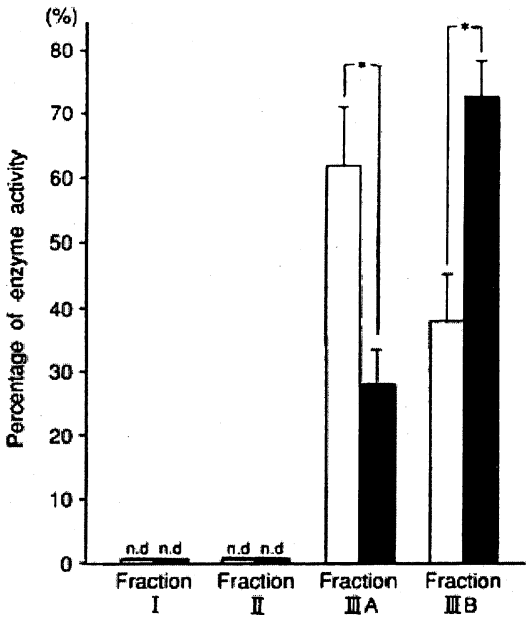

Figure 3. Activity of prostatic acid phosphatase (PAP) in four fractions collected from concanavalin A and phytohemagglutinin columns. The open columns represent PAP activity in patients with benign prostate hyperplasia, and the closed columns represent patients diagnosed with prostate cancer. The term ''n.d.'' indicates that no activity was detected. [Reproduced with permission from Yoshida et al. (1997).]

Other glycoproteins have also been studied and quantitated by lectin affinity chromatography. For instance, low-performance columns based on concanavalin A have been used to separate apoA- and apoB-containing lipoproteins in human plasma (Tavella et al., 1991), to study the microheterogeneity of serum transferrin during alcoholic liver disease (Inoue et al., 1996), to examine glycoproteins produced at postsynaptic sites (Villanueva and Steward, 2001), and to characterize the carbohydrate structure of follicle-stimulating hormone and luteinizing hormone under various clinical conditions (Papandreou et al., 1993). Wheat germ agglutinin columns have been used to develop and validate HPLC methods to distinguish between liver- and bone-derived isoenzymes of alkaline phosphatase (Anderson et al., 1990; Gonchoroff et al., 1989, 1991). Immobilized jakalin on low-performance supports has been employed to characterize the rabbit homolog of human MUC1 glycoprotein (Higuchi et al., 2000). And the lectin *Ricinus communis* agglutinin has been coupled to a polymeric support

for the high-performance affinity chromatography of glycoproteins and oligosaccharides (Cartellieri et al., 2001).

An interesting application of lectin affinity chromatography was demonstrated during the isolation of glycoprotein G from viral particles. Envelope glycoproteins from the outer surface of viruses play a vital role during infection, making them the focus of both structural and functional research. This has resulted in a need for chromatographic methods that maintain the activity and structure of these glycoproteins. In one study, a fish rhabdovirus known as viral haemorrhagic septicaemia virus (VHSV) was isolated and sonicated in polyethylene glycol (PEG) to liberate glycoprotein G from the viral particles. The samples were then passed over an immobilized concanavalin A column to purify glycoprotein G from the detergent-solubilized virion and virion-free PEG supernatants. The glycoprotein G was eluted using 1 M glucopyranoside and 1 M methyl-D-mannopyranoside in pH 7.5 sodium acetate buffer. The purified glycoprotein G was shown to retain its phosphatidyleserine-binding properties and was able to bind antiglycoprotein G antibodies. This suggested that purification by a concanavalin A column retained most of the native properties and conformation of this glycoprotein (Perez et al., 1998).

Lectin affinity chromatography has been combined with other analytical methods for a variety of applications. Proteomic analysis of glycoproteins has been performed by combining immobilized lectin columns with reversed-phase liquid chromatography, with the collected fractions then being analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The proteins in these fractions were identified by comparing their mass spectra to a spectral database of protein digests (Geng et al., 2001). Other reports have used lectin affinity chromatography as the basis for affinity capture prior to the MALDI analysis of microorganisms (Bundy and Fenselau, 2001). Finally, research in the production of designer lectins has produced a synthetic ligand, based on the coupling of a triazine with 5-aminoindan, that demonstrates selective binding toward glycoproteins that parallels the behavior of concanavalin A (Palanisamy et al., 2000).

PROTEIN A AND PROTEIN G AFFINITY CHROMATOGRAPHY

A third class of ligands used for affinity chromatography is antibody-binding proteins like protein A and protein G. These are bacterial cell wall proteins produced by *Staphylococcus aureus* and group G *streptococci*, respectively (Bjorck and Kronvall, 1984; Ey et al., 1978; Lindmark et al., 1981). These ligands bind the constant region of many types of

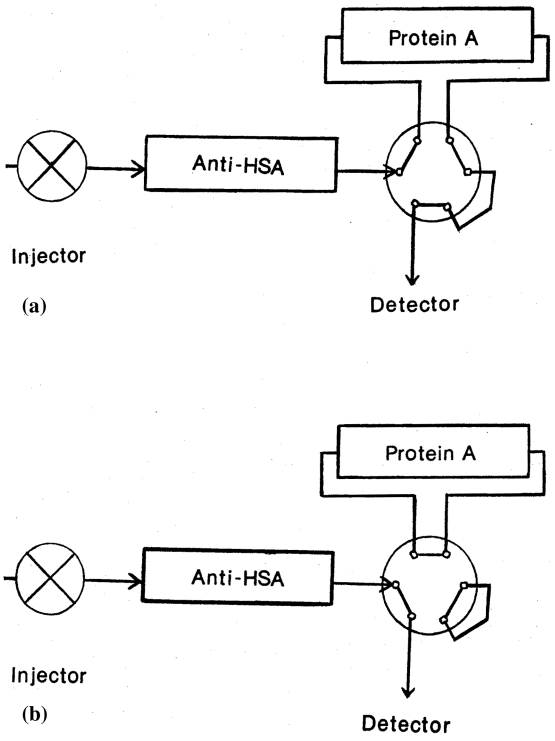

Figure 4. A dual-column system based on an antihuman serum albumin (anti-HSA) antibody column and a protein A column for the detection of HSA and immunoglobulin G (IgG) in serum samples. The diagram in (a) shows both columns on-line during sample application. The scheme in (b) shows the valve configuration used to elute the retained HSA with a pH 3.0 phosphate buffer, followed by a change back to the top configuration for the elution of IgG with the same buffer.

immunoglobulins. Protein A and protein G bind to immunoglobulins most strongly at or near neutral pH and readily dissociate from these proteins when placed into a lower pH buffer. These two ligands are different in the strength with which they bind to antibodies from different species and classes (Aakerstrom and Bjoerck, 1986; Hermanson et al., 1992; Lindmark et al., 1981). For instance, human IgG_3 binds much more strongly to protein G than to protein A, and human IgM shows no binding to protein G but does have weak interactions with protein A (Hermanson et al., 1992). A recombinant protein known as protein A/G that blends the activities of these ligands is also available for use in affinity columns (Eliasson et al., 1988; Hermanson et al., 1992).

