1999

Affinity Chromatography: A Review of Clinical Applications

David S. Hage
University of Nebraska - Lincoln, dhage1@unl.edu

Clinical Chemistry 45:5
593–615 (1999)

Review

Affinity Chromatography: A Review of Clinical Applications

David S. Hage

Affinity chromatography is a type of liquid chromatography that makes use of biological-like interactions for the separation and specific analysis of sample components. This review describes the basic principles of affinity chromatography and examines its use in the testing of clinical samples, with an emphasis on HPLC-based methods. Some traditional applications of this approach include the use of boronate, lectin, protein A or protein G, and immunoaffinity supports for the direct quantification of solutes. Newer techniques that use antibody-based columns for on- or off-line sample extraction are examined in detail, as are methods that use affinity chromatography in combination with other analytical methods, such as reversed-phase liquid chromatography, gas chromatography, and capillary electrophoresis. Indirect analyte detection methods are also described in which immunoaffinity chromatography is used to perform flow-based immunoassays. Other applications that are reviewed include affinity-based chiral separations and the use of affinity chromatography for the study of drug or hormone interactions with binding proteins. Some areas of possible future developments are then considered, such as tandem affinity methods and the use of synthetic dyes, immobilized metal ions, molecular imprints, or aptamers as affinity ligands for clinical analytes.

Liquid chromatographic methods, and especially those based on HPLC, are an important group of techniques in modern clinical laboratories. Clinical chemists are generally familiar with the most common forms of liquid chromatography, including reversed-phase, normal-phase, size-exclusion, and ion-exchange chromatographic methods. However, there is another category of liquid chromatography that is often overlooked by clinical chemists. This technique, known as "affinity chromatography", is rapidly becoming the separation method of choice in other biologically related fields such as pharmaceutical science and biotechnology. Similar developments are beginning to occur in clinical laboratories, thus creating a need for clinical chemists to be aware of this technique. The goal of this review is to acquaint the reader with affinity chromatography and to discuss the current or potential applications of this technique in the field of clinical chemistry. Although several types of affinity chromatography will be considered, an emphasis will be placed on those methods in which affinity columns are used as part of HPLC systems.

According to the International Union of Pure and Applied Chemistry *(1)*, affinity chromatography is defined as a liquid chromatographic technique that makes use of a "biological interaction" for the separation and analysis of specific analytes within a sample. Examples of these interactions include the binding of an enzyme with an inhibitor or of an antibody with an antigen. Such binding processes are used in affinity chromatography by first obtaining a binding agent, known as the "affinity ligand", that selectivity interacts with the desired analyte and then placing this ligand onto a solid support within a column. [See Refs. *(2, 3)* for reviews of supports and immobilization methods that can be used in making affinity columns.] Once this immobilized ligand has been prepared, it can be used for isolation or quantification of the analyte.

The immobilized ligand is the key factor that determines the success of any affinity chromatographic method. As implied by the definition given earlier for affinity chromatography, most of these ligands are of biological origin; however, the term "affinity chromatography" has also been used throughout the years to describe some columns that contain selective ligands of nonbiological origin. Examples of these nonbiological ligands are boronates, immobilized metal ion complexes, and synthetic dyes (e.g., triazine-related compounds). Terms such as "bioaffinity chromatography" and "biospecific adsorption" are occasionally used to specify whether the affinity ligand is really a biological compound. Regardless of the origin of the ligand, the type of ligand can

Department of Chemistry, 738 Hamilton Hall, University of Nebraska, Lincoln, NE 68588-0304. Fax 402-472-9402; e-mail dhage@unlinfo.unl.edu.

Received August 10, 1998; accepted March 2, 1999.

be used to divide affinity techniques into various subcategories, such as lectin, immunoaffinity, dye ligand, and immobilized metal ion affinity chromatography, to name a few *(2, 3)*. These and other affinity techniques will be examined in more detail later.

Another factor that can be used to distinguish between one affinity method and another is the type of support used within the column. In "low-performance (or column) affinity chromatography", the support usually is a large diameter, nonrigid gel, such as agarose, dextran, or cellulose. In "high-performance affinity chromatography" (HPAC),[1] the support consists of small, rigid particles based on silica or synthetic polymers that are capable of withstanding the flow rates and/or pressures that are characteristic of HPLC systems *(2, 4)*. Both low- and high-performance methods have been used in clinical methods. Low-performance affinity chromatography commonly is used for sample extraction and pretreatment because it is relatively easy to set up and inexpensive to use. However, the better flow and pressure stability of high-performance supports makes HPAC easier to incorporate into instrumental systems, which in turn gives it better speed and precision for the automated quantification of analytes.

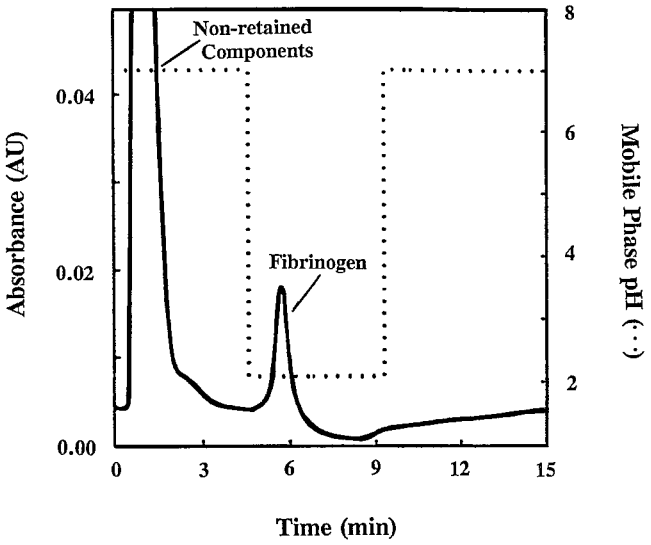


Fig. 1. Typical operating scheme for affinity chromatography, as illustrated by the determination of fibrinogen in human plasma, using an anti-fibrinogen immobilized antibody column and HPIAC.

The *dotted line* indicates the times during which the application buffer (pH 7.0) and elution buffer (pH 2.1) were passed through the column. Adapted with permission from McConnell and Anderson *(49)*.

Direct Analyte Detection by Affinity Chromatography

An example of the simplest and most common operating scheme for affinity chromatography is shown in Fig. 1. In this scheme, the sample of interest is first injected onto the affinity column under conditions in which the analyte will bind strongly to the immobilized ligand. This is usually done 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 solutes in the sample tend to have little or no binding to the ligand and quickly wash from the column. After these nonretained solutes have been removed, an elution buffer is applied to dissociate the retained analyte; this commonly involves changing the pH or buffer composition of the mobile phase (to decrease the strength of the analyte-ligand interaction) or adding a competing agent to the mobile phase (to displace the analyte from the ligand). As the analyte elutes, it is then detected or collected for further use. Later, the initial application buffer is reapplied to the system, and the column is allowed to regenerate before the next sample injection. The overall result is a separation that is selective and easy to perform. It is this feature that makes this format so appealing for solute purification or for the quantification of sample components.

In addition to its simplicity, there are several other advantages to using the direct detection mode of affinity chromatography. For example, when this mode is performed on an HPLC system, the precision is generally in the range of 1–5% and the run times are often as low as 5–6 min per sample (for an example, see Fig. 1) *(2, 4, 5)*. The greater speed of these systems compared with many other ligand-based techniques (e.g., traditional immunoassays) largely can be attributed to the better mass transfer properties and increased analyte-ligand binding rates that are produced by the supports used in affinity columns. The precision of this approach is partly the result of the reproducible sample volumes, flow rates, and column residence times that are possible with modern HPLC equipment. Another factor that leads to the good precision in HPLC-based affinity methods is the reduced batch-to-batch variation, which is the result of using the same ligand for the analysis of multiple samples and calibrators. It has been reported in many studies that several hundred injections can be performed on the same affinity column, provided that proper elution and regeneration conditions have been selected. In some cases, there have been reports were affinity columns have been used for >1000 injections with no serious signs of degradation *(2, 5, 6)*.

One limitation of the direct detection format in affinity chromatography is that this requires the presence of enough analyte to allow the measurement of this substance as it elutes from the affinity column; in HPLC-based systems this is usually performed by on-line ultraviolet/visible absorbance or fluorescence detectors. Such a requirement tends to make the direct detection mode

[1] Nonstandard abbreviations: HPAC, high-performance affinity chromatography; HSA, human serum albumin; IAC, immunoaffinity chromatography; HPIAC, high-performance IAC; PTH, parathyrin; GC, gas chromatography; CE, capillary electrophoresis; RPLC, reversed-phase liquid chromatography; AChE, acetylcholinesterase; AGP, α_1-acid glycoprotein; and BSA, bovine serum albumin.

most useful when dealing with intermediate-to-high concentration solutes in clinical samples. However, it is also possible to use direct detection with trace sample components if the affinity column is combined with precolumn sample derivatization and/or more sensitive detection schemes, such as an off-line immunoassay or a suitable postcolumn reactor *(5)*.

A second potential limitation of the direct detection mode is that samples and calibrators are analyzed sequentially by the affinity column rather than in batch mode. This makes the direct detection format most valuable in situations where low-to-moderate numbers of samples are being processed and/or fast turnaround times per sample are desired. It should be noted, however, that sequential analysis has the advantage of making affinity chromatography easier to troubleshoot than batch-mode techniques and easier to determine whether the assay is operating satisfactorily before patient samples are tested.

BORONATE AFFINITY CHROMATOGRAPHY

Affinity methods that use boronic acid or boronates as ligands are one group of chromatographic techniques that have been used successfully with clinical samples. This group of methods, known collectively as "boronate affinity chromatography", includes one of the earliest reported quantitative applications of affinity chromatography in the clinical laboratory—namely, the determination of glycohemoglobin for the assessment of long-term diabetes management (Fig. 2) *(7–15)*. At a pH above 8, most boronate derivatives form covalent bonds with compounds that contain cis-diol groups in their structure. Because sugars such as glucose possess cis-diol groups, boronates are valuable for resolving glycoproteins (e.g., glycohemoglobin) from non-glycoproteins (e.g., normal hemoglobin).

The first use of a boronate affinity column for the determination of glycohemoglobin was by Mallia et al. *(8)* in 1981, where a low-performance agarose gel was used as the support and absorbance detection at 414 nm was used to quantify the retained and nonretained hemoglobin fractions in human hemolysate samples. Elution was performed by passing through the column a soluble diol-containing agent (i.e., sorbitol) that displaced the retained glycohemoglobin from the column; alternatively, a decrease in mobile phase pH could also be used for elution *(9)*. After the initial report by Mallia et al., similar low-performance methods were reported or evaluated by other groups *(10–13)*. The same approach has since been adapted for use in HPAC and HPLC-based systems *(9, 11, 14, 15)*.

In addition to hemoglobin, it is possible to use boronate columns to look at other types of glycoproteins in samples. For example, by monitoring the absorbance at 280 nm instead of 410–415 nm, the technique used for glycohemoglobin can easily be modified to determine the relative amount of all glycated proteins in a sample *(14)*. 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, such as is done by using absorbance measurements at 410–415 nm for the quantification of glycohemoglobin. Examples of this later approach include the use of boronic acid columns followed by an immunoassay for the detection of glycated albumin in serum and urine *(16)* or for the determination of glycated apolipoprotein B in serum *(17)*.

LECTIN AFFINITY CHROMATOGRAPHY

Lectins are another class of ligands that have been used for the direct detection of clinical analytes by affinity chromatography. The lectins are non-immune system proteins that have the ability to recognize and bind certain types of carbohydrate residues *(18)*. Two lectins that are often placed into affinity columns are concanavalin A, which binds to α-D-mannose and α-D-glucose residues, and wheat germ agglutinin, which binds to D-*N*-acetylglucosamine. Other lectins that can be used are jacalin and lectins found in peas, peanuts, or soybeans. These ligands

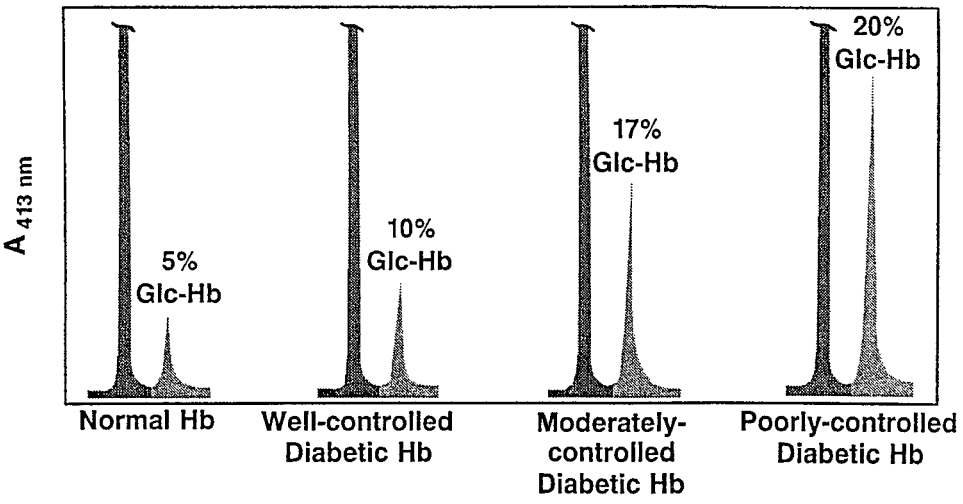


Fig. 2. Determination of glycohemoglobin (*Glc-Hb*) by HPAC for 10-μL samples of diluted whole blood.

Adapted with permission from Singhal and DeSilva *(14)*.

commonly are used in the isolation of many carbohydrate-containing compounds, such as polysaccharides, glycoproteins, and glycolipids *(2, 3)*.

One clinical application of lectin affinity chromatography has been in the separation and analysis of isoenzymes. This is illustrated in Fig. 3, where an HPLC column containing immobilized wheat germ agglutinin was used to distinguish between the liver- and bone-derived isoenzymes of alkaline phosphatase in human serum *(19)*. This method showed improved resolution of the isoenzymes vs a low-performance affinity column *(20)* and gave good correlation for a variety of patient samples when compared with a solid-phase immunoassay for alkaline phosphatase *(21)*.

A variety of other glycoproteins also have been studied and quantified by the use of lectin affinity columns. For example, low-performance columns based on concanavalin A have been used to separate apolipoprotein A- and apolipoprotein B-containing lipoproteins in human plasma *(22)*, to study the microheterogeneity of serum transferrin during alcoholic liver disease *(23)*, and to characterize the carbohydrate structure of follicle-stimulating hormone and luteinizing hormone under various clinical conditions *(24)*. A combination of concanavalin A and wheat germ agglutinin columns also has been used to identify changes that occur in asparagine-linked sugars on human prostatic acid phosphatase during prostate cancer *(25)*.

PROTEIN A OR PROTEIN G AFFINITY CHROMATOGRAPHY

A third class of ligands that have been used in direct analyte detection by affinity chromatography are antibody-binding proteins such as protein A and protein G. These are bacterial cell wall proteins produced by *Staphylococcus aureus* and group G streptococci, respectively *(26–28)*. These ligands have the ability to bind to the constant region of many types of immunoglobulins. Protein A and protein G bind most strongly to immunoglobulins at or near neutral pH, but readily dissociate from these solutes when placed in a buffer with a lower pH. These two ligands differ in their ability to bind to antibodies from different species and classes *(3, 26, 29)*; for example, human IgG_3 binds much more strongly to protein G than protein A, and human IgM shows no binding to protein G but does interact weakly with protein A *(3)*. A recombinant protein known as protein A/G, which blends the activities of these ligands, also is available for use in affinity columns *(3, 30)*.