The ability of protein A and G to bind antibodies makes them valuable for the analysis of immunoglobulins, especially IgG-class antibodies, in humans. The first clinical applications of these ligands in HPLC systems were methods for the analysis of IgG in serum (Cassulis et al., 1991; Crowley and Walters, 1983; Ohlson, 1983). Another study used a combination of two affinity columns, one containing immobilized protein A and the other anti-human serum albumin antibodies, for the simultaneous analysis of IgG and albumin in serum for the determination of albumin/IgG ratios (see Figure 4) (Hage and Walters, 1987). An additional application of protein A and protein G has been their use as secondary ligands for the adsorption of antibodies onto supports for immunoaffinity chromatography. This method, which will be considered more in the next chapter, is advantageous when high activities or frequent regeneration is needed for antibody-based columns (de Frutos and Regnier, 1993; Hage, 1998b; Phillips, 1985).

OTHER TYPES OF AFFINITY LIGANDS

The largest and most diverse group of affinity methods in clinical testing utilizes antibodies or antibody fragments as ligands. The term *immunoaffinity chromatography* (*IAC*) is used for chromatographic methods in which the stationary phase consists of such binding agents (Hage, 1998b; Phillips, 1985). If this technique is performed as part of an HPLC system, the resulting approach is referred to as *high-performance immunoaffinity chromatography* (*HPIAC*) (Hage, 1998b; Phillips, 1985). Owing to the wide variety of clinical applications for IAC and HPIAC, these topics will be discussed separately in the next chapter.

Besides the ligands that have already been discussed, there have been several other binding agents that have been used for the detection of clinical analytes by affinity chromatography. For instance, an immobilized heparin column has been employed for the determination of antithrombin III in human plasma (Dawidowicz et al., 1993, 1994). *S*-Octylglutathione has been reported as a ligand for the separation and analysis of glutathione *S*-transferase isoenzymes in human lung and liver samples (Wheatley et al., 1994a,b). In addition, immobilized *p*-aminobenzamidine has been used for the separation of human plasminogen species, with the addition of an immobilized urokinase column for on-line detection (Abe et al., 1991).

Another example that demonstrates the flexibility of affinity chromatography is the use of avian ovomucoid as a receptor analog for Shiga-like toxin type 1 (Stx1) (Miyake et al., 2000). Shiga and Shiga-like toxins are important factors in the pathogenesis of infections caused by *Shigella dysenteriae* type 1 and certain serotypes of enterotoxic *Escherichia coli*.

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These toxins not only bind a cell surface glycolipid but also bind to the P1 antigen that is contained in avian ovomucoid. To make use of this, an ovomucoid glycoprotein fraction was prepared from pigeon egg whites and coupled to cyanogen bromide-activated Sepharose 4B. This column was then used to purify Stx1 from an *E. coli* cell lysate with almost 100% of the toxin's activity being retained during this process.

AFFINITY EXTRACTION

Affinity extraction refers to the use of affinity chromatography for the isolation of a specific analyte or group of analytes from a sample prior to their determination by a second method. The general operating scheme for this is the same as for other types of affinity chromatography, but it now involves combining the affinity column either off-line or on-line with some other method for the actual quantitation of analytes. Affinity extraction represents one of the most commonly employed uses of affinity chromatography in chemical analysis. This section will discuss several examples of affinity extraction, including both off-line methods and those that involve the on-line coupling of affinity columns with techniques like HPLC, gas chromatography, and capillary electrophoresis.

Off-Line Affinity Extraction

Off-line extraction is the easiest way of combining an affinity column with another analytical technique. This usually involves placing an affinity ligand onto a low-performance support (e.g., activated agarose) that is packed into a small disposable syringe or solid-phase extraction cartridge. After conditioning the affinity column with the necessary application buffer and conditioning solvents, the sample is applied, and the nonbound sample components are washed away. An elution buffer is then applied, and the analyte is collected as it is removed from the column. Occasionally this eluted fraction is analyzed directly by a second technique, but usually the collected fraction is first dried down and reconstituted in a solvent that is more compatible with the method to be used for quantitation. If necessary, the collected solute fraction may be derivatized before it is analyzed to obtain improved detectability or more appropriate physical properties (e.g., an increase in solute volatility prior to separation and analysis by GC).

The most common ligands in affinity extraction are antibodies, as discussed in the next chapter. But these are not the only ligands used for such an approach. For example, off-line boronic acid columns have been used for the reversed-phase analysis of modified nucleosides in patients with

gastrointestinal cancer (Nakano et al., 1985) and in the purification of human platelet glycoclicin before analysis by anion-exchange HPLC (DeCristofaro et al., 1988). Agarose beads derivatized with 3-aminophenylboronic acid were used to extract glycohemoglobin from a complex mixture prior to analysis (Fiechtner et al., 1992). Wheat germ agglutinin extraction columns have been used to extract phospholipase A2 (Sribar et al., 2001) and have been combined with anion-exchange chromatography to purify and analyze angiotensinase A and aminopeptidase M in human urine and kidney samples (Scherberich et al., 1990). Another lectin, concanavalin A, has been utilized to purify human paraoxonase 1 prior to analysis (Brushia et al., 2001). Additionally, sample extraction by an organomercurial agarose column followed by RPLC analysis has been used for the assessment of urinary 2-thioxothiazolidine-4-carboxylic acid, a proposed indicator of environmental exposure to carbon disulfide (Thienpont et al., 1990).

Another application of affinity extraction involves its use to remove specific interferences from samples. One example is the use of protein A and antimouse immunoglobulin supports for the removal of human antimouse antibodies prior to a sample's determination by an immunoassay (Madry et al., 1997). Another illustration of this is the use of antihuman immunoglobulin immunoaffinity chromatography or protein A supports to adsorb selectively enzyme-immune complexes (i.e., macroenzymes) from patient samples (Remaley and Wilding, 1989).