The ability of protein A and protein G to bind to antibodies make these good ligands for the analysis of immunoglobulins, especially IgG-class antibodies, in humans. The first clinical uses of these ligands in an HPLC system were methods based on immobilized protein A for the analysis of IgG in serum samples *(31, 32)*. A similar method for the determination of IgG in serum has been developed based on high-performance protein G columns *(33)*. Yet another study used a combination of two affinity columns, one containing immobilized protein A and the other containing anti-human serum albumin (HSA) antibodies, for the simultaneous analysis of IgG and albumin in serum for the determination of albumin/IgG ratios (Fig. 4) *(34)*. An additional application of protein A and protein G has been as secondary ligands for the adsorption of antibodies onto supports to be used in immunoaffinity chromatography, as discussed in the following section. This particular method can be used when high antibody activities are needed or if it is desirable to

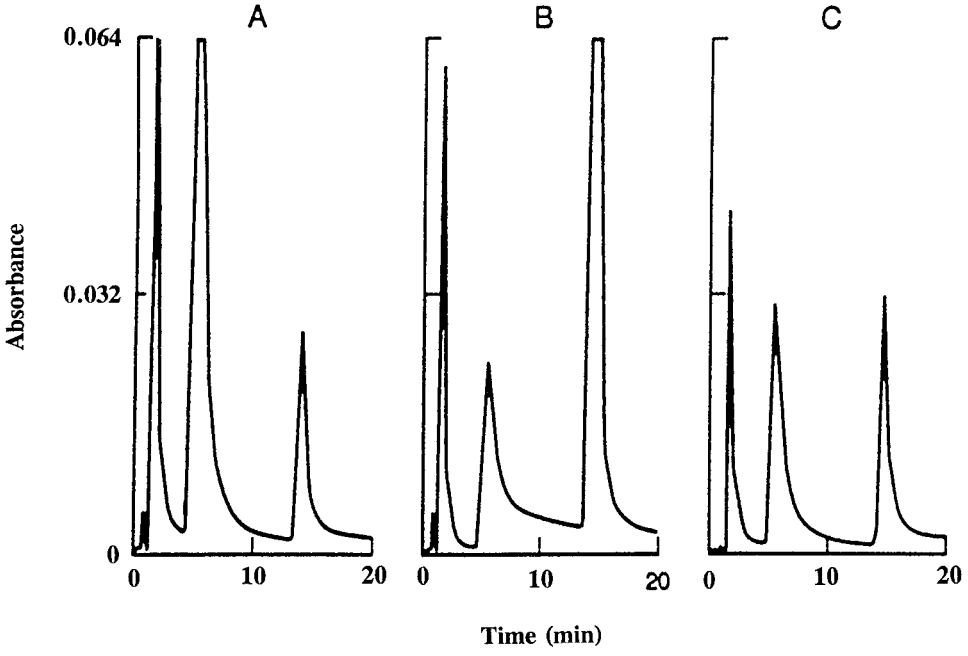


Fig. 3. Determination of liver and bone-derived isoenzymes of alkaline phosphatase by HPAC, using an immobilized wheat germ agglutinin column for 50-μL injections of serum from patients with liver (*A*) or bone (*B*) disease, and healthy individuals (*C*).

The peaks at 5.6–5.7 min and 15.0–15.2 min are produced by the liver- and bone-derived isoenzymes, respectively. Adapted with permission from Gonchoroff et al. *(21)*.

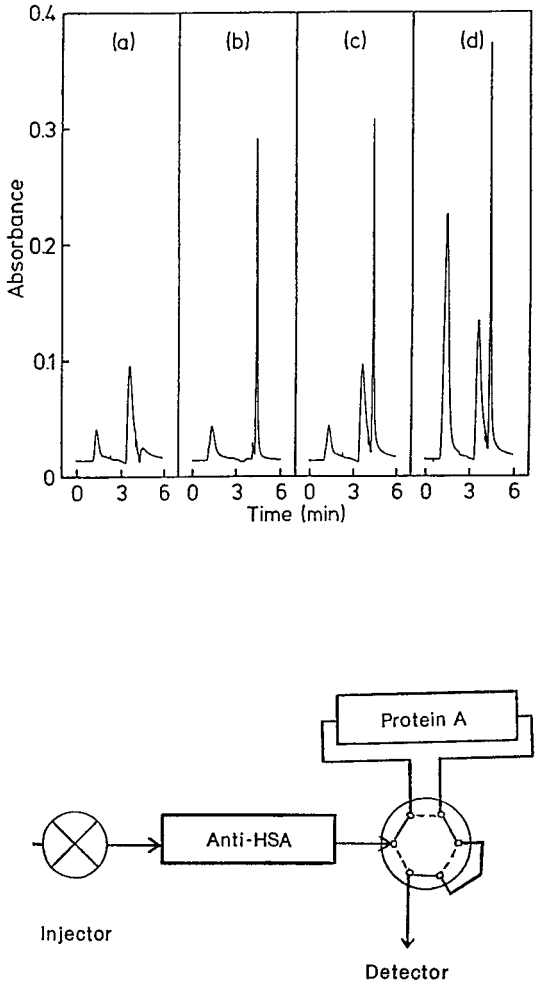


Fig. 4. Chromatograms (*top*) and valve switching system (*bottom*) for 10-μL injections of an HSA calibrator (*a*), an IgG calibrator (*b*), a mixture of HSA and IgG (*c*), and a 1:5 dilution of serum (*d*) on a dual column HPAC system containing anti-HSA antibodies and immobilized protein A.

The *solid lines* within the six-port valve shown at the *bottom* represent the configuration of the system during sample injection and later elution of IgG from the protein A column; the *dashed lines* show the position of the valve during the elution of albumin from the anti-HSA column. Adapted with permission from Hage and Walters *(34)*.

replace the antibodies in the affinity column frequently *(5, 6, 35)*.

IMMUNOAFFINITY CHROMATOGRAPHY

Of all the types of affinity chromatography, those that use antibodies or antibody fragments as ligands make up the largest and most diverse group of affinity methods in clinical testing. This is a combined result of the specificity of antibodies and the relative ease with which they can be obtained to a wide variety of analytes. The term "immunoaffinity chromatography" (IAC) is used for an affinity chromatographic method in which the stationary phase consists of an antibody or antibody-related reagent *(5, 35)*. When such a technique is performed as part of an HPLC system, the resulting method can be referred to as "high-performance immunoaffinity chromatography" (HPIAC) *(5, 35)*.

Several examples of direct analyte detection by HPLC-based IAC are described in Ref. *(5)*. Some clinical applications that have been reported include methods developed for anti-idiotypic antibodies *(36, 37)*, glucose-containing tetrasaccharides *(38, 39)*, granulocyte colony-stimulating factor *(40)*, HSA *(34, 41)*, IgG *(42)*, immunoglobulin E *(43)*, interferon *(44, 45)*, tumor necrosis factor-α *(45)*, interleukins *(45, 46)*, β_2-microglobulin *(47)*, and transferrin *(48)*. One such example (i.e., the determination of fibrinogen in human plasma) is illustrated in Fig. 1 *(49)*. In this particular case, the amount of fibrinogen in the retained peak was determined by the measurement of its absorbance at 280 nm. The sample was a 20-μL aliquot of plasma diluted 1:10. The retained peak appeared at 6 min, and the time between sample injections was 15 min, which included 9 min for column reequilibration *(49)*.

Both large and small analytes can be determined by the use of direct detection in IAC. Furthermore, it is possible to utilize immunoaffinity columns either separately or in combination with other affinity columns. This was demonstrated in the previous section in the discussion of the dual-column immunoaffinity/protein A method for the analysis of HSA and IgG in serum *(34)*. A similar approach recently has been used with fluorescent labeled samples and up to 10 separate immunoaffinity columns connected in series for the simultaneous determination of various cytokines in clinical samples (Fig. 5) *(45)*. In theory, this multicolumn format could be used with even larger numbers of compounds and with other combinations of analytes. The fact that a single sample aliquot is required for all of the columns makes this technique appealing in situations where only a limited amount of a patient's sample may be available for analysis.

When used as part of an HPLC system, the direct detection of analytes as they elute from immunoaffinity columns usually involves monitoring their ultraviolet/visible absorbance. However, special methods for the detection of low-concentration analytes have also been devised that use precolumn derivatization to place fluorescent tags *(40, 45, 46)* or radiolabels *(39)* onto sample solutes before injection. Alternatively, the column eluate can be collected in fractions and later analyzed by an immunoassay *(43, 47)* or receptor assay *(46)* that is specific for the species of interest. In addition, specialized methods can be combined with IAC to monitor compounds that elute in the nonretained fraction of the sample. This latter approach was used recently in a method that combined an HPIAC column and flow injection analysis for the determination of urinary albumin/creatinine ratios. This technique used an anti-albumin immunoaffinity column for the capture and detection of HSA and a Jaffé-based colorimetric reactor for the quantification of creatinine in the portion of the sample that was not bound by the antibody column *(30)*. This is yet

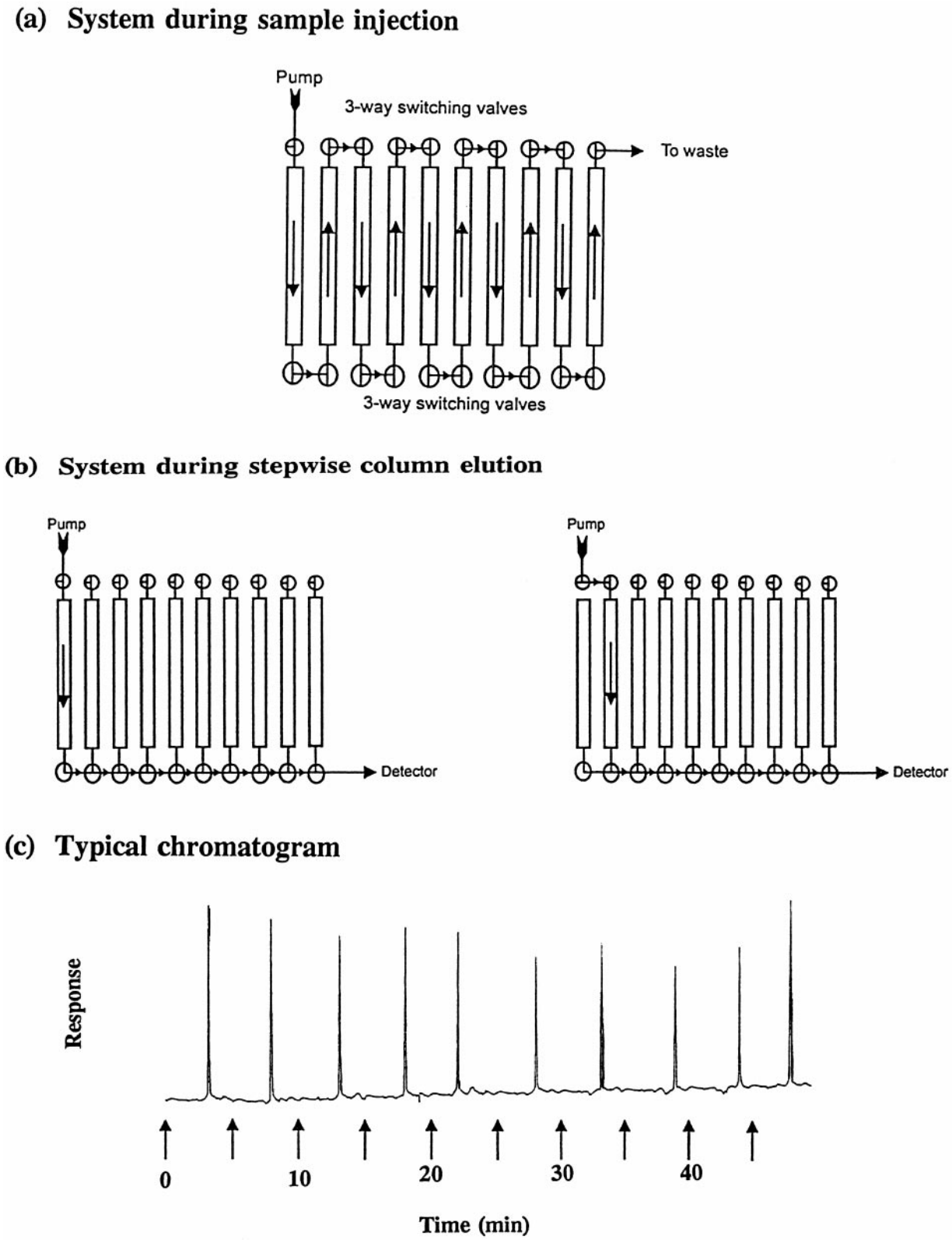


Fig. 5. Diagram of a recycling immunoaffinity system for the determination of multiple analytes during sample application (*a*) and stepwise analyte elution (*b*) from each column in the system; and chromatogram (*c*) showing results obtained for the analysis of a calibration mixture.

In (*c*), the sample contains (*left* to *right*) interleukin-1 (IL-1), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, tumor necrosis factor-α, and γ-interferon, each present at a concentration of 100 ng/L in the injected sample. The *arrows* in (*c*) indicate the times at which the valve configuration was changed for stepwise analyte elution. Adapted with permission from Phillips and Krum *(45)*.

another approach that could be useful when only small amounts of sample are available and information on several clinical analytes is desired.

COLUMNS BASED ON MISCELLANEOUS LIGANDS

In addition to the ligands that have been mentioned already, several other types of ligands have also been used for the direct detection of clinical analytes by affinity chromatography. For example, an immobilized heparin column has been used for the determination of antithrombin III in human plasma *(50, 51)*. *S*-Octylglutathione has been reported as a ligand for the separation and analysis of glutathione *S*-transferase isoenzymes in human lung and liver samples *(52, 53)*. And finally, 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 *(54)*.

Chromatographic Immunoassays

One relatively new area of affinity chromatography that has received increasing attention in recent years has been the use of both low- and high-performance immunoaffinity columns to perform various types of immunoassays. Such an approach is known as a "chromatographic (or flow-injection) immunoassay". This technique has been reviewed recently *(5, 6, 55–58)*. This format is particularly valuable in the determination of trace analytes that by themselves may not produce a readily detectable signal when analyzed directly by affinity chromatography. This problem is overcome in chromatographic immunoassays by the use of a labeled antibody or analyte analog that can be used for indirect analyte detection.

Many of the same labels that have been used in traditional immunoassays have also been used within chromatographic-based immunoassays. For example, enzyme labels such as horseradish peroxidase, alkaline phosphatase, and glucose oxidase have all been used in such methods. Other labels that have been reported include fluorescent tags such as fluorescein, Texas red, or lucifer yellow; chemiluminescent labels based on acridinium esters; and liposomes impregnated with fluorescent dye molecules *(5)*. The detection of these labels generally is performed on line as they elute in the nonretained or retained peaks of the immunoaffinity column; however, fraction collection and off-line detection can also be used when required.

COMPETITIVE BINDING IMMUNOASSAYS

There are several different methods for performing chromatographic immunoassays, but the most common method uses a competitive binding format. The easiest approach is to mix the sample with a labeled analyte analog (i.e., the label) and to inject the mixture simultaneously onto an immunoaffinity column that contains a relatively small amount of antibody. This format, known as a "simultaneous injection competitive binding immunoassay", is the most common approach for chromatographic immunoassays. A specific example of this method is shown in Fig. 6, in which theophylline was measured in serum by a flow-injection competitive binding immunoassay that used carboxyfluorescein-impregnated liposomes as the label. The injected samples contained 50 μL of serum diluted 1:100 and combined in a 1:2 ratio with a working solution of the label. The total cycle time between samples was 16 min, and good correlation was noted vs a fluorescence polarization immunoassay *(59)*. Other clinical analytes that have been measured by simultaneous injection competitive binding immunoassays include human chorionic gonadotropin *(60)*, thyroid-stimulating hormone *(60)*, HSA *(61)*, IgG *(62, 63)*, testosterone *(64)*, and transferrin *(61, 65)*; additional studies with theophylline have also been reported *(66–69)*.

An alternative format that has been explored for clinical testing involves the application of only sample to the immunoaffinity column, followed later by a separate injection of the label. This method is known as a "sequential injection competitive binding immunoassay" *(61, 70)*.

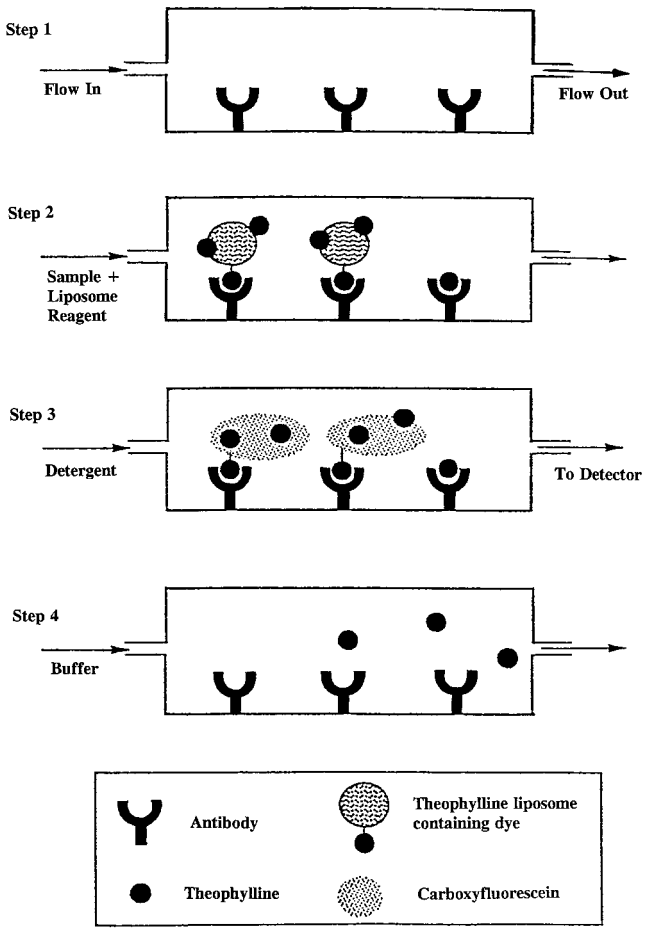


Fig. 6. Scheme for a theophylline flow-based simultaneous injection competitive binding immunoassay, using a label that consists of liposomes impregnated with carboxyfluorescein as a fluorescent marker.

Reproduced with permission from Locascio-Brown et al. *(59)*.

One advantage of the sequential injection approach is that even an unlabeled preparation of analyte potentially can be used as the label, provided that this species produces a sufficient signal for detection; this method is particularly useful for complex samples that contain analytes at moderate-to-high concentrations in complex mixtures. Another advantage of this technique over the simultaneous injection format is that there are no matrix interferences present during detection of the label because it is never in contact with the actual sample. However, the sequential injection method does require an additional step vs the simultaneous injection method for the separate application of label to the immunoaffinity column. One strength of both the simultaneous and sequential injection competitive binding methods is that they can be used equally well for either small or large analytes.

SANDWICH IMMUNOASSAYS

The sandwich immunoassay, or two-site immunometric assay, can also be performed as part of an affinity chromatographic system *(71–74)*. In this technique, two different types of antibodies that bind to the analyte of interest are used. The first of these two antibodies is attached to a chromatographic support and is used to extract the analyte from samples. The second antibody contains an easily measured tag and is added in solution to the analyte either before or after sample injection; this second antibody serves to place a label onto the analyte, thus allowing the amount of analyte on the immunoaffinity support to be quantified as it and the label are eluted from the column.

One clinical application in which a chromatographic sandwich immunoassay has been used is in the determination of intact parathyrin (PTH) in plasma *(73, 74)*. Fig. 7 shows a typical calibration curve and correlation plot for this technique. This particular method involved incubation of plasma samples combined in a 2:1 ratio with a working solution of anti-(1–34 PTH) acridinium ester-labeled antibodies for 1 h. After the incubation, a 100-μL aliquot of this mixture was injected onto an immunoaffinity column containing anti-(44–68 PTH) antibodies, thus producing formation of sandwich immune complex within the column. The amount of retained PTH was then determined by examination of the signal produced by the labeled antibodies as they were eluted at pH 3.0 and passed through a postcolumn chemiluminescence reactor. The total time per sample injection was 6.0–6.5 min, and the limit of detection was 0.2 pmol/L PTH *(73, 74)*. Other analytes that have been examined by chromatographic sandwich immunoassays are some antigen-specific antibodies *(71)* and IgG *(72)*.

Like its traditional solid-phase counterpart, an important advantage of the chromatographic sandwich immunoassay is that it produces a signal for the bound label that is directly proportional to the amount of injected analyte (Fig. 7a). The use of two types of antibodies in a sandwich immunoassay gives this technique higher selectivity than chromatographic-based competitive binding immunoassays. The main disadvantage of the chromatographic sandwich immunoassay is that it can be used only for analytes such as large peptides or proteins that are large enough to bind simultaneously to two separate antibodies.

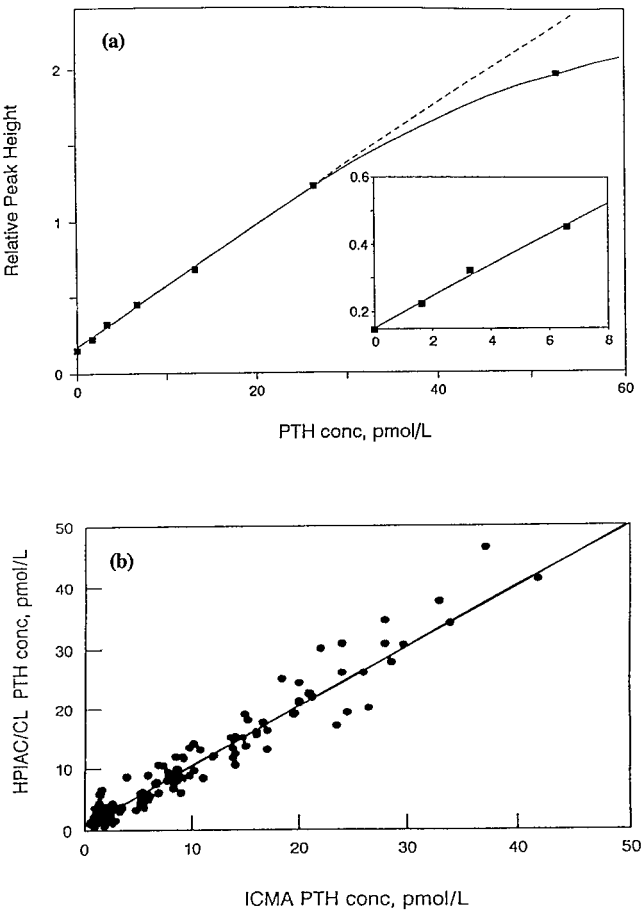


Fig. 7. Calibration curve (*a*) and correlation plot (*b*) vs a manual immunochemiluminometric assay (*ICMA*) for the determination of intact PTH in human plasma by use of a sandwich immunoassay format in HPIAC with chemiluminescence detection (*HPIAC/CL*).
The *inset* in (*a*) shows an expanded view of the lower end of the calibration curve. Reproduced with permission from Hage et al. *(75)*.

ONE-SITE IMMUNOMETRIC ASSAYS

This is the third format that has been used to automate immunoassays by affinity chromatography. This approach has been used in determining such agents as thyroxine *(55)* and α-(difluoromethyl)ornithine *(75)*. In this technique, the sample first is incubated with a known excess of labeled antibodies or Fab fragments that are specific for the analyte of interest. After binding between the analyte and antibodies has occurred, this mixture is applied to a column that contains an immobilized analog of the analyte. This column serves to extract any antibodies or Fab fragments that are not bound to the original analyte. Meanwhile, those antibodies or Fab fragments

that are bound to analyte from the sample will pass through the column in the nonretained peak, which is then detected and used for analyte quantification. Like the chromatographic competitive binding immunoassays, this method is able to detect both small and large solutes. However, like a chromatographic sandwich immunoassay, it also gives a signal for the nonretained label that is directly proportional to the amount of analyte in the original sample. One disadvantage of this approach is that relatively pure and highly active labeled antibodies/Fab fragments must be used to provide a low background signal.

Affinity Extraction

The technique of "affinity extraction" refers to the use of affinity chromatography for the isolation of a specific solute or group of solutes from a sample before their determination by a second analytical method. This uses the same general operating scheme as other types of affinity chromatography, but now involves combining the affinity column either off-line or on-line with some other method for the actual quantification of analytes. Affinity extraction represents one of the most common uses of affinity chromatography in chemical analysis. This section will examine some applications of affinity extraction, including both off-line methods and those that involve the on-line coupling of affinity columns with techniques such as HPLC, gas chromatography (GC), or capillary electrophoresis (CE).

off-line affinity extraction

Off-line extraction is the easiest method for combining an affinity column with another analytical technique. This approach typically involves the use of an affinity ligand that is immobilized onto a low-performance support (e.g., activated agarose) that is packed into a small disposable syringe or solid-phase extraction cartridge. After the affinity column is conditioned with the necessary application buffer or conditioning solvents, the sample is applied and nonbound sample components are washed off of the packing, as shown in Fig. 1. An elution buffer is then applied, and the analyte is collected as it elutes from the column. In some cases, this eluted fraction is analyzed directly by a second technique, but in most situations the collected fraction is first dried and reconstituted in a solvent that is compatible with the method to be used for quantification. If needed, the collected solute fraction may also be derivatized before it is examined by other techniques to obtain improved detection or more appropriate physical properties (e.g., an increase in solute volatility before separation and analysis by GC).

The most common ligands in affinity extraction are antibodies, with the terms "immunoextraction" or "immunoaffinity extraction" often being used to refer to this particular extraction technique. Sample preparation by off-line immunoextraction has been the subject of several recent reviews *(5, 76–79)*, most of which have emphasized its applications in the area of drug residue analysis. Examples involving human samples include the use of immunoextraction before reversed-phase liquid chromatography (RPLC) in the determination of albuterol in plasma *(80)*, human chorionic gonadotropin in urine *(81)*, and ochratoxin A in human serum, plasma, or milk *(82)*. Off-line immunoextraction also has been used for sample clean-up before analysis by GC or GC–mass spectrometry in the determination of prostaglandins and thromboxanes *(83–86)* or alkylated DNA adducts *(87, 88)* in human urine. The same approaches have been used in several animal studies involving off-line immunoextraction and RPLC or GC for the detection of alkylated DNA adducts in DNA extracts from rats *(89)*, chloramphenicol in urine and tissue samples from pigs *(90)*, dexamethasone and flumethasone in equine urine *(91, 92)*, ivermectin and avermectin in sheep serum *(93)*, and estrogens *(94, 95)*, nortestosterone *(96)*, or trenbolone *(97)* in bovine urine and bile samples.

Although antibodies are the most popular ligands in off-line affinity extraction, they are not the only ligands used for this approach. For example, 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 *(98)*. Off-line boronic acid columns similarly have been used for the reversed-phase analysis of modified nucleosides in patients with gastrointestinal cancer *(99)* and in the purification of human platelet glycocalicin before analysis by anion-exchange HPLC *(100)*. A method based on a wheat germ agglutinin extraction column combined with high-performance anion-exchange chromatography has been reported as a means to purify and analyze angiotensinase A and aminopeptidase M in human urine and kidney samples *(101)*. Another application of affinity extraction is in the removal of specific interferences from samples. Examples include the use of protein A and anti-mouse immunoglobulin supports for the removal of human anti-mouse antibodies before the analysis of a sample by immunoassay *(102)* and with the use of anti-human immunoglobulin IAC or protein A supports to selectively adsorb enzyme-immune complexes (i.e., macroenzymes) from patient samples *(103)*.

It should always be kept in mind when using affinity extraction that many ligands (even antibodies) will show some binding or cross-reactivity with solutes that are closely related to the desired analyte in structure. Each affinity extraction method should be evaluated for such cross-reactivity by the use of 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, even if several solutes do bind to the same extraction column, this does not present a problem as long as the analyte can be resolved or discriminated from these other compounds by the method that is used for quantification. In many cases, this can even be used to

an advantage because it allows several species in the same class of compounds to be determined in a single analytical run. For example, the ability of antibodies to cross-react with a parent compound and related agents or metabolites has been used for the development of immunoextraction methods for 17α- and 17β-trenbolone *(97)*, 17α- and 17β-nortestosterone *(96)*, and diethylstilbestrol, dienestrol, and hexestrol *(94)*. This idea can be taken one step further by the use of multiple types of antibodies in the same column. This has been used in the HPLC analysis of testosterone, nortestosterone, methyltestosterone, trenbolone, zeranol, estradiol, diethylstilbestrol, and related compounds in urine, where samples were extracted off-line with an affinity column that contained seven different types of immobilized antibodies *(76)*.

One advantage of off-line affinity extraction is that the samples collected from the extraction column can be derivatized readily or placed into a different solvent between the sample purification and quantification steps. This advantage is particularly important when affinity extraction is combined with GC, where it is desirable to remove any water from the collected sample before injection onto the GC system and solute derivatization is often required to improve solute volatility or detection. Another advantage of off-line affinity extraction is that it is relatively easy to set up once an appropriate ligand preparation has been selected or obtained. The cost of an affinity extraction cartridge is typically much higher than for conventional solid-phase extraction; however, this difference can be minimized by the careful selection of application and elution conditions so that the same affinity cartridge can be used for multiple samples *(76)*.

on-line affinity extraction

The direct coupling of affinity extraction with other analytical methods is yet another area that has been the subject of increasing research. The use of immunoextraction columns as part of HPLC systems has been of particular interest *(5, 6)*. The relative ease with which immunoaffinity columns can be incorporated into an HPLC system makes this appealing as a means for automating immunoextraction methods and for reducing the time required for sample pretreatment. In addition, the relatively high precision of HPLC pumps and injection systems provides on-line immunoextraction with better precision than off-line extraction methods, because the on-line approach has more tightly controlled sample application and elution conditions.

Clinical applications of on-line immunoextraction in HPLC have been developed for such analytes as α_1-antitrypsin *(104)*, cortisol *(105)*, digoxin *(106)*, estrogens *(107, 108)*, human epidermal growth factor *(109)*, lysergic acid diethylamide, lysergic acid diethylamide analogs and metabolites *(110, 111)*, phenytoin *(112)*, propranolol *(110)*, Δ^9-tetrahydrocannabinol *(113)*, and transferrin *(104, 114)*. Additional details on these methods are provided in Ref. *(5)*. All of these particular examples have used immunoaffinity columns combined with standard analytical columns for RPLC; however, there have also been reports from the field of biotechnology that have described the use of on-line immunoextraction with size exclusion *(115)* or ion-exchange chromatography *(116, 117)*.