Yet another unique application of affinity extraction is its use to bind to several related compounds in the same sample. Many ligands show some binding or cross-reactivity with solutes that are closely related to the desired analyte in structure. This cross-reactivity should be evaluated for each affinity extraction method by performing binding and interference studies with any solutes or metabolites that are similar to the analyte and that may be present in the samples of interest. However, this does not present a problem as long as the analyte of interest can be resolved or discriminated from cross-reacting compounds by the method used for quantitation. In many cases this can even be used to an advantage by allowing several species in the same class of compounds to be determined in a single run. For example, it will be shown in the next chapter how this approach has been used with antibody columns to look at anabolic steroids (Bagnati et al., 1990; van Ginkel et al., 1988, 1989).

It is further possible to place multiple types of specific affinity ligands into the same column, as has again been reported for the analysis of anabolic steroids using immunoextraction columns (van Ginkel, 1991). In this case, a column containing up to seven different ligands was used to extract simultaneously testosterone, nortestosterone, methyl testosterone, trenbolone, zeranol, estradiol, diethylstilbestrol, and related compounds in urine. This

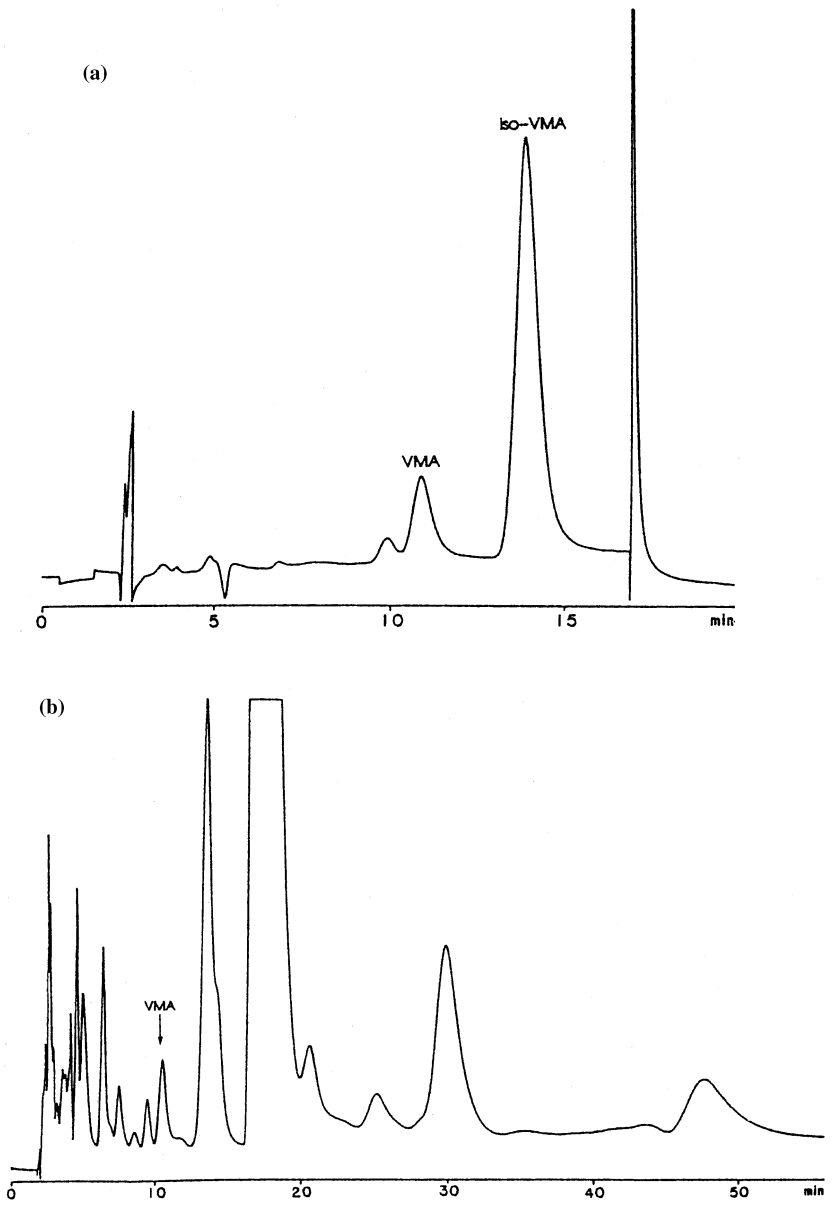

Figure 5. Analysis of vanilmandelic acid (VMA) in human urine using (a) a boronate extraction column on-line with an anion exchange column and (b) an anion exchange column alone. The peak labeled iso-VMA is isovanilmandelic acid, which was added to the samples as an internal standard. [Reproduced with permission from Eriksson and Wikstrom (1991).]

was followed by quantitation using GC–MS. This approach allowed for extensive sample cleanup without losing any important components of the sample or prolonging the time needed for sample preparation.

An advantage of off-line extraction is that the samples collected from the extraction column can be readily derivatized or placed into a different solvent between the sample purification and quantitation steps. This is particularly important when combining affinity extraction with GC, where it is desirable to remove any water from the collected sample before injection onto the GC system and where solute derivatization is often required to improve solute volatility or detection. This advantage has been demonstrated in the analysis of anabolic steroids (Dubois et al., 1998) and THC metabolites (Feng et al., 2000) by affinity extraction and GC–MS. Another advantage of off-line affinity extraction is that it is easier than on-line extraction to set up once an appropriate ligand has been obtained.

On-Line Affinity Extraction

The direct coupling of affinity extraction with other analytical methods is one other area that has been the subject of increasing research. The use of affinity extraction columns as part of HPLC systems is particularly attractive because this is compatible with automation and reduces the time needed for sample pretreatment. In addition, the high precision of HPLC pumps and injection systems provides on-line affinity extraction with better precision than off-line extraction methods, since the on-line approach has more tightly controlled sample application and elution conditions.

As with off-line affinity extractions, antibodies are generally the ligand of choice for on-line extractions (see next chapter). However, other ligands, particularly boronates, have been shown to be valuable in performing on-line affinity extraction with HPLC. Examples include several methods in which boronate columns have been combined with HPLC columns for the analysis of catechol-related compounds like epinephrine, norepinephrine, and dopamine (Boos et al., 1987; Edlund and Westerlund, 1984; Ni et al., 1989), dihydroxyphenylalanine (Edlund, 1986), dihydroxyphenylacetic acid (Edlund, 1986; Hansson et al., 1983), and 5-*S*-cysteinyldopa (Hansson et al., 1987). This same approach has been adapted for profiling and quantitating ribonucleotides in urine and serum (Larsson et al., 1983; Hagemeier et al., 1983a,b).