One reason for the large number of reports involving the combination of on-line immunoextraction with RPLC undoubtedly has to do with the popularity of RPLC in routine analytical separations. Another, more fundamental, reason arises from the fact that the elution buffer for an immunoaffinity column is an aqueous solvent that generally contains little or no organic modifier, a feature that makes this same buffer act as a weak mobile phase for RPLC. This means that as a solute elutes from an antibody-based column, it will tend to have strong retention on any on-line reversed-phase support, thus leading to analyte reconcentration. This effect is valuable for analytes that desorb slowly from immobilized antibody columns and thus are difficult to analyze by the direct detection mode of affinity chromatography.

One common format for on-line immunoextraction in RPLC (Fig. 8) involves injecting the sample onto an immunoaffinity extraction column, with the nonretained components being flushed into a waste container. The immunoaffinity column is then switched on-line with a RPLC precolumn, and an elution buffer is applied to the antibody support to dissociate any retained analyte. As these analytes elute, they are captured and reconcentrated at the head of the RPLC precolumn. After all solutes have left the immunoaffinity column, this column is then switched back off-line and regenerated by washing with the initial application buffer. Meanwhile, the RPLC precolumn is placed on-line with a larger analytical RPLC column, and both are developed with an isocratic or gradient elution scheme involving the application of a solvent with an increased organic modifier content. This causes analytes at the head of the RPLC precolumn to move through the analytical column and to be separated on the basis of their differences in polarity. As these solutes elute, they are monitored and quantified through the use of a flow-through detector.

Other ligands besides antibodies, particularly boronates, have been shown to be valuable in performing on-line affinity extraction with HPLC. Examples include several separation methods in which boronate columns have been combined with HPLC columns for the clinical analysis of catechol-related compounds such as epinephrine, norepinephrine, and dopamine *(118–120)*, dihydroxyphenylalanine *(121)*, dihydroxyphenylacetic acid *(121, 122)*, 5-*S*-cysteinyldopa *(123)*, and vanillylmandelic acid *(124)*. This same approach has been adapted for profiling *(125)* and quantifying ribonucleotides in urine *(126, 127)* and serum *(127)*.

Although not as common as on-line extraction in HPLC, there has been some work investigating the use of affinity extraction coupled directly with GC for the deter-

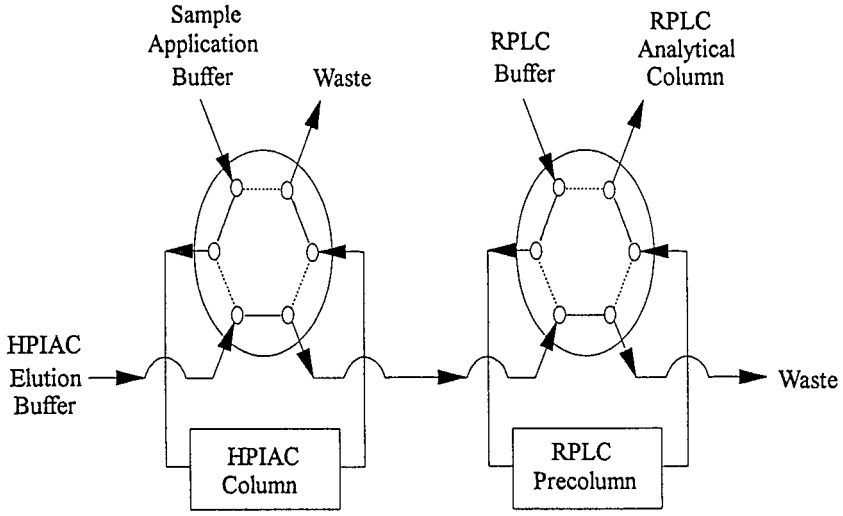


Fig. 8. Scheme for coupling on-line immunoextraction by HPIAC with compound separation and analysis by RPLC.

Reproduced with permission from Hage *(55)*.

mination of β-19-nortestosterone and related steroids in urine *(128)*. In this case, a RPLC precolumn was again used to capture and reconcentrate retained analytes as they eluted from an immunoaffinity extraction column. However, this RPLC precolumn also now served to remove any water from the analytes and to place them into a volatile organic solvent (ethyl acetate, which was used as the elution mobile phase). A portion of the analytes that eluted from the RPLC precolumn was then passed into the injection gap of a GC system. Once the solute/organic solvent plug had entered the GC system, a temperature program was initiated for solute separation. One advantage of this approach (and also of immunoextraction/HPLC) is that large volumes of sample can be applied to the immunoaffinity column, thus providing low detection limits. The main disadvantage of on-line immunoextraction in GC is the greater complexity of this method vs off-line immunoextraction or on-line immunoextraction/HPLC.

Several recent studies have considered the additional possibility of combining on-line immunoextraction with CE. For example, immunoextraction based on immobilized Fab fragments was used to extract and concentrate tear samples for the CE analysis of cyclosporin A and its metabolites in samples from corneal transplant patients (Fig. 9) *(129)*. In another study, antibodies were covalently immobilized in microcapillary bundles or in laser-drilled glass rods that were then connected to a CE capillary for the on-line immunoextraction and detection of immunoglobulin E in serum *(130)*. Finally, a capillary packed with a protein G chromatographic support has been used to adsorb antibodies for the extraction and concentration of insulin from serum before quantification by CE *(131)*.

Postcolumn Affinity Detection

Yet another way in which affinity columns can be used is for monitoring the elution of specific solutes from other chromatographic columns. This involves the use of a postcolumn reactor and an affinity column attached to the exit of an analytical HPLC column. A large number of affinity ligands can be used for this purpose. One specific example is the reported use of anion-exchange chromatography followed by an HPLC boronate column for the determination of glycated albumin in serum samples *(132)*. Another example is the recent use of immobilized

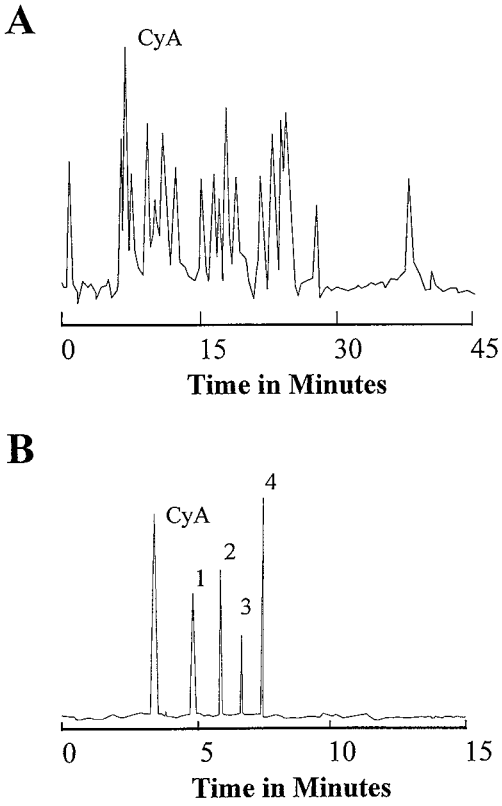


Fig. 9. Analysis of cyclosporin A in tear samples by routine CE (*a*) and immunoextraction coupled on-line with CE (*b*).

CyA, cyclosporin A; *peaks 1–4* in *panel b* represent various cyclosporin A metabolites. Adapted with permission from Phillips and Chmielinska *(129)*.

receptors for the detection of bioactive interleukin-2 as it eluted from an anti-interleukin immunoaffinity HPLC column *(47)*. As in many other affinity methods, most research in the area of postcolumn affinity detection has used immobilized antibody (or immobilized antigen) columns. This has given rise to a specific type of detection scheme known as "postcolumn immunodetection" *(5, 133)*.

The direct detection mode of affinity chromatography represents the simplest approach for postcolumn quantification of an analyte, provided that the solute is capable of generating a sufficiently strong signal for detection. One example of this approach involved the use of size exclusion chromatography and postcolumn immunodetection for the analysis of acetylcholinesterase (AChE) in amniotic fluid *(134)*. The method in this report used an immunoaffinity column containing anti-AChE antibodies to capture AChE as it eluted from the analytical column. After the AChE was adsorbed to the immunoaffinity column, a substrate solution for AChE was passed through the column, and the resulting colored product was detected by an on-line absorbance detector.

Other formats also are possible for postcolumn immunodetection, including techniques based on competitive binding immunoassays *(133, 135)* and sandwich immunoassays *(136)*. However, the one-site immunometric assay is the most common format for immunodetection, and it is the only additional approach that has been used previously in clinical applications. The basic operation of this format involves taking the eluate from the HPLC analytical column and combining this with a solution of labeled antibodies or Fab fragments that bind to the analyte of interest. The mixture of column eluate and antibody or Fab fragments is then allowed to react in a mixing coil and passed through an immunodetection column that contains an immobilized analog of the analyte. The antibodies or Fab fragments that are bound to the analyte will pass through this column and onto the detector, where they will provide a signal that is proportional to the amount of bound analyte. If desired, the immunodetection column can be washed later with an eluting solvent to dissociate the retained antibodies or Fab fragments; but a sufficiently high binding capacity is generally used so that a reasonably large amount of analytical column eluate can be analyzed before the immunodetection column must be regenerated.

One-site immunometric detection originally was used to quantify digoxin and digoxigenin as they eluted from a standard RPLC column by the use of fluorescein-labeled Fab fragments (raised against digoxigenin) and an immobilized digoxin support in the postcolumn detection system *(137)*. This method was then used to successfully monitor both digoxin and its metabolites in plasma and urine samples *(137)*. The same general system was later used along with a restricted-access RPLC column to monitor digoxin, digoxigenin, and related metabolites in serum samples *(138)*.

Affinity-based Chiral Separations

Another important application of affinity ligands has been in the analysis of chiral compounds *(139)*. Because of pressure from regulatory agencies such as the US Food and Drug Administration, there has been increasing interest in the pharmaceutical field for methods capable of discriminating between the individual chiral forms of drugs *(140)*. This has also touched on the field of clinical chemistry, where the ability to quantify the different chiral forms of a drug or its metabolites is increasingly used in studies of drug metabolism and in therapeutic monitoring. HPLC methods that include chiral stationary phases make up one set of tools that have been shown to be particularly valuable in the quantification and separation of chiral compounds *(139, 141)*. Because many of the ligands used in affinity chromatography are inherently chiral, this makes them logical choices as stationary phases for such separations.

As will be seen later, various naturally occurring proteins and carbohydrates have been used as ligands for chiral separations of clinical analytes *(142–161)*. Other, synthetic ligands that have also been used for chiral separations with clinical samples, such as derivatives of amylose or cellulose and Pirkle-type stationary phases *(162–189)*, but these other ligands will not be considered in this present review. Most clinical separations that will be discussed were performed by routine liquid-liquid or solid-phase extraction of the sample, with the content of this extract later being injected onto the chiral column of interest. However, this is not the only approach that can be used. In some cases, a chiral column was first used to resolve the enantiomers of a particular solute, followed by collection of these fractions and their on-line or off-line injection onto a second, achiral column for further separation and quantification (Fig. 10) *(150, 156)*. Alternatively, an achiral column, such as a reversed- or normal-phase support, was sometimes used to isolate the compounds from the sample, and a chiral column was then used on-line or off-line to resolve the enantiomers in each peak of interest *(151, 152)*.

protein-based stationary phases

Proteins are one group of affinity ligands that have received some attention as chiral HPLC stationary phases. Although all proteins are chiral, only one [α_1-acid glycoprotein (AGP)] has seen any significant use in the analysis of chiral drugs in clinical samples. AGP (also known as AAG or orosomucoid) is a human serum protein involved in the transport of many small solutes throughout the body. AGP differs from HSA (another drug-binding protein in serum) in that AGP has a lower isoelectric point and contains carbohydrate residues as part of its structure. The lower isoelectric point makes AGP more useful than serum albumin in binding cationic compounds, whereas the carbohydrate residues may play a role in determining the stereoselectivity of the binding properties of AGP *(141)*. There are many drugs and related solutes

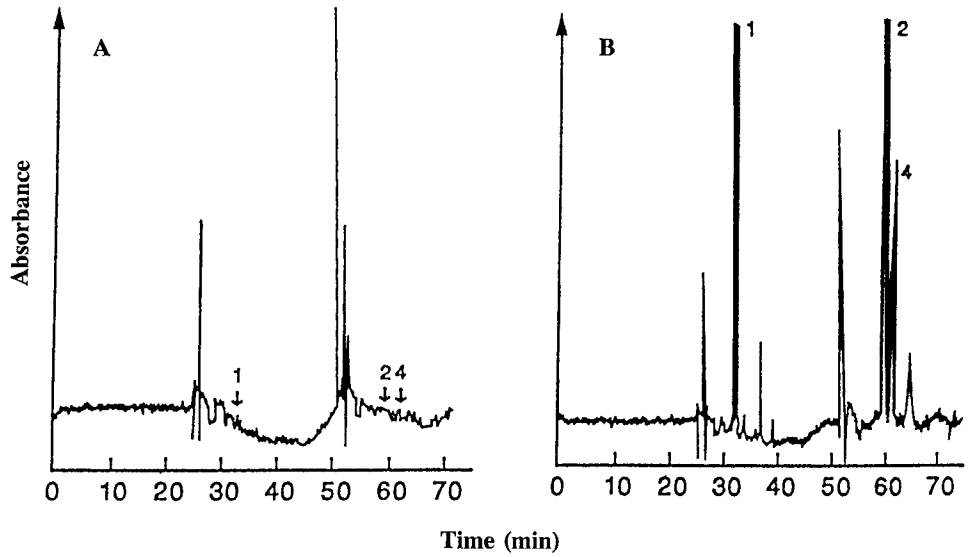


Fig. 10. Analysis of a blank plasma sample (*A*) and a plasma sample taken 1 h after intravenous administration of leucovorin (*B*) by a coupled-column HPLC system using a column containing a BSA chiral stationary phase and a C_{18} reversed-phase analytical column.
Peak 1, (6*S*)-leucovorin; *peak 2*, (6*R*)-leucovorin; *peak 4*, (6*R*)-5-methyltetrahydrofolate. Reproduced with permission from Silan et al. *(156)*.

that have been separated by AGP in human urine, serum, or plasma. Examples of clinical interest include bunolol *(142)*, citalopram *(143)*, fenoprofen *(144)*, flurbiprofen *(145)*, ibuprofen *(144, 146)*, ketamine *(147)*, ketoprofen *(144)*, methadone *(148–150)*, norketamine *(147)*, norverapamil *(151)*, pindolol *(152)*, thiopentone *(153)*, vamicamide *(154)*, and verapamil *(151, 155)*.

Other proteins that have received some attention in clinical applications of chiral HPLC are bovine serum albumin (BSA) and ovomucoid. Ovomucoid is a glycoprotein obtained from egg whites that has been shown to be useful in the separation of cationic solutes *(141)*. BSA is a member of the serum albumin family, which constitute most of the protein content of serum in mammals and are involved in the transport of a wide range of small organic and inorganic compounds throughout the body, including many pharmaceutical agents *(190, 191)*. BSA, and the related protein HSA, tend to bind best to neutral or anionic compounds, thus making these proteins complementary to AGP and ovomucoid in their applications *(139, 141)*. In clinical work, BSA has been used for the chiral separation of leucovorin in plasma *(156)*, and ovomucoid has been used for separating the individual forms of pentazocine in serum samples *(157)*.

carbohydrate-based stationary phases

One class of natural carbohydrates that can be used as stereoselective ligands in HPLC are the cyclodextrins *(158–161)*. 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 *(139, 141)*. The cone-shaped structure and hydrophobic interior cavity of cyclodextrins give them the ability to form inclusion complexes with numerous small, aromatic solutes. Furthermore, the well-defined arrangement of hydroxyl groups about the upper and lower faces of the cyclodextrins provide these agents with the ability to discriminate between various chiral compounds. Examples of some clinical applications for cyclodextrins in HPLC include methods reported for chlorpheniramine *(158)*, citalopram, desmethylcitalopram, and didesmethylcitalopram *(159)*, hexobarbital *(158)*, the M1 and M2 metabolites of moguisteine *(160)*, and propranolol *(161)*.

Characterization of Drug- and Hormone-Protein Interactions

In addition to its applications as a method for quantifying or isolating specific solutes, affinity chromatography can also be used in studying the interactions that take place between biomolecules. Such an approach is known as "analytical" or "quantitative affinity chromatography". This area has been the subject of several past reviews and has been used to examine a variety of biological systems, including lectin/sugar, enzyme/inhibitor, protein/protein, and DNA/protein interactions *(2, 192)*. However, most work in the clinical arena has focused on the use of this technique in the study of the binding of drugs or hormones to serum proteins *(193–195)*. In some instances, this type of protein binding occurs with general ligands, such as the interaction of many drugs with HSA or AGP *(196–199)*. In other cases, this binding is highly specific in nature, such as, the interactions of L-thyroxine with thyroxine-binding globulin or the binding of corticosteroids and sex hormones to steroid-binding globulins *(200, 201)*. This protein binding is of interest because it plays a role in determining the final biological activity, metabolism, and

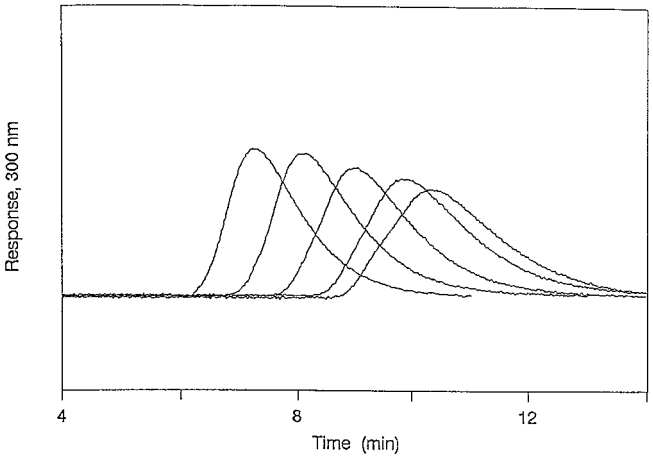
elimination of many drugs and hormones. In addition, the competition between drugs or between drugs and endogenous compounds (e.g., fatty acids or bilirubin) for protein binding sites can be an important source of drug-drug or drug displacement interactions *(196–199, 202)*.

Drug- and hormone-protein binding has been examined in affinity chromatography by the use of both immobilized drugs and immobilized proteins, but protein-based columns currently are more common *(195)*. One advantage of using an immobilized protein column for binding studies is the ability to reuse the same ligand preparation for multiple experiments (e.g., up to 500-1000 injections per column in some HPLC studies) *(203–205)*. It is important in such experiments to first consider and evaluate how effectively the immobilized protein models the behavior of the same protein in its soluble form. Fortunately, there is growing evidence that at least some immobilized proteins, particularly HSA, can be used quite successfully for the study of drug-protein interactions. For example, it has been shown that association constants measured by equilibrium dialysis for soluble HSA with *R*- and *S*-warfarin or L-tryptophan (i.e., solutes that interact with one of the two major binding regions of HSA) are in close agreement with values determined using immobilized HSA columns *(206–208)*. It has also been found that displacement phenomena and allosteric interactions observed for HSA columns are representative of behavior noted for HSA in solution *(208–213)*.

ZONAL ELUTION STUDIES

The method of zonal elution is the technique that has been used most frequently to study the binding of drugs and other solutes on immobilized protein columns *(193, 214)*. This generally is done by injecting a small sample of the drug or solute of interest into the presence of buffer only or a fixed concentration of a competing agent in the mobile phase. Analysis of the results is performed by determining how the elution time, or retention factor (k', also known as the capacity factor) of the injected solute changes as a function of the concentration of the competing agent (Fig. 11). Alternatively, similar experiments can be used to examine how various solvent conditions affect drug-protein interactions *(141, 207, 215–221)* or to develop quantitative structure-retention relationships that describe these binding processes *(222–224)*.

The most common application of zonal elution and HPLC-based affinity chromatography in drug- and hormone-protein studies has been in the examination of the displacement of drugs and hormones from proteins by other solutes *(195, 225)*. Examples include the use of zonal elution to examine the displacement of D,L-thyronine and D,L-tryptophan from HSA by bilirubin or caprylate *(226)*; the competition of *R/S*-warfarin with racemic oxazepam, lorazepam, and their hemisuccinate derivatives on an HSA column *(211)*; 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 *(213)*; the competition of *R*-warfarin and L-tryptophan with D-tryptophan *(207)* or L-thyroxine and related thyronine compounds on HSA *(203, 208)*; and the displacement of *R*- and *S*-ibuprofen by one another at their binding regions on HSA *(227)*. The same technique has been used to characterize the binding sites of nonsteroidal antiinflammatory drugs on HSA *(228)* and the displacement of nonsteroidal antiinflammatory drugs and benzodiazepines by phenylbutazone, *R/S*-ibuprofen, or 2,3,5-triiodobenzoic acid from serum albumin columns *(229)*. This type of work can provide not only qualitative information on binding and displacement, but also quantitative information on the equilibrium constants for these processes and retention mechanisms *(203, 208, 213, 222, 224, 227–229)*. Information on the kinetics of these solute-protein interactions can also be obtained if appropriate



Association constants for the binding of thyroid hormones and related analogs to HSA at 37°C

Compound	Association constant, K *(L/mol x 10^5)*	
	Warfarin site	Indole site
L-T_4	1.4 ± 0.1	5.7 ± 0.8
L-T_3	0.170 ± 0.001	0.25 ± 0.02
L-rT_3	1.99 ± 0.07	3.3 ± 0.6
L-T_2	0.63 ± 0.03	1.16 ± 0.06
L-T_0	0.18 ± 0.02	——
D-T_4	5.5 ± 0.9	29 ± 2
D-T_3	1.45 ± 0.06	1.2 ± 0.2

Fig. 11. Typical chromatograms (*top*) and association equilibrium constants (*bottom*) obtained by zonal elution studies examining the competitive binding of thyroid hormones with site-specific probe compounds for the warfarin and indole regions of HSA.

The chromatograms were obtained for the injection of *R*-warfarin into the presence of mobile phases containing (*left* to *right*) 1.90, 0.97, 0.49, 0.24, or 0.0 μmol/L of L-reverse triiodothyronine. T_4, thyroxine; T_3, triiodothyronine; rT_3, reverse triiodothyronine; T_0, thyronine. *L* and *D* in the *table* refer to the L- or D-enantiomers of each compound. Reproduced with permission from Loun and Hage *(203)*.

data are collected on the width and retention for solute peaks under various flow-rate conditions, as demonstrated recently for *R*- and *S*-warfarin *(204)* and D,L-tryptophan *(205)* on HSA columns.

FRONTAL ANALYSIS STUDIES

The technique of "frontal analysis" or "breakthrough analysis" is a second method that can be used in affinity chromatography to study biological interactions *(195)*. In this method, a solution containing a known concentration of the solute to be studied is applied continuously to an affinity column. As the solute binds to the immobilized ligand, the ligand becomes saturated and the amount of solute eluting from the column gradually increases, forming a characteristic breakthrough curve. If fast association and dissociation kinetics are present in the system, then the mean positions of the breakthrough curves can be related to the concentration of applied solute, the amount of ligand in the column, and the association equilibrium constants for solute-ligand binding. Regarding systems of clinical interest, frontal analysis and affinity chromatography have been used to investigate the binding of HSA to *R*- or *S*-warfarin *(206, 208)* and D- or L-tryptophan *(205, 207, 208, 213)*; to determine the binding capacities of monomeric vs dimeric HSA for salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulfamethizole, and sulfonylureas *(230)*; and to examine the competition of sulfamethizole with salicylic acid for HSA binding regions *(231)*. This same approach recently was used to characterize the binding of chemically modified HSA to various site-specific probe compounds *(232)*. Although frontal analysis generally requires more of a drug or hormone for study than zonal elution, this technique does tend to provide binding constants that are more precise and accurate than those measured by zonal elution methods *(195)*.

Future Trends and Developments

Although it is clear that affinity chromatography can be used in a variety of ways within clinical chemistry, there remains plenty of room for new growth and development in this method. One trend that has always been present in affinity chromatography has been the search for more selective, robust, and/or reproducible ligands. The availability of such ligands will become particularly important if affinity chromatography is to be accepted as a routine method in clinical laboratories.

There are several likely candidates of alternative ligands that should be useful in clinical testing but that have not yet been used for such applications. One example is a group of ligands based on synthetic dyes, such as triazine or triphenylmethane compounds, which are used in a technique known as "dye-ligand affinity chromatography". Specific ligands used in this method include Cibacron Blue F3G-A, Procion Blue MX-3G or MX-R, Procion Red HE-3B, and Thymol Blue or Phenol Red *(2, 3)*. Although these compounds are all synthetic in nature, they are still classified as affinity ligands because they interact with the active sites of many proteins and enzymes by mimicking the structure of the substrates, cofactors, or binding agents for these biomolecules. For example, Cibacron Blue F3G-A consists of a chlorotriazine ring that has several side groups attached, one of which is an anthraquinone that interacts with enzymes that have a binding site for NAD^+, $NADP^+$, or ATP. Some advantages of these dye ligands include their selectivity, reproducibility, and ability to be produced in large quantities. These properties have made them useful for the large-scale purification of dehydrogenases, kinases, albumin, α-fetoprotein, CoA-dependent enzymes, hydrolases, IgG, lipoproteins, nucleases, polymerases, synthetases, and transferases *(2, 3, 233, 234)*. It probably is only a matter of time until these dyes appear in affinity methods for the quantification of similar proteins and enzymes in clinical samples.

"Immobilized metal ion affinity chromatography", also known as "metal chelate affinity chromatography", is another method that has been widely used in purification processes but that has not yet received much attention in clinical testing. In this approach, the affinity ligand is a metal ion that is complexed with an immobilized chelating agent. Iminodiacetic acid is the most common chelating agent used, but carboxymethylaspartic acid, tris-carboxymethylethylenediamine, tris(2-aminoethyl)amine, or dipicolylamine sometimes are also used. The metal ions placed within these chelating groups are Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, or Fe^{3+}. This method separates proteins and peptides on the basis of interactions between certain amino acid residues (such as histidine, tryptophan, or cysteine) and the metal ions within the immobilized metal chelate *(235–237)*. Since its discovery, several peptides, proteins, and amino acids have been purified commercially by this method. Like dye-ligand affinity chromatography, immobilized metal ion affinity chromatography is quite selective and is based on ligands that can be made reproducibly on a large scale, again making this approach attractive for future work in clinical applications.

Two other types of ligands that may become important in clinical testing by affinity chromatography are those based on aptamers and molecular imprints. Aptamers are polymers of nucleotides that have well-defined sequences and three-dimensional structures. These are of current interest in research because it has been shown that a large number of aptamers can be generated randomly in an oligonucleotide library and then those ligands that bind to a given target solute can be enriched selectively for use in applications such as affinity chromatography *(238–240)*. A molecular imprint is an affinity ligand that is actually part of the surface or internal structure of the support used in the affinity column. These are usually made by combining the analyte of interest with a series of monomers that contain side chains capable of forming various interactions with the analyte. As these interactions take

place, the monomers are fixed in position about the analyte by polymerization. After polymerization has occurred, the support is ground into a powder, the retained analyte is released by the application of an appropriate solvent, and the imprinted support is placed in a column for use. In this way, an affinity support is created that has known specificity and binding/elution properties *(241, 242)*. Some appealing characteristics of both aptamers and molecular imprints are their ability to be custom-designed for a given analyte, their stability over long-term use, and their moderate-to-high selectivity *(238–242)*. However, more research and development in optimizing the use and production of these ligands is still needed before their full potential can be realized in clinical assays.

A second trend that is expected to continue in affinity chromatography is the search for improved system designs and formats that will give this technique greater speed, selectivity, and higher sample throughput. This is needed to make this approach competitive with more common clinical methods, such as batch-mode immunoassays. One way of obtaining increased selectivity while also increasing the number of solutes that are examined per assay is by using affinity chromatography in combination with other analytical techniques. This can already be seen in the growing popularity of the use of off-line affinity extraction with HPLC or GC and the use of on-line affinity extraction with HPLC. In the future, continued progress probably will be made in the development of such tandem methods, as well as in the further combination of on-line affinity extraction with GC *(128)* or CE *(129–131)* and mass spectrometry *(111, 243)*. As discussed earlier, another approach for obtaining increased sample throughput is to use an array of affinity columns in series for the determination of a battery of clinical analytes (Fig. 5). Alternatively, a group of identical affinity columns might be operated in parallel for determining the same solute in multiple samples.

The applications described in this review clearly demonstrate that affinity chromatography is an attractive alternative to traditional methods for the selective quantification and study of clinical samples. This combination of the large number of ligands that are available for affinity chromatography and the various operating formats that can be used for direct or indirect solute determination allows the creation of an affinity system for almost any compound of clinical interest. Affinity chromatography should become especially valuable to clinical laboratories as greater importance is placed on more specialized tests, such as the analysis of chiral drugs or the examination of drug- and hormone-protein binding. In the years to come, even more applications for this method should appear in clinical chemistry, as workers in this field become more familiar with affinity chromatography and the information that it can provide on clinical samples.

This work was supported in part by the National Institutes of Health (Grant GM44931).

References

1. International Union of Pure and Applied Chemistry. Nomenclature for chromatography. http://wingate.merck.de/english/services/chromatographie/iupac/chrnom.htm.
2. Hage DS. Affinity chromatography. In: Katz E, Eksteen R, Shoenmakers P, Miller N, eds. Handbook of HPLC. New York: Marcel Dekker, 1998:483–98.
3. Hermanson GT, Mallia AK, Smith PK. Immobilized affinity ligand techniques. New York: Academic Press, 1992:454 pp.
4. Larsson PO. High-performance liquid affinity chromatography. Methods Enzymol 1987;104:212–23.
5. Hage DS. A survey of recent advances in analytical applications of immunoaffinity chromatography. J Chromatogr 1998;715: 3–28.
6. de Frutos M, Regnier FE. Tandem chromatographic-immunological analyses. Anal Chem 1993;65:17A–25A.
7. Mayer TK, Freedman ZR. Protein glycosylation in diabetes mellitus: a review of laboratory measurements and of their clinical utility. Clin Chim Acta 1983;127:147–84.
8. Mallia AK, Hermanson GT, Krohn RI, Fujimoto EK, Smith PK. Preparation and use of a boronic acid affinity support for the separation and quantitation of glycosylated hemoglobins. Anal Lett 1981;14:649–61.
9. Hjerten S, Li JP. High-performance liquid chromatography of proteins on deformed non-porous agarose beads: fast boronate affinity chromatography of haemoglobin at neutral pH. J Chromatogr 1990;500:543–53.
10. Fluckiger R, Woodtli T, Berger W. Quantitation of glycosylated hemoglobin by boronate affinity chromatography. Diabetes 1984;33:73–6.
11. Gould BJ, Hall PM, Cook JGH. Measurement of glycosylated haemoglobins using an affinity chromatography method. Clin Chim Acta 1982;125:41–8.
12. Klenk DC, Hermanson GT, Krohn RI, Fujimoto EK, Mallia AK, Smith PK, et al. Determination of glycosylated hemoglobin by affinity chromatography: comparison with colorimetric and ion-exchange methods, and effects of common interferences. Clin Chem 1982;28:2088–94.
13. Johnson RN, Baker JR. Inaccuracy in measuring glycated albumin concentration by thiobarbituric acid colorimetry and by boronate chromatography. Clin Chem 1988;34:1456–9.
14. Singhal RP, DeSilva SSM. Boronate affinity chromatography. Adv Chromatogr 1992;31:293–335.
15. Kitagawa N, Treat-Clemens LG. Chromatographic study of immobilized boronate stationary phases. Anal Sci 1991;7:195–8.
16. Silver AC, Lamb E, Cattell WR, Dawnay ABSJ. Investigation and validation of the affinity chromatography method for measuring glycated albumin in serum and urine. Clin Chim Acta 1991;202: 11–22.
17. Panteghini M, Bonora R, Pagani F. Determination of glycated apolipoprotein B in serum by a combination of affinity chromatography and immunonephelometry. Ann Clin Biochem 1994;31: 544–9.
18. Liener IE, Sharon N, Goldstein IJ. The lectins: properties, functions and applications in biology and medicine. London: Academic Press, 1986:600 pp.
19. Anderson DJ, Branum EL, O'Brien JF. Liver- and bone-derived isoenzymes of alkaline phosphatase in serum as determined by high-performance affinity chromatography. Clin Chem 1990;36: 240–6.
20. Gonchoroff DG, Branum EL, O'Brien JF. Alkaline phosphatase

isoenzymes of liver and bone origin are incompletely resolved by wheat-germ-lectin affinity chromatography. Clin Chem 35;1989: 29–32.