The analysis of vanilmandelic acid by boronate affinity extraction and anion exchange chromatography is a good example of a clinical use of on-line affinity extraction (see Figure 5). Vanilmandelic acid is a major metabolite of epinephrine and norepinephrine and is often analyzed in urine to determine the presence of excess catecholamines in cases of neuroblastoma. When samples are passed through a boronate affinity column under acidic conditions,

vanilmendelic acid (VMA) and an internal standard (i.e., isovanilmandelic acid) are retained while the remaining urine contents wash through. The analytes are eluted at a higher pH from the boronate column and passed to an on-line anion exchange column followed by electrochemical detection. This approach eliminates the need for preanalytical sample extraction and allows VMA to be measured in only 15 min (Eriksson and Wikstrom, 1991).

POSTCOLUMN AFFINITY DETECTION

Another way in which affinity columns can be used is to have them monitor the elution of specific analytes from other chromatographic columns. This involves the use of a postcolumn reactor and an affinity column attached to the exit of an analytical column. Several affinity ligands have been used for this purpose. One example is the use of anion-exchange chromatography followed by an HPLC boronate column for the determination of glycated albumin in serum samples (Yasukawa et al., 1992). Using this approach, analysis of glycated albumin was achieved in 10 min for the analysis of samples from both diabetic and nondiabetic patients. Another example is the use of immobilized receptors for the detection of bioactive interleukin-2 as it eluted from an anti-interleukin immunoaffinity HPLC column (Mogi et al., 1989).

AFFINITY-BASED CHIRAL SEPARATIONS

Affinity ligands are also important in the separation of chiral compounds (Armstrong, 1987). Owing to differing pharmacological activities for enantiomeric drugs and pressure from regulatory agencies such as the U.S. Food and Drug Administration, there has been great interest in the pharmaceutical field for methods capable of discriminating between the individual chiral forms of drugs (Chiral drugs). This has influenced the field of clinical chemistry, where the ability to quantitate the different forms of a chiral drug and its metabolites is seeing more use in metabolism studies and therapeutic drug monitoring. HPLC methods with chiral stationary phases have been shown to be valuable in quantitating and separating chiral compounds (Allenmark, 1991; Armstrong, 1987). Since many of the ligands used in affinity chromatography are inherently chiral, this makes them logical choices as stationary phases for such separations.

Various naturally occurring proteins and carbohydrates, as well as derivatives of these compounds, have been used as ligands for chiral separations of clinical analytes (Beck et al., 1991; Castoldi et al., 1994; Chu

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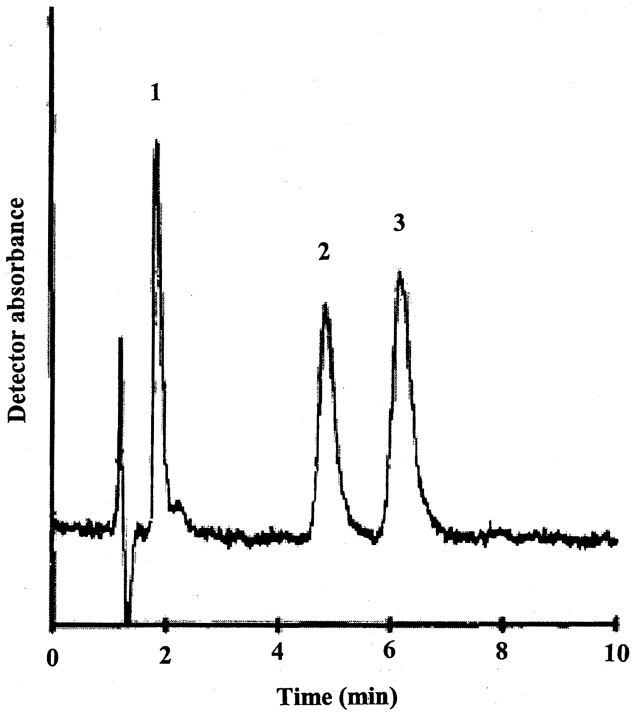

Figure 6. Analysis of R-(+)-thiopentone (peak 2) and S-(−)-thiopentone (peak 3) in an extract of plasma injected onto an immobilized AGP column. Ketamine (peak 1) was used as the internal standard. [Reproduced with permission from Jones et al. (1996).]

and Wainer, 1989; Fieger and Blaschke, 1992; Geisslinger et al., 1991, 1992; Haginaka and Wakai, 1990; Haupt, 1996; Jones et al., 1996; Kelly et al., 1994; Kristensen et al., 1994; Li et al., 1994; Mangani et al., 1997; Menzel-Soglowek et al., 1990; Pettersson and Olsson, 1991; Pham-Huy et al., 1995; Rochat et al., 1995; Schmidt et al., 1992; Silan et al., 1990; Suzuki et al., 1993). Most of these separations are performed by carrying out a routine liquid–liquid or solid-phase extraction of the sample, followed by injection of the extracted contents onto a chiral affinity column. However, other approaches are possible. In some cases, a chiral column is first used to resolve the different forms of the analyte, with fractions then being collected and applied either on- or off-line to a second achiral column for further separation and quantitation (Kristensen et al., 1994; Silan et al., 1990). Alternatively, an achiral column can be used first to isolate the

compounds from a sample, and a chiral column can then be employed on- or off-line to resolve the different chiral forms in each peak of interest (Chu and Wainer, 1989; Mangani et al., 1997).

Protein-Based Stationary Phases

The use of proteins as chiral stationary phases has received some degree of attention. Although all proteins are chiral, only one (α_1-acid glycoprotein) has seen any significant use for the analysis of drugs in a clinical setting. α_1-Acid glycoprotein (also known as AGP, AAG, or orosomucoid) is a human serum protein that is involved in the transport of many small solutes throughout the body. AGP differs from human serum albumin in that AGP has a lower isoelectric point and contains carbohydrate residues as part of its structure. The lower isoelectric point makes AGP useful in binding cationic compounds, while the presence of carbohydrate residues may play a role in determining the steroeselectivity of AGP (Allenmark, 1991). Many drugs and related analytes have been separated by AGP in human urine, serum, or plasma. Examples of clinical interest include bunolol (Li et al., 1994), citalopram (Haupt, 1996), fenoprofen (Menzel-Soglowek et al., 1990), flurbiprofen (Geisslinger et al., 1992), ibuprofen (Menzel-Soglowek et al., 1990; Pettersson and Olsson, 1991), ketamine (Geisslinger et al., 1991), ketoprofen (Menzel-Soglowek et al., 1990), methadone (Beck et al., 1991; Kristensen et al., 1994; Schmidt et al., 1992), norketamine (Geisslinger et al., 1991), norverapamil (Chu and Wainer, 1989), pindolol (Mangani et al., 1997), vamicamide (Suzuki et al., 1993), and verapamil (Chu and Wainer, 1989; Fieger and Blaschke, 1992).