21. Gonchoroff DG, Branum EL, Cedel SL, Riggs BL, O'Brien JF. Clinical evaluation of high-performance affinity chromatography for the separation of bone and liver alkaline phosphatase isoenzymes. Clin Chim Acta 1991;199:43–50.

22. Tavella M, Alaupovic P, Knight-Gibson C, Tournier H, Schinella G, Mercuri O. Separation of apoA- and apoB-containing lipoproteins of human plasma by affinity chromatography on concanavalin A. Prog Lipid Res 1991;30:181–7.

23. Inoue T, Yamauchi M, Toda G, Ohkawa K. Microheterogeneity with concanavalin A affinity of serum transferrin in patients with alcoholic liver disease. Alcohol Clin Exp Res 1996;20:363A–5A.

24. Papandreou MJ, Asteria C, Pettersson K, Ronin C, Beck-Peccoz P. Concanavalin A affinity chromatography of human serum gonadotropins: evidence for changes in carbohydrate structure in different clinical conditions. J Clin Endocrinol Metab 1993;76: 1008–13.

25. Yoshida KI, Honda M, Arai K, Hosoya Y, Moriguchi H, Sumi S, et al. Serial lectin affinity chromatography with concanavalin A and wheat germ agglutinin demonstrates altered asparagine-linked sugar-chain structures of prostatic acid phosphatase in human prostate carcinoma. J Chromatogr B 1997;695:439–43.

26. Lindmark R, Biriell C, Sjoequist J. Quantitation of specific IgG antibodies in rabbits by a solid-phase radioimmunoassay with ^{125}I-protein A from *Staphylococcus aureus*. Scand J Immunol 1981;14:409–20.

27. Ey PL, Prowse SJ, Jenkin CR. Isolation of pure IgG, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry 1978;15:429–36.

28. Bjorck L, Kronvall G. Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J Immunol 1984;133:969–74.

29. Aakerstrom B, Bjoerck L. A physiochemical study of protein G, a molecule with unique immunoglobulin G-binding properties. J Biol Chem 1986;261:10240–7.

30. Eliasson M, Olsson A, Palmcrantz E, Wibers K, Inganas M, Guss B, et al. Chimeric IgG-binding receptors engineered from staphylococcal protein A and streptococcal protein G. J Biol Chem 1988;263:4323–7.

31. Ohlson S. High performance liquid affinity chromatography (HPLAC) with protein A-silica. In: Chaiken IM, Wilchek M, Parikh I, eds. Affinity chromatography and biological recognition. New York: Academic Press, 1983:255–6.

32. Crowley SC, Walters RR. Determination of immunoglobulins in blood serum by high-performance affinity chromatography. J Chromatogr 1983;266:157–62.

33. Cassulis P, Magasic MV, DeBari VA. Ligand affinity chromatographic separation of serum IgG on recombinant protein G-silica. Clin Chem 1991;37:882–6.

34. Hage DS, Walters RR. Dual-column determination of albumin and immunoglobulin G in serum by high-performance affinity chromatography. J Chromatogr 1987;386:37–49.

35. Phillips TM. High performance immunoaffinity chromatography. LC Magazine 1985;3:962–972.

36. Phillips TM. High-performance immunoaffinity chromatographic detection of immunoregulatory anti-idiotypic antibodies in cancer patients receiving immunotherapy. Clin Chem 1988;34:1689–92.

37. Phillips TM, Babashak JV. Isolation of anti-idiotypic antibodies by immunoaffinity chromatography on Affinichrom beads. J Chromatogr 1990;512:387–94.

38. Wang WT, Kumlien J, Ohlson S, Lundblad A, Zopf D. Analysis of a glucose-containing tetrasaccharide by high-performance liquid affinity chromatography. Anal Biochem 1989;182:48–53.

39. Zopf D, Ohlson S, Dakour J, Wang W, Lundblad A. Analysis and purification of oligosaccharides by high-performance liquid affinity chromatography. Methods Enzymol 1989;179:55–64.

40. Phillips TM. Immunoaffinity measurement of recombinant granulocyte colony stimulating factor in patients with chemotherapy-induce neutropenia. J Chromatogr B 1994;662:307–13.

41. Ruhn PF, Taylor JD, Hage DS. Determination of urinary albumin using high-performance immunoaffinity chromatography and flow injection analysis. Anal Chem 1994;66:4265–71.

42. Phillips TM, More NS, Queen WD, Holohan TV, Kramer NC, Thompson AM. High-performance affinity chromatography: a rapid technique for the isolation and quantitation of IgG from cerebral spinal fluid. J Chromatogr 1984;317:173–9.

43. Phillips TM, More NS, Queen WD, Thompson AM. Isolation and quantitation of serum IgE levels by high-performance immunoaffinity chromatography. J Chromatogr 1985;327:205–11.

44. Phillips TM. Measurement of recombinant interferon levels by high performance immunoaffinity chromatography in body fluids of cancer patients on interferon therapy. Biomed Chromatogr 1992;6:287–90.

45. Phillips TM, Krum JM. Recycling immunoaffinity chromatography for multiple analyte analysis in biological samples. J Chromatogr B 1998;715:55–63.

46. Phillips TM. Measurement of total and bioactive interleukin-2 in tissue samples by immunoaffinity-receptor affinity chromatography. Biomed Chromatogr 1997;11:200–4.

47. Mogi M, Harada M, Adachi T, Kojima K, Nagatsu T. Selective removal of β_2-microglobulin from human plasma by high-performance immunoaffinity chromatography. J Chromatogr 1989; 496:194–200.

48. Ohlson S, Gudmundsson B-M, Wikstrom P, Larsson, P-O. High-performance liquid affinity chromatography: rapid immunoanalysis of transferrin in serum. Clin Chem 1988;34:2039–43.

49. McConnell JP, Anderson DJ. Determination of fibrinogen in plasma by high-performance immunoaffinity chromatography. J Chromatogr 1993;615:67–75.

50. Dawidowicz AL, Rauckyte T, Rogalski J. The preparation of sorbents for the analysis of human antithrombin III by means of high performance affinity chromatography. Chromatographia 1993;37:168–72.

51. Dawidowicz AL, Rauckyte T, Rogalski J. High performance affinity chromatography for analysis of human antithrombin III. J Liq Chromatogr 1994;17:817–31.

52. Wheatley JB, Kelley MK, Montali JA, Berry COA, Schmidt DE Jr. Examination of glutathione *S*-transferase isoenzyme profiles in human liver using high-performance affinity chromatography. J Chromatogr A 1994;663:53–63.

53. Wheatley JB, Montali JA, Schmidt DE Jr. Coupled affinity-reversed-phase high-performance liquid chromatography systems for the measurement of glutathione *S*-transferase in human tissues. J Chromatogr A 1994;676:65–79.

54. Abe I, Ito N, Noguchi K, Kazama M, Kasai KI. Immobilized urokinase column as part of a specific detection system for plasminogen species separated by high-performance affinity chromatography. J Chromatogr 1991;565:183–95.

55. Hage DS. Chromatographic approaches to immunoassays. J Clin Ligand Assay 1998;20:293–301.

56. Mattiasson B, Nilsson M, Berden P, Hakanson H. Flow-ELISA: binding assays for process control. Trends Anal Chem 1990;9: 317–21.

57. Gubitz G, Shellum C. Flow-injection immunoassays. Anal Chim Acta 1993;283:421–8.

58. Pollema CH, Ruzicka J, Lernmark A, Christian GD. Flow-injection

immunoassays: present and future. Microchem J 1992;45: 121–8.

59. Locascio-Brown L, Plant AL, Chesler R, Kroll M, Ruddel M, Durst RA. Liposome-based flow-injection immunoassay for determining theophylline in serum. Clin Chem 1993;39:386–91.

60. Johns MA, Rosengarten LK, Jackson M, Regnier FE. Enzyme-linked immunosorbent assays in a chromatographic format. J Chromatogr A 1996;743:195–206.

61. Cassidy SA, Janis LJ, Regnier FE. Kinetic chromatographic sequential addition immunoassays using protein A affinity chromatography. Anal Chem 1992;64:1973–7.

62. De Alwis U, Wilson GS. Rapid heterogeneous competitive electrochemical immunoassay for IgG in the picomole range. Anal Chem 1987;59:2786–9.

63. Valencia-Gonzalez MJ, Diaz-Garcia ME. Flow-through fluorescent immunosensing of IgG. Ciencia 1996;4:29–40.

64. Palmer DA, Evans M, Miller JN, French MT. Rapid fluorescence flow injection immunoassay using a novel perfusion chromatographic support. Analyst 1994;119:943–7.

65. Palmer DA, Xuezhen R, Fernandez-Hernando P, Miller JN. A model on-line flow injection fluorescence immunoassay using a protein A immunoreactor and lucifer yellow. Anal Lett 1993;26: 2543–53.

66. Durst RA, Locascio-Brown L, Plant AL. Automated liposome-based flow injection immunoassay system. In: Schmid RD, ed. Flow injection analysis (FIA) based on enzymes or antibodies. New York: VCH, 1991:181–90.

67. Yap WT, Locascio-Brown L, Plant AL, Choquette SJ, Horvath V, Durst RA. Liposome flow injection immunoassay: model calculations of competitive immunoreactions involving univalent and multivalent ligands. Anal Chem 1991;63:2007–11.

68. Rico CM, Del Pilar Fernandez M, Guiterrez AM, Conde MCP, Camara C. Development of a flow fluoroimmunosensor for determination of theophylline. Analyst 1995;120:2589–91.

69. Palmer DA, Edmonds TE, Seare NJ. Flow injection immunosensor for theophylline. Anal Lett 1993;26:1425–39.

70. Hage DS, Thomas DH, Beck MS. Theory of a sequential addition competitive binding immunoassay based on high-performance immunoaffinity chromatography. Anal Chem 1993;65:1622–30.

71. De Alwis WU, Wilson GS. Rapid sub-picomole electrochemical enzyme immunoassay for immunoglobulin G. Anal Chem 1985; 57:2754–6.

72. Gunaratna PC, Wilson GS. Noncompetitive flow injection immunoassay for a hapten, α-(difluoromethyl)ornithine. Anal Chem 1993;65:1152–7.

73. Hacker A, Hinterleitner M, Shellum C, Gubitz G. Development of an automated flow injection chemiluminescence immunoassay for human immunoglobulin G. Fresenius J Anal Chem 1995;352: 793–6.

74. Hage DS, Kao PC. High-performance immunoaffinity chromatography and chemiluminescent detection in the automation of a parathyroid hormone sandwich immunoassay. Anal Chem 1991; 63:586–95.

75. Hage DS, Taylor B, Kao PC. Intact parathyroid hormone: performance and clinical utility of an automated assay based on high-performance immunoaffinity chromatography and chemiluminescence detection. Clin Chem 1992;38:1494–500.

76. van Ginkel LA. Immunoaffinity chromatography, its applicability and limitations in multi-residue analysis of anabolizing and doping agents. J Chromatogr 1991;564:363–84.

77. Haagsma N, van de Water C. Immunochemical methods in the analysis of veterinary drug residues. In: Agarwal VK, ed. Analysis of antibiotic drug residues in food products of animal origin. New York: Plenum Press, 1992:81–97.

78. Katz SE, Siewierski M. Drug residue analysis using immunoaffinity chromatography. J Chromatogr 1992;624:403–9.

79. Katz SE, Brady MS. High-performance immunoaffinity chromatography for drug residue analysis. J Assoc Off Anal Chem 1990; 73:557–60.

80. Ong H, Adam A, Perreault S, Marleau S, Bellemare M. Du Souich P. Analysis of albuterol in human plasma based on immunoaffinity chromatographic clean-up combined with high-performance liquid chromatography with fluorometric detection. J Chromatogr 1989;497:213–21.

81. Liu CL, Bowers LD. Immunoaffinity trapping of urinary human chorionic gonadotropin and its high-performance liquid chromatographic–mass spectrometric determination. J Chromatogr B 1996;687:213–20.

82. Zimmerli B, Dick R. Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. J Chromatogr B 1995;666:85–99.

83. Bachi A, Zuccato E, Baraldi M, Fanelli R, Chiabrando C. Measurement of urinary 8-epi-prostaglandin $F_{2\alpha}$, a novel index of lipid peroxidation in vivo, by immunoaffinity extraction/gas chromatography–mass spectrometry. Basal levels in smokers and nonsmokers. Free Radic Biol Med 1996;20:619–24.

84. Mackert G, Reinke M, Schweer H, Seyberth HW. Simultaneous determination of the primary prostanoids prostaglandin E_2, prostaglandin $F_{2\alpha}$ and 6-oxoprostaglandin $F_{1\alpha}$ by immunoaffinity chromatography in combination with negative ion chemical ionization gas chromatography-tandem mass spectrometry. J Chromatogr 1989;494:13–22.

85. Chiabrando C, Pinciroli V, Campoleoni A, Benigni A, Piccinelli A, Fanelli R. Quantitative profiling of 6-ketoprostaglandin $F_{1\alpha}$, 2,3-dinor-6-ketoprostaglandin $F_{1\alpha}$, thromboxane B_2 and 2,3-dinor-thromboxane B_2 in human and rat urine by immunoaffinity extraction with gas chromatography–mass spectrometry. J Chromatogr 1989;495:1–11.

86. Ishibashi M, Watanabe K, Ohyama Y, Mizugaki M, Hayashi Y, Takasaki W. Novel derivatization and immunoextraction to improve microanalysis of 11-dehydrothromboxane B_2 in human urine. J Chromatogr 1991;562:613–24.

87. Prevost V, Shuker DEG, Friesen MD, Eberle G, Rajewsky MF, Bartsch H. Immunoaffinity purification and gas chromatography–mass spectrometric quantitation of 3-alkyladenines in urine: metabolism studies and basal excretion levels in man. Carcinogenesis 1993;14:199–204.

88. Friesen MD, Garren L, Prevost V, Shuker DEG. Isolation of urinary 3-methyladenine using immunoaffinity columns prior to determination by low-resolution gas chromatography–mass spectrometry. Chem Res Toxicol 1991;4:102–6.

89. Bonfanti M, Magagnotti C, Galli A, Bagnati R, Moret M, Gariboldi P, Fanelli R, Airoldi L. Determination of O^6-butylguanine in DNA by immunoaffinity extraction/gas chromatography–mass spectrometry. Cancer Res 1990;50:6870–5.

90. Gude T, Preiss A, Rubach K. Determination of chloramphenicol in muscle, liver, kidney and urine of pigs by means of immunoaffinity chromatography and gas chromatography with electron-capture detection. J Chromatogr B 1995;673:197–204.

91. Stanley SMR, Wilhelmi BS, Rodgers JP. Comparison of immunoaffinity chromatography combined with gas chromatography-negative ion chemical ionisation mass spectrometry and radioimmunoassay for screening dexamethasone in equine urine. J Chromatogr 1993;620:250–3.

92. Stanley SMR, Wilhelmi BS, Rodgers JP, Bertschinger H. Immunoaffinity chromatography combined with gas chromatography-negative ion chemical ionisation mass spectrometry for the

confirmation of flumethasone abuse in the equine. J Chromatogr 1993;614:77–86.

93. Li J, Zhang S. Immunoaffinity column cleanup and liquid chromatographic method for determining ivermectin in sheep serum. J AOAC Int 1996;79:1300–2.

94. Bagnati R, Castelli MG, Airoldi L, Oriundi MP, Ubaldi A, Fanelli R. Analysis of diethylstilbestrol, dienestrol and hexestrol in biological samples by immunoaffinity extraction and gas chromatography-negative-ion chemical ionization mass spectrometry. J Chromatogr 1990;527:267–78.

95. Bagnati R, Oriundi MP, Russo V, Danese M, Berti F, Fanelli R. Determination of zeranol and β-zeranol in calf urine by immunoaffinity extraction and gas chromatography–mass spectrometry after repeated administration of zeranol. J Chromatogr 1991; 564:493–502.

96. van Ginkel LA, Stephany RW, van Rossum HJ, van Blitterswijk H, Zoontjes PW, Hooijshuur RCM, Zuydendorp J. Effective monitoring of residues of nortestosterone and its major metabolite in bovine urine and bile. J Chromatogr 1989;489:95–104.

97. van Ginkel LA, van Blitterswijk H, Zoontjes PW, van den Bosch D, Stephany RW. Assay for trenbolone and its metabolite 17α-trenbolone in bovine urine based on immunoaffinity chromatographic clean-up and off-line high-performance liquid chromatography–thin-layer chromatography. J Chromatogr 1988;445:385–92.

98. Thienpont LM, Depourcq GC, Nelis HJ, De Leenheer AP. Liquid chromatographic determination of 2-thioxothiazolidine-4-carboxylic acid isolated from urine by affinity chromatography on organomercurial agarose gel. Anal Chem 1990;62:2673–5.

99. Nakano K, Shindo K, Yasaka T, Yamamoto H. Reversed-phase liquid chromatographic investigation of nucleosides and bases in mucosa and modified nucleosides in urines from patients with gastrointestinal cancer. J Chromatogr 1985;332:127–37.

100. DeCristofaro R, Landolfi R, Bizzi B, Castagnola M. Human platelet glycocalicin purification by phenyl boronate affinity chromatography coupled to anion-exchange high-performance liquid chromatography. J Chromatogr 1988;426:376–80.

101. Scherberich JE, Wiemer J, Herzig C, Fischer P, Schoeppe W. Isolation and partial characterization of angiotensinase A and aminopeptidase M from urine and human kidney by lectin affinity chromatography and high-performance liquid chromatography. J Chromatogr 1990;521:279–89.

102. Madry N, Auerbach B, Schelp C. Measures to overcome HAMA interferences in immunoassays. Anticancer Res 1997;17:2883.

103. Remaley AT, Wilding P. Macroenzymes: biochemical characterization, clinical significance, and laboratory detection. Clin Chem 1989;35:2261–70.

104. Flurer CL, Novotny M. Dual microcolumn immunoaffinity liquid chromatography: an analytical application to human plasma proteins. Anal Chem 1993;65:817–21.

105. Nilsson B. Extraction and quantitation of cortisol by use of high-performance liquid affinity chromatography. J Chromatogr 1983;276:413–7.

106. Reh E. Determination of digoxin in serum by on-line immunoadsorptive clean-up high-performance liquid chromatographic separation and fluorescence-reaction detection. J Chromatogr 1988;433:119–30.

107. Farjam A, Brugman AE, Lingeman H, Brinkman UAT. On-line immunoaffinity sample pre-treatment for column liquid chromatography: evaluation of desorption techniques and operating conditions using an anti-estrogen immuno-precolumn as a model system. Analyst 1991;116:891–6.

108. Farjam A, Brugman AE, Soldaat A, Timmerman P, Lingeman H, de Jong GJ, et al. Immunoaffinity precolumn for selective sample pretreatment in column liquid chromatography: immunoselective desorption. Chromatographia 1991;31:469–77.

109. Hayashi T, Sakamoto S, Wada I, Yoshida H. HPLC analysis of human epidermal growth factor using immunoaffinity precolumn. II. Determination of hEGFs in biological fluids. Chromatographia 1989;27:574–80.

110. Rule GS, Henion JD. Determination of drugs from urine by on-line immunoaffinity chromatography-high-performance liquid chromatography–mass spectrometry. J Chromatogr 1992;582:103–12.

111. Cai J, Henion J. On-line immunoaffinity extraction-coupled column capillary liquid chromatography/tandem mass spectrometry: trace analysis of LSD analogs and metabolites in human urine. Anal Chem 1996;68:72–8.

112. Johansson B. Simplified quantitative determination of plasma phenytoin: on-line pre-column high-performance liquid immunoaffinity chromatography with sample pre-purification. J Chromatogr 1986;381:107–13.

113. Kircher V, Parlar H. Determination of Δ^9-tetrahydrocannabinol from human saliva by tandem immunoaffinity chromatography-high-performance liquid chromatography. J Chromatogr B 1996; 677:245–55.

114. Janis LJ, Regnier FE. Dual-column immunoassays using protein G affinity chromatography. Anal Chem 1989;61:1901–6.

115. Riggin A, Sportsman JR, Regnier FE. Immunochromatographic analysis of proteins: identification, characterization and purity determination. J Chromatogr 1993;632:37–44.

116. Janis LJ, Regnier FE. Immunological-chromatographic analysis. J Chromatogr 1988;444:1–11.

117. Janis LJ, Grott A, Regnier FE, Smith-Gill SJ. Immunological-chromatographic analysis of lysozyme variants. J Chromatogr 1989;476:235–44.

118. Edlund PO, Westerlund D. Direct injection of plasma and urine in automated analysis of catecholamines by coupled-column liquid chromatography with post-column derivatization. J Pharm Biomed Anal 1984;2:315–33.

119. Ni P, Guyon F, Caude M, Rosset R. Automated determination of catecholamines using on-column extraction of diphenylboronate-catecholamine complexes and high-performance liquid chromatography with electrochemical detection. J Liq Chromatogr 1989; 12:1873–88.

120. Boos KS, Wilmers B, Sauerbrey R, Schlimme E. Development and performance of an automated HPLC-analyzer for catecholamines. Chromatographia 1987;24:363–70.

121. Edlund PO. Determination of dihydroxyphenylalanine and dihydroxyphenylacetic acid in biological samples by coupled-column liquid chromatography with dual coulometric-amperometric detection. J Pharm Biomed Anal 1986;4:625–39.

122. Hansson L, Glad M, Hansson C. Boronic acid-silica: a new tool for the purification of catecholic compounds on-line with reversed-phase high-performance liquid chromatography. J Chromatogr 1983;265:37–44.

123. Hansson C, Kagedal B, Kallberg M. Determination of 5-*S*-cysteinyldopa in human urine by direct injection in coupled-column high-performance liquid chromatography. J Chromatogr 1987;420:146–51.

124. Eriksson B-M, Wikstrom M. Determination of vanillylmandelic acid in urine by coupled-column liquid chromatography combining affinity to boronate and separation by anion exchange. J Chromatogr 1991;567:1–9.

125. Larsson P-O, Glad M, Hansson L, Mansson M-O, Ohlson S, Mosbach K. High-performance liquid affinity chromatography. Adv Chromatogr 1983;21:41–85.

126. Hagemeier E, Boos K-S, Schlimme E, Lechtenboerger K, Kettrup A. Synthesis and application of a boronic acid-substituted silica

for high-performance liquid affinity chromatography. J Chromatogr 1983;268:291–5.

127. Hagemeier E, Kemper K, Boos K-S, Schlimme E. On-line high-performance liquid affinity chromatography-high-performance liquid chromatography analysis of monomeric ribonucleoside compounds in biological fluids. J Chromatogr 1983;282:663–9.

128. Farjam A, Vreuls JJ, Cuppen WJGM, Brinkman UAT, de Jong GJ. Direct introduction of large-volume urine samples into an on-line immunoaffinity sample pretreatment-capillary gas chromatography system. Anal Chem 1991;63:2481–7.

129. Phillips TM, Chmielinska JJ. Immunoaffinity capillary electrophoresis analysis of cyclosporin in tears. Biomed Chromatogr 1994;8:242–6.

130. Guzman NA. Biomedical applications of on-line preconcentration-capillary electrophoresis using an analyte concentrator: investigation of design options. J Liq Chromatogr 1995;18:3751–68.

131. Cole LJ, Kennedy RT. Selective preconcentration for capillary zone electrophoresis using protein G immunoaffinity capillary chromatography. Electrophoresis 1995;16:549–56.

132. Yasukawa K, Abe F, Shida N, Koizumi Y, Uchida T, Noguchi K, Shima K. High-performance affinity chromatography system for the rapid, efficient assay of glycated albumin. J Chromatogr 1992;597:271–5.

133. Irth H, Oosterkamp AJ, Tjaden UR, van der Greef J. Strategies for on-line coupling of immunoassays to high-performance liquid chromatography. Trends Anal Chem 1995;14:355–61.

134. Vanderlaan M, Lotti R, Siek G, King D, Goldstein M. Perfusion immunoassay for acetylcholinesterase: analyte detection based on intrinsic activity. J Chromatogr A 1995;711:23–31.

135. Oosterkamp AJ, Irth H, Tjaden UR, van der Greef J. On-line coupling of liquid chromatography to biochemical assays based on fluorescent-labeled ligands. Anal Chem 1994;66:4295–301.

136. Cho BY, Zou H, Strong R, Fisher DH, Nappier J, Krull IS. Immunochromatographic analysis of bovine growth hormone releasing factor involving reversed-phase high-performance liquid chromatography-immunodetection. J Chromatogr A 1996; 743:181–94.

137. Irth H, Oosterkamp AJ, van der Welle W, Tjaden UR, van der Greef J. On-line immunochemical detection in liquid chromatography using fluorescein-labelled antibodies. J Chromatogr 1993;633: 65–72.

138. Oosterkamp AJ, Irth H, Beth M, Unger KK, Tjaden UR, van der Greef J. Bioanalysis of digoxin and its metabolites using direct serum injection combined with liquid chromatography and on-line immunochemical detection. J Chromatogr B 1994;653:55–61.

139. Armstrong DW. Optical isomer separation by liquid chromatography. Anal Chem 1987;59:84A–91A.

140. Chiral drugs. Chem Eng News 1993;71(Sept. 27):38–65.

141. Allenmark S. Chromatographic enantioseparation: methods and applications. New York: Ellis Horwood, 1991:282 pp.

142. Li F, Cooper SF, Cote M, Ayotte C. Determination of the enantiomers of bunolol in human urine by high-performance liquid chromatography on a chiral AGP stationary phase and identification of their metabolites by gas chromatography–mass spectrometry. J Chromatogr B 1994;660:327–39.

143. Haupt D. Determination of citalopram enantiomers in human plasma by liquid chromatographic separation on a Chiral-AGP column. J Chromatogr B 1996;685:299–305.

144. Menzel-Soglowek S, Geisslinger G, Brune K. Stereoselective high-performance liquid chromatographic determination of ketoprofen, ibuprofen and fenoprofen in plasma using a chiral α_1-acid glycoprotein column. J Chromatogr 1990;532:295–303.

145. Geisslinger G, Menzel-Soglowek S, Schuster O, Brune K. Stereoselective high-performance liquid chromatographic determination of flurbiprofen in human plasma. J Chromatogr 1992;573: 163–7.

146. Pettersson K-J, Olsson A. Liquid chromatographic determination of the enantiomers of ibuprofen in plasma using a chiral AGP column. J Chromatogr 1991;563:414–8.

147. Geisslinger G, Menzel-Soglowek S, Kamp H-D, Brune K. Stereoselective high-performance liquid chromatographic determination of the enantiomers of ketamine and norketamine in plasma. J Chromatogr 1991;568:165–76.

148. Schmidt N, Brune K, Geisslinger G. Stereoselective determination of the enantiomers of methadone in plasma using high-performance liquid chromatography. J Chromatogr 1992;583: 195–200.

149. Beck O, Boreus LO, LaFolie P, Jacobsson G. Chiral analysis of methadone in plasma by high-performance liquid chromatography. J Chromatogr 1991;570:198–202.

150. Kristensen K, Angelo HR, Blemmer T. Enantioselective high-performance liquid chromatographic method for the determination of methadone in serum using an AGP and a CN column as chiral and analytical column, respectively. J Chromatogr A 1994; 666:283–7.

151. Chu Y-Q, Wainer IW. Determination of the enantiomers of verapamil and norverapamil in serum using coupled achiral-chiral high-performance liquid chromatography. J Chromatogr 1989; 497:191–200.

152. Mangani F, Luck G, Fraudeau C, Verette E. On-line column switching high-performance liquid chromatography analysis of cardiovascular drugs in serum with automated sample clean-up and zone cutting technique to perform chiral separation. J Chromatogr A 1997;762:235–41.

153. Jones DJ, Nguyen KT, McLeish MJ, Crankshaw DP, Morgan DJ. Determination of (*R*)-(+)- and (*S*)-(−)-isomers of thiopentone in plasma by chiral high-performance liquid chromatography. J Chromatogr 1996;675:174–9.

154. Suzuki A, Takagaki S, Suzuki H, Noda K. Determination of the R,R- and S,S-enantiomers of vamicamide in human serum and urine by high-performance liquid chromatography on a Chiral-AGP column. J Chromatogr 1993;617:279–84.

155. Fieger H, Blaschke G. Direct determination of the enantiomeric ratio of verapamil, its major metabolite norverapamil and gallopamil in plasma by chiral high-performance liquid chromatography. J Chromatogr 1992;575:255–60.

156. Silan L, Jadaud P, Whitfield LR, Wainer IW. Determination of low levels of the stereoisomers of leucovorin and 5-methyltetrahydrofolate in plasma using a coupled chiral-achiral high-performance liquid chromatographic system with post-chiral column peak compression. J Chromatogr 1990;532:227–36.

157. Kelly JW, Stewart JT, Blanton CD. HPLC separation of pentazocine enantiomers in serum using an ovomucoid chiral stationary phase. Biomed Chromatogr 1994;8:255–7.

158. Haginaka J, Wakai J. β-Cyclodextrin bonded silica for direct injection analysis of drug enantiomers in serum by liquid chromatography. Anal Chem 1990;63:997–1000.

159. Rochat B, Amey M, Baumann P. Analysis of enantiomers of citalopram and its demethylated metabolites in plasma of depressive patients using chiral reverse-phase liquid chromatography. Ther Drug Monit 1995;17:273–9.