A specific example of a chiral separation based on AGP is the resolution of the *R*-(+)- and *S*-(−)-isomers of thiopentone (see Figure 6) (Jones et al., 1996). Thiopentone is a common anaesthetic agent that is administered as a racemate either through bolus injection or continuous intravenous infusion. Preliminary studies have shown that the *S*-(−)-isomer is more potent and more slowly eliminated than the *R*-(+)-isomer, but pharmacokinetic data for these compounds are difficult to obtain due to difficulties in differentiating between these two isomers in samples. To overcome this, an immobilized AGP column was used with UV detection at 280 nm to separate and quantitate the enantiomers of thiopentone. Using a pH 5.0 phosphate buffer as the mobile phase and a flow rate of 0.9 mL/min, the isomers were resolved in under 8 min for injections of plasma samples.

Other proteins that have received attention in clinical applications of chiral HPLC are bovine serum albumin (BSA) and ovomucoid. Ovomucoid is a glycoprotein obtained from egg whites that has shown promise in the

separation of cationic solutes (Allenmark, 1991). BSA is a member of the serum albumin family, which is involved in the transport of a wide range of small organic and inorganic compounds throughout the body, including many pharmaceutical agents (Carter and Ho, 1994; Kragh-Hansen, 1981). BSA tends to bind best to neutral or anionic compounds, making this protein complementary to AGP and ovomucoid in its applications (Allenmark, 1991; Armstrong, 1987). In clinical work, BSA has been used for the chiral separation of leucovorin in plasma (Silan et al., 1990), and ovomucoid has been used for separating the individual forms of pentazocine in serum samples (Kelly et al., 1994).

Carbohydrate-Based Stationary Phases

Cyclodextrins are natural carbohydrates that can be employed as stereoselective ligands for HPLC (Castoldi et al., 1994; Pham-Huy et al., 1995; Rochat et al., 1995). These are circular polymers of α-1,4-D-glucose that are produced through the degradation of starch by the microorganism *Bacillus macerans*. The most common forms of these polymers are α-, β-, and γ-cyclodextrin, which contain six, seven, or eight glucose units, respectively (Allenmark, 1991; Armstrong, 1987). The cone-shaped structure and hydrophobic interior cavity of cyclodextrins give them the ability to form inclusion complexes with numerous small aromatic solutes. In addition,

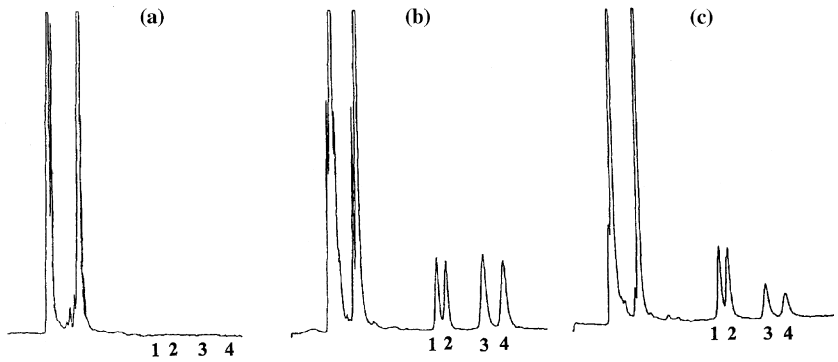

Figure 7. Separation of *S*- and *R*-alprenolol (peaks 1 and 2) and *S*- and *R*-propranolol (peaks 3 and 4) in a urine extract using a β-cyclodextrin column. Chromatogram (a) is blank urine sample, (b) is human urine spiked with 200 ng/mL racemic propranolol plus a fixed amount of racemic alprenolol (the internal standard), and (c) is urine from a healthy volunteer after oral administration of 80 mg of racemic propranolol. These compounds were monitored by using fluorescence detection at an excitation wavelength of 222 nm and an emission wavelength of 340 nm. [Reproduced with permission from Pham-Huy et al. (1995).]

the well-defined arrangement of hydroxyl groups about the upper and lower faces of cyclodextrins provides these agents with the ability to discriminate between various chiral compounds. Clinical applications for cyclodextrins in HPLC include methods reported for chlorpheniramine (Haginaka and Wakai, 1990), citaloprom, desmethylcitalopram, and didesmethylcitalopram (Rochat et al., 1995), hexobarbital (Kelly et al., 1994), and the M1 and M2 metabolites of moguisteine (Castoldi et al., 1994).

The most commonly used cyclodextrin, β-cyclodextrin, has been used to separate the (*R*)- and (*S*)-enantiomers of propranolol from both human plasma and urine samples, as shown in Figure 7 (Pham-Huy et al., 1995). Propranolol is a β-adrenergic blocking agent used in the management of patients with cardiovascular diseases. The (*S*)-enantiomer is 100-fold more active than its (*R*)-counterpart. In order to perform pharmacokinetic studies after the oral administration of racemic propranolol, a method was created to separate and quantitate the propranolol enantiomers using a β-cyclodextrin column and fluorescence detection at 340 nm. After extraction from 200 μL of plasma or urine, 50 μL of the extract was injected onto the cyclodextrin column with a polar organic mobile phase, giving a separation within 16–18 min that provided a limit of detection of 1.5 ng/mL for each enantiomer.

CHARACTERIZATION OF BIOLOGICAL INTERACTIONS

In addition to its application as a method for quantitating or isolating specific solutes, affinity chromatography can also be used to study the interactions in biological systems. Such an approach is known as *analytical* or *quantitative affinity chromatography*. This technique has been used to examine a variety of biological systems, including lectin/sugar, enzyme/inhibitor, protein/protein, and DNA/protein interactions (Chaiken, 1987; Hage, 1998a). A recent study has used affinity chromatography to study magnesium and calcium binding to human serum albumin (Guillaume et al., 1999). However, most work in the clinical setting has focused on the use of affinity chromatography to study the binding of drugs or hormones to serum proteins (Cserhati and Valko, 1994; Hage and Tweed, 1997; Wainer, 1994). In some cases, this type of protein binding is general, as in the interaction of many different drugs with melanin (Knorle et al., 1998), human serum albumin, or AAG (Ascoli et al., 1998; Barre et al., 1987; Hage and Sengupta, 1998, 1999; Kwong, 1985; Lindup, 1987; Russeva and Zhivkova, 1999; Russeva et al., 1999; Svensson et al., 1986; Zhivkova and Russeva, 1998). But on other occasions, this binding is highly specific in nature, such as the interaction of L-thyroxine with thyroxine-binding globulin or the

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binding of corticosteroids and sex hormones to steroid-binding globulins (Refetoff and Larsen, 1989; Westphal, 1971). Protein binding of drugs and hormones is interesting because it plays a role in determining the final biological activity, metabolism, and elimination of these compounds. Also, the competition between drugs or between drugs and endogenous compounds (e.g., fatty acids or bilirubin) for protein binding sites can be an important source of drug–drug or drug displacement interactions (Barre et al., 1987; Kwong, 1985; Levy and Moreland, 1984; Lindup, 1987; Svensson et al., 1986).

Affinity chromatography has been used to examine drug–protein binding by using both immobilized drugs and immobilized proteins, but protein-based columns are more common (Hage and Tweed, 1997). One advantage of employing protein columns for binding studies is the ability to reuse the same ligand for multiple experiments, with up to 500 to 1000 injections per column being reported in some HPLC studies (Loun and Hage, 1995, 1996; Yang and Hage, 1996). However, it is important when using an immobilized protein column to consider how effectively this models the behavior of the same protein in its soluble form. Fortunately, there are now many studies that show that immobilized proteins, particularly human serum albumin (HSA), can successfully be used as such models. For example, the association constants measured by equilibrium dialysis for soluble HSA with *R*- and *S*-warfarin or L-tryptophan are in close agreement with values obtained using immobilized HSA columns (Loun and Hage, 1992, 1994; Yang and Hage, 1993). Studies have also shown that displacement phenomena and allosteric interactions found with immobilized HSA are representative of behavior observed for HSA in solution (Ascoli et al., 1998; Domenici et al., 1990a,b, 1991; Loun and Hage, 1992; Noctor et al., 1992a,b).

There are a number of different ways in which affinity chromatography can be used to investigate solute–protein interactions. Two techniques used for this purpose are zonal elution and frontal analysis. The next few sections will discuss each of these approaches in more detail and go over some of their applications in the study of protein binding by drugs and other solutes.

Zonal Elution

Zonal elution is the method most frequently used to study the binding of drugs and other solutes to immobilized protein columns (Sebille et al., 1990; Wainer, 1994). The general format for these studies is to inject a small sample of the drug or analyte of interest onto a protein column in the presence of only buffer or a fixed concentration of a competing agent in the

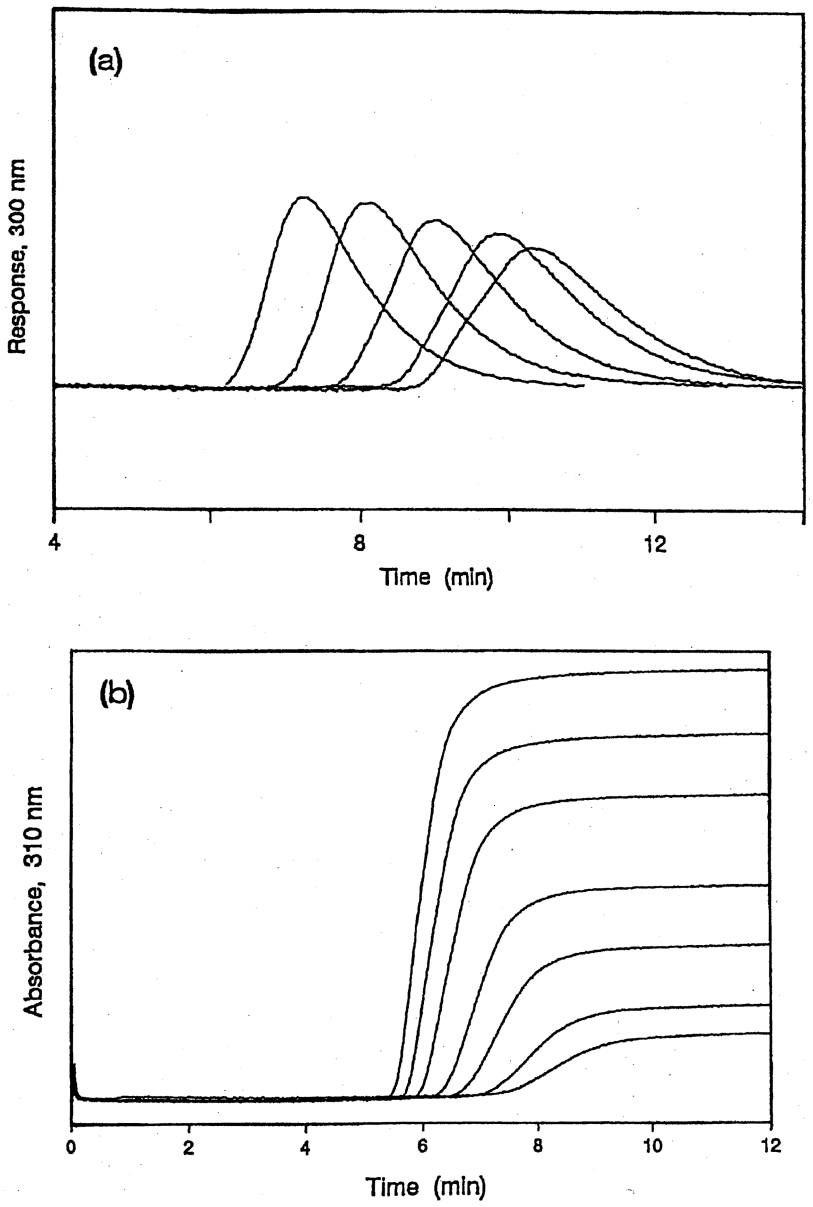

Figure 8. (a) Zonal elution experiment for the injection of *R*-warfarin onto an immobilized HSA column in the presence of various concentrations of a competing agent (increasing in concentration from right to left); (b) frontal analysis curves for the application of various concentrations of *R*-warfarin (increasing in concentration from right to left) on an immobilized HSA column. [Reproduced with permission from Loun and Hage (1994, 1995).]

mobile phase. The injected analyte's elution time or retention factor (also known as capacity factor, k or k') is then examined to see how this changes as a function of the competing agent concentration. An example of this type of experiment is shown in Figure 8a (Loun and Hage, 1992). Similar experiments can be used to study how changes in temperature or various solvent conditions affect solute–protein interactions (Allenmark, 1991; Allenmark et al., 1983, 1984, 1988; Fornstedt et al., 1996; Hermansson, 1983; Miwa et al., 1987; Schill et al., 1986; Yang and Hage, 1993) or to develop quantitative structure–retention relationships that describe these binding processes (Kaliszan, 1998; Kaliszan et al., 1992; Noctor et al., 1992a).