160. Castoldi D, Oggioni A, Renoldi MI, Ratti E, DiGiovine S, Bernareggi A. Assay of moguisteine metabolites in human plasma and urine: conventional an chiral high-performance liquid chromatographic methods. J Chromatogr B 1994;655:243–52.

161. Pham-Huy C, Radenen B, Sahui-Gnassi A, Claude JR. High-performance liquid chromatographic determination of (*S*)- and (*R*)-propranolol in human plasma and urine with a chiral β-cyclodextrin bonded phase. J Chromatogr B 1995;665:125–32.

162. Tan SC, Jackson SHD, Swift CG, Hutt AJ. Stereospecific analysis of the major metabolites of ibuprofen in urine by sequential achiral-chiral high-performance liquid chromatography. J Chromatogr B 1997;701:53–63.

163. Elsing B, Blaschke G. Achiral and chiral high-performance liquid chromatographic determination of tramadol and its major metabolites in urine after oral administration of racemic tramadol. J Chromatogr 1993;612:223–30.

164. Shibukawa A, Wainer IW. Simultaneous direct determination of the enantiomers of verapamil and norverapamil in plasma using a derivatized amylose high-performance liquid chromatographic chiral stationary phase. J Chromatogr 1992;574:85–92.

165. Hartmann C, Krauss D, Spahn H, Mutschler E. Simultaneous determination of (*R*)- and (*S*)-celiprolol in human plasma and urine: high-performance liquid chromatographic assay on a chiral stationary phase with fluorometric detection. J Chromatogr 1989;496:387–96.

166. Fernandez C, Gimenez F, Baune B, Maradeix V, Thuillier A, Farinotti R. Determination of the enantiomers of zopiclone and its two chiral metabolites in urine using an automated coupled achiral-chiral chromatographic system. J Chromatogr 1993;617: 271–8.

167. Echizen H, Ochiai K, Kato Y, Chiba K, Ishizake T. Simultaneous determination of disopyramide and mono-*N*-dealkyldisopyramide enantiomers in plasma and urine by use of a chiral cellulose-derivative column. Clin Chem 1990;36:1300–4.

168. Soons PA, Roosemalen MCM, Breimer DD. Enantioselective determination of felodipine and other chiral dihydropyridine calcium entry blockers in human plasma. J Chromatogr 1990; 528:343–56.

169. Pichini S, Pacifici R, Altieri I, Pellegrini M, Zuccaro P. Stereoselective determination of fluoxetine and norfluoxetine enantiomers in plasma samples by high-performance liquid chromatography. J Liq Chromatogr Relat Technol 1996;19:1927–35.

170. Takahashi H, Kashima T, Kimura S, Muramoto N, Nakahata H, Kubo S, et al. Determination of unbound warfarin enantiomers in human plasma and 7-hydroxywarfarin in human urine by chiral stationary-phase liquid chromatography with ultraviolet or fluorescence and on-line circular dichroism detection. J Chromatogr B 1997;701:71–80.

171. Aboul-Enein HY, Islam MR. Enantiomeric separation of ketamine hydrochloride in pharmaceutical formulation and human serum by chiral liquid chromatography. J Liq Chromatogr 1992;15: 3285–93.

172. Yamaguchi M, Yamashita K, Aoki I, Tabata T, Hirai S-I, Yashiki T. Determination of manidipine enantiomers in human serum using chiral chromatography and column-switching liquid chromatography. J Chromatogr 1992;575:123–9.

173. Rutledge DR, Garrick C. Rapid high-performance liquid chromatographic method for the measurement of the enantiomers of metoprolol in serum using a chiral stationary phase. J Chromatogr 1989;497:181–90.

174. Straka R, Johnson KA, Marshall PS, Remmel RP. Analysis of metoprolol enantiomers in human serum by liquid chromatography on a cellulose-based chiral stationary phase. J Chromatogr 1990;530:89–93.

175. Chiarotto JA, Wainer IW. Determination of metyrapone and the enantiomers of its chiral metabolite metyrapol in human plasma and urine using coupled achiral-chiral liquid chromatography. J Chromatogr B 1995;665:147–54.

176. Heinig R, Muschalek V, Ahr G. Determination of the enantiomers of nisoldipine in human plasma using high-performance liquid chromatography on a chiral stationary phase and gas chromatography with mass-selective detection. J Chromatogr B 1994; 655:286–92.

177. Zhang H, Stewart JT, Ujhelyi M. High-performance liquid chromatographic analysis of pindolol enantiomers in human serum and urine using a reversed-phase cellulose-based chiral column. J Chromatogr B 1995;668:309–13.

178. Liu J, Stewart JT. High-performance liquid chromatography determination of praziquantel enantiomers in human serum using a reversed-phase cellulose-based chiral stationary phase and disc solid-phase extraction. J Chromatogr B 1997;692:141–7.

179. Aboul-Enein HY, Bakr SA. Direct enantiomeric high performance liquid chromatographic separation of propafenone and its major metabolites in serum on a cellulose tris-3,5-dimethylphenyl carbamate chiral stationary phase. Biomed Chromatogr 1993; 7:38–40.

180. Takahashi H, Kanno S, Ogata H, Kashiwada K, Ohira M, Someya K. Determination of propranolol enantiomers in human plasma and urine and in rat tissues using chiral stationary-phase liquid chromatography. J Pharm Sci 1988;77:993–5.

181. DePuy ME, Demetriades JL, Musson DG, Rogers JD. Stereoselective determination of *R*-(+)- and *S*-(−)-remoxipride, a dopamine D_2-receptor antagonist, in human plasma by chiral high-performance liquid chromatography. J Chromatogr B 1997;700: 165–73.

182. Ceccato A, Chiap P, Hubert P, Crommen J. Automated determination of tramadol enantiomers in human plasma using solid-phase extraction in combination with chiral liquid chromatography. J Chromatogr B 1997;698:161–70.

183. Fernandez C, Baune B, Gimenez F, Thuillier A, Farinotti R. Determination of zopiclone enantiomers in plasma by liquid chromatography using a chiral cellulose carbamate column. J Chromatogr 1991;572:195–202.

184. Adams AG, Stewart JT. A high performance liquid chromatographic method for the determination of albuterol enantiomers in human serum using solid phase extraction and a Sumichiral-OA chiral stationary phase. J Liq Chromatogr 1993;16:3863–75.

185. Siluveru M, Stewart JT. Stereoselective determination of mepivacaine in human serum using a brush-type chiral stationary phase and solid-phase extraction. J Chromatogr B 1997;690: 359–62.

186. Doyle TD, Brunner CA, Vick JA. Enantiomeric analysis of phenylpropanolamine in plasma via resolution of dinitrophenylurea derivatives on a high performance liquid chromatographic chiral stationary phase. Biomed Chromatogr 1991;5:43–6.

187. Boulton DW, Fawcett JP. Determination of salbutamol enantiomers in human plasma and urine by chiral high-performance liquid chromatography. J Chromatogr B 1995;672:103–9.

188. Egginger G, Lindner W, Kahr S, Stoschitzky K. Stereoselective HPLC bioanalysis of atenolol enantiomers in plasma: application to a comparative human pharmacokinetic study. Chirality 1993; 5:505–12.

189. Jortani SA, Poklis A. Determination of thioridazine enantiomers in human serum by sequential achiral and chiral high-performance liquid chromatography. J Anal Toxicol 1993;17:374–7.

190. Kragh-Hansen U. Molecular aspects of ligand binding to serum albumin. Pharmacol Rev 1981;33:17–53.

191. Carter DC, Ho JX. Structure of serum albumin. Adv Protein Chem 1994;45:153–203.

192. Chaiken IM, ed. Analytical affinity chromatography. Boca Raton, FL: CRC Press, 1987:196 pp.

193. Wainer, IW. Enantioselective high-performance liquid affinity chromatography as a probe of ligand-biopolymer interactions: an overview of a different use for high-performance liquid chromatographic chiral stationary phases. J Chromatogr A, 1994;666: 221–34.

194. Cserhati T, Valko K. Chromatographic determination of molecular interactions. Boca Raton, FL: CRC Press, 1994:341 pp.

195. Hage DS, Tweed SA. Recent advances in chromatographic and electrophoretic methods for the study of drug-protein interactions. J Chromatogr B 1997;699:499–525.

196. Lindup WE. Progress in drug metabolism, Vol. 10. New York: Taylor & Francis, 1987:354 pp.

197. Kwong TC. Free drug measurements: methodology and clinical significance. Clin Chim Acta 1985;151:193–216.

198. Svensson CK, Woodruff MN, Baxter JG, Lalka D. Free drug concentration monitoring in clinical practice: rationale and current status. Clin Pharmacokinet 1986;11:450–69.

199. Barre J, Hamberger C, Didey F, Duche JC, Tillement JP. Principles of methods for drug determination in biological fluids applied to therapeutic monitoring. Feuill Biol 1987;28:47–55.

200. Refetoff S, Larsen PR. Transport, cellular uptake, and metabolism of thyroid hormone. In: DeGroot LJ, ed. Endocrinology. Philadelphia, PA: WB Saunders, 1989.

201. Westphal U. Steroid-protein interactions. New York: Springer-Verlag, 1971:567 pp.

202. Levy R H, Moreland TA. Rationale for monitoring free drug levels. Clin Pharmacokinet 1984;9:1–9.

203. Loun B, Hage DS. Characterization of thyroxine-albumin binding using high-performance affinity chromatography. 2. Comparison of the binding of thyroxine, triiodothyronines and related compounds at the warfarin and indole sites of human serum albumin. J Chromatogr B 1995;665:303–14.

204. Loun B, Hage DS. Chiral separation mechanisms in protein based HPLC columns. 2. Kinetic studies of (*R*)- and (*S*)-warfarin binding to immobilized human serum albumin. Anal Chem 1996; 68:1218–25.

205. Yang J, Hage DS. Role of binding capacity versus binding strength in the separation of chiral compounds on protein-based high-performance liquid chromatographic columns: interactions of D- and L-tryptophan with human serum albumin. J Chromatogr B 1996;725:273–85.

206. Loun B, Hage DS. Chiral separation mechanisms in protein-based HPLC columns. 1. Thermodynamic studies of (*R*)- and (*S*)-warfarin binding to immobilized human serum albumin. Anal Chem 1994;66:3814–22.

207. Yang J, Hage DS. Characterization of the binding and chiral separation of D- and L-tryptophan on a high-performance immobilized human serum albumin column. J Chromatogr, 1993;645: 241–50.

208. Loun B, Hage DS. Characterization of thyroxine-albumin binding using high-performance affinity chromatography. 1. Interactions at the warfarin and indole sites of albumin. J Chromatogr 1992;579:225–35.

209. Domenici E, Bertucci C, Salvadori P, Motellier S, Wainer IW. Immobilized serum albumin: rapid HPLC probe of stereoselective protein-binding interactions. Chirality 1990;2:263–8.

210. Domenici E, Bertucci C, Salvadori P, Felix G, Cahagne I, Motellier S, Wainer IW. Synthesis and chromatographic properties of an HPLC chiral stationary phase based upon human serum albumin. Chromatographia 1990;29:170–6.

211. Domenici E, Bertucci C, Salvadori P, Wainer IW. Use of a human serum albumin-based high-performance liquid chromatography chiral stationary phase for the investigation of protein binding: detection of the allosteric interaction between warfarin and benzodiazepine binding sites. J Pharm Sci 1991;80:164–6.

212. Noctor TAG, Pham CD, Kaliszan R, Wainer IW. Stereochemical aspects of benzodiazepine binding to human serum albumin. I. Enantioselective high performance liquid affinity chromatographic examination of chiral and achiral binding interactions between 1,4-benzodiazepines and human serum albumin. Mol Pharmacol 1992;42:506–11.

213. Noctor TAG, Wainer IW, Hage DS. Allosteric and competitive displacement of drugs from human serum albumin by octanoic acid, as revealed by high-performance liquid affinity chromatography, on a human serum albumin-based stationary phase. J Chromatogr 1992;577:305–15.

214. Sebille B, Zini R, Madjar CV, Thuaud N, Tillement JP. Separation procedures used to reveal and follow drug-protein binding. J Chromatogr 1990;531:51–77.

215. Schill G, Wainer IW, Barkin SA. Chiral separations of cationic and anionic drugs on an α_1-acid glycoprotein-bonded stationary phase (Enantiopac). II. Influence of mobile phase additives and pH on chiral resolution and retention. J Chromatogr 1986;365: 73–88.

216. Allenmark S, Bomgren B, Boren H. Direct liquid chromatographic separation of enantiomers on immobilized protein stationary phases. IV. Molecular interaction forces and retention behavior in chromatography on bovine serum albumin as a stationary phase. J Chromatogr 1984;316:617–24.

217. Allenmark S, Bomgren B, Boren H. Direct LC separation of enantiomers on immobilized protein stationary phases. III. Optical resolution of a series of *N*-aroyl D,L-amino acids by high-performance liquid chromatography on bovine serum albumin covalently bound to silica. J Chromatogr 1983;264:63–8.

218. Hermansson J. Direct liquid chromatographic resolution of racemic drugs using α_1-acid glycoprotein as the chiral stationary phase. J Chromatogr 1983;269:71–80.

219. Miwa T, Miyakawa T, Kayano M, Miyake Y. Application of an ovomucoid-conjugated column for the optical resolution of some pharmaceutically important compounds. J Chromatogr 1987; 408:316–22.

220. Allenmark S, Andersson S, Bojarski J. Direct liquid chromatographic separation of enantiomers on immobilized protein stationary phases. VI. Optical resolution of a series of racemic barbiturates: studies of substituent and mobile phase effects. J Chromatogr 1988;436:479–83.

221. Fornstedt T, Zhong G, Bensetiti Z, Guiochon G. Experimental and theoretical study of the adsorption behavior and mass transfer kinetics of propranolol enantiomers on cellulase protein as the selector. Anal Chem 1996;68:2370–8.

222. Noctor TAG, Pham CD, Kaliszan R, Wainer IW. Stereochemical aspects of benzodiazepine to human serum albumin. I. Enantioselective high-performance liquid affinity chromatographic examination of chiral and achiral binding interactions between 1,4-benzodiazepines and human serum albumin. Mol Pharmacol 1992;42:506–11.

223. Kaliszan R, Noctor TAG, Wainer IW. Stereochemical aspects of benzodiazepine binding to human serum albumin. II. Quantitative relationships between structure and enantioselective retention in high performance liquid affinity chromatography. Mol Pharmacol 1992;42:512–7.

224. Kaliszan R. Retention data from affinity high-performance liquid chromatography in view of chemometrics. J Chromatogr B 1998; 715:229–44.

225. Noctor TAG, Wainer IW. The use of displacement chromatography to alter retention and enantioselectivity on a human serum albumin-based HPLC chiral stationary phase: a mini-review. J Liq Chromatogr 1993;16:783–800.

226. Dalgaard L, Hansen JJ, Pedersen JL. Resolution and binding site determination of D,L-thyronine by high-performance liquid chromatography using immobilized albumin as chiral stationary phase. Determination of the optical purity of thyroxine in tablets. J Pharm Biomed Anal 1989;7:361–8.

227. Hage DS, Noctor TAG, Wainer IW. Characterization of the protein binding of chiral drugs by high-performance affinity chromatography. Interactions of *R*- and *S*-ibuprofen with human serum albumin. J Chromatogr A 1995;693:23–32.

228. Rahim S, Aubry A-F. Location of binding sites in immobilized human serum albumin for some nonsteroidal anti-inflammatory drugs. J Pharm Sci 1995;84:949–52.
229. Aubry A-F, Markoglou N, McGann A. Comparison of drug binding interactions on human, rat and rabbit serum albumin using high-performance displacement chromatography. Comp Biochem Physiol