The most common application of zonal elution in drug–protein binding studies is its use to examine the displacement of drugs by other solutes (Hage and Tweed, 1997; Noctor and Wainer, 1993). Examples include displacement studies of DL-thyronine and DL-tryptophan from HSA by bilirubin or caprylate (Dalgaard et al., 1989); the competition of *R*/*S*-warfarin with racemic oxazepam, lorazepam, and their hemisuccinate derivatives on an HSA column (Domenici et al., 1991); the direct or allosteric competition of octanoic acid on immobilized HSA for the binding sites of *R*/*S*-warfarin, phenylbutazone, tolbutamide, *R*/*S*-oxazepam, hemisuccinate, ketoprofen A/B, and suprofen A/B (Noctor et al., 1992b; Zhivkova and Russeva, 1998); the binding of L-thyroxine and related thyronine compounds to HSA (Loun and Hage, 1992, 1995); and the displacement of *R*- and *S*-ibuprofen by each other at their common binding regions on HSA (Hage et al., 1995). This same technique has been used to characterize the binding sites of nonsteroidal anti-inflammatory drugs on HSA (Rahim and Aubry, 1995; Russeva and Zhivkova, 1999), as well as the binding of digitoxin and acetyldigitoxin (Hage and Sengupta, 1999), *cis*- and *trans*-clomiphene (Hage and Sengupta, 1998), and piroxicam (Russeva et al., 1999) to HSA. In addition, this has been used to examine the displacement of nonsteroidal anti-inflammatory drugs and benzodiazepines by phenylbutazone, *R*/*S*-ibuprofen or 2,3,5-triiodobenzoic acid from serum albumin columns (Aubry et al., 1995).

Besides providing qualitative information on binding and displacement effects, zonal elution can provide quantitative information on the equilibrium constants for these interactions. Many examples of this can be found in the cases already cited (Ascoli et al., 1998; Aubry et al., 1995; Hage et al., 1995; Kaliszan, 1998; Loun and Hage, 1992, 1995; Noctor et al., 1992a, b; Rahim and Aubry, 1995; Russeva et al., 1999; Zhang et al., 2000). With proper experimental design, kinetic information on the rates of solute–protein interactions can also be obtained, provided that appropriate data are collected on the width and retention for solute peaks under various flow rate conditions. This latter case has been demonstrated for the binding of *R*/*S*-warfarin (Loun and Hage, 1996) and DL-tryptophan (Yang and Hage, 1996) to HSA columns.

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Frontal Analysis

A second affinity method for studying biological interactions is *frontal analysis* (Hage and Tweed, 1997). In this approach, a solution containing a known concentration of analyte is continuously applied to an affinity column. As the column becomes saturated, the amount of solute eluting from the column gradually increases, forming a characteristic breakthrough curve, as shown in Figure 8b. As long as the association and dissociation kinetics of this system are fast, the mean positions of the breakthrough curves can be related to the concentration of applied analyte, the amount of ligand in the column, and the association equilibrium constants for analyte–ligand binding.

Frontal analysis and affinity chromatography have been used to study various systems. One example is the binding of HSA to *R*- or *S*-warfarin (Loun and Hage, 1992, 1994) and D- or L-tryptophan (Loun and Hage, 1992; Noctor et al., 1992b; Yang and Hage, 1993, 1996). This approach has further been used to determine the binding capacities of monomeric versus dimeric HSA for salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulphamethizole, and sulphonylureas (Nakano et al., 1982), and to examine the competition of sulphamethizole with salicylic acid for HSA binding regions (Nakano et al., 1980). Another application of this approach has been in characterizing the binding between chemically modified HSA and various site-specific probe compounds (Chattopadhyay et al., 1998).

One drawback of frontal analysis is that it generally requires a larger amount of applied solute than zonal elution. However, this is largely offset by the fact that frontal analysis tends to provide binding constants that are more precise and accurate than those obtained by zonal elution methods (Hage and Tweed, 1997). Another advantage of frontal analysis is that it is easy with this method to obtain information on both the binding capacity and affinity of an immobilized ligand.

FUTURE TRENDS AND DEVELOPMENTS

Although the application of affinity chromatography has found a wide variety of uses within clinical chemistry, there are still numerous ways this method can be improved or further adapted for work in this field. One area of ongoing research is in the creation of alternative ligands for clinical separations by affinity chromatography. For instance, ligands based on synthetic dyes, like triazine or triphenylmethane compounds, have been used for many years in enzyme and protein purification in a technique known as *dye–ligand affinity chromatography* (Alberghina et al., 1999; Clonis et al., 2000; Hage, 1998a; Hermanson et al., 1992; Jones, 1991;

Kaminska et al., 1999; Koch et al., 1998; Mori et al., 2000; Scaween, 1991). However, these ligands have not yet seen any significant use in clinical labs.

Another example of a technique that is just beginning to appear in clinical labs is *immobilized metal ion affinity chromatography* (*IMAC*). This uses a metal ion complexed with an immobilized chelating agent as the affinity ligand, such as iminodiacetic acid complexed with Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, or Fe^{3+}. This method separates proteins and peptides based on the interaction between amino acids like histidine, tryptophan, or cysteine with the metal ions within the immobilized chelate (Lopatin and Varlamov, 1995; Porath, 1992; Winzerling et al., 1992). IMAC has been used in isolating human single-chain Fv antibodies (Laroche-Traineau et al., 2000) and fusion proteins (Liu et al., 2001). This technique has also been employed in the separation of human blood components, including protein C and prothrombin (Wu and Bruley, 1999). In addition, IMAC has been used to study the interaction of human granulocyte-colony stimulating factor (rhG-CSF) with metal ions (Zaveckas et al., 2000).

Two new affinity ligands that may soon become important in clinical testing are *aptamers* and *molecular imprints*. An aptamer is a polymer of nucleotides that has a well-defined sequence and three-dimensional structure. This is obtained by screening a random oligonucleotide library for the binding of such ligands to a given target compound; any oligonucleotides that show binding are then amplified and used in applications such as affinity chromatography (Ellington and Szostak, 1990; McGown et al., 1995; Romig et al., 1999; Turek and Gold, 1990). A molecular imprint is an affinity ligand that is actually part of a support surface. This is prepared by combining the analyte of interest with a series of monomers that contain side chains capable of forming various interactions with the analyte. Polymerization is then initiated and the monomers are fixed in position about the analyte. This provides a material with binding pockets that have a known specificity (Kriz et al., 1997; Sellergran, 1997) and that can be placed into a column for affinity chromatography. Both aptamers and molecular imprints are appealing candidates for affinity ligands owing to their ability to be custom designed for a given analyte, their stability over long-term use, and their moderate-to-high selectivity (Ellington and Szostak, 1990; Kriz et al., 1997; McGown et al., 1995; Romig et al., 1999; Sellergran, 1997; Turek and Gold, 1990; Vallano and Remcho, 2000).

One specific application of aptamers in affinity chromatography has been their use in the isolation of a fusion protein (Romig et al., 1999). In this study, an aptamer that could bind L-selectin was generated and immobilized within an affinity column. This column was then utilized in the purification of a recombinant human L-selectin–IgG fusion protein. This resulted in a 1500-fold purification and 83% recovery of the fusion

protein in a single step. Similarly, a molecular imprint based on nortryptiline has been used as a stationary phase for the separation of structurally similar tricyclic antidepressant drugs (Vallano and Remcho, 2000). This was based on a capillary HPLC column packed with nortriptyline-imprinted particles that were used to screen a simulated combinatorial library consisting of tricyclic antidepressants and related compounds. Using a mobile phase that contained 0.02% TFA and 0.015% TEA in acetonitrile, the retention factor for each compound was compared with its structure. This demonstrated the selectivity of the material, since compounds that shared the major structural features of nortriptyline were also the most strongly retained on the column (see Figure 9).

Other novel ligands have appeared in recent affinity work involving clinical analytes. Immobilized synthetic oligosaccharide tumor antigens have been used to assess the response of cancer patients to carbohydrate-based vaccines (Wang et al., 2000). There has also been a study that utilized red blood cells as an affinity stationary phase by adsorbing them to wheat germ lectin agarose gel beads (Gottschalk et al., 2000). This procedure was developed because previous methods of red cell adsorption were plagued with significant hemolysis, whereas immobilization to the wheat germ lectin left the majority of red cells intact. Using this cellular-based stationary phase and frontal analysis chromatography, interactions of D-glucose and cytochalasin B with the glucose transporter Glut1 were studied. Dissociation constants were measured for both analytes, and the affinity of the analytes for the Glut1 in red cells was determined to

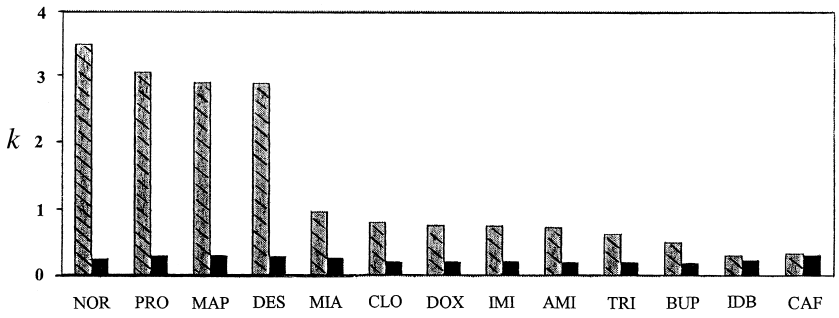

Figure 9. Comparison of retention factors (k) for various compounds injected onto a nortriptyline molecular imprint support (dashed boxes) and a similar support with no imprint (solid boxes). The compounds used in this study were as follows: nortriptyline (NOR), protriptyline (PRO), maprotiline (MAP), desiprimine (DES), mianserin (MIA), clomipramine (CLO), doxepin (DOX), imipramine (IMI), amitriptyline (AMI), trimipramine (TRI), bupropion (BUP), iminodibenzyl (IDB), and caffeine (CAF). [Reproduced with permission from Vallano and Remcho (2000).]

be higher than for Glut1 in cytoskeleton-depleted membrane vescicles or proteoliposomes.

Another novel affinity application involved the immobilization of liposomes containing a photosynthetic reaction center to study the binding of c-type cytochromes to this center. The liposomes were immobilized by integrating biotinyl phosphatidylethanolamine into their structures and adsorbing them to streptavidin-coupled gel beads (Yang et al., 2000). In other work, a column with immobilized N2,N2,7-trimethyl guanosine (TMG) was used to purify TMG-binding proteins from human cells (Espuny et al., 1999), and sulphate affinity chromatography was conducted to purify viral particles from crude cellular lysates (O'Riordan et al., 2000).

Another ongoing trend in affinity chromatography is the design of improved systems and formats that will give this technique greater speed, greater selectivity, and higher sample throughput. These improvements must be made for affinity chromatography to be competitive with common clinical methods such as batch-mode immunoassays. One approach to increasing selectivity and/or increasing the number of analytes examined per assay is to combine affinity chromatography with other analytical techniques. This is already reflected in the growing popularity of using off-line affinity extraction with HPLC or GC and on-line affinity extraction with HPLC. Development of these tandem methods is expected to continue, as is further combination of on-line affinity extraction with GC, capillary electrophoresis, and mass spectrometry (Cai and Henion, 1996; Cole and Kennedy, 1995; Creaser et al., 1996; Farjam et al., 1991; Guzman, 1995; Phillips and Chmielinska, 1994).

Affinity chromatography clearly possesses several attributes that make it a viable alternative to current methods of clinical analysis. It can be used for a wide variety of analyses, due to the large number of available ligands and variety of operating formats. This makes affinity chromatography a very flexible technique that can be developed for almost any analyte of clinical interest. When combined with HPLC or other methods, affinity chromatography can also be used to create both robust and reproducible analyses, which is important for the development of any clinical method. It is for these reasons that as more emphasis is placed on rapid specialized testing, affinity chromatography should play an increasing role as an important tool in clinical laboratories.

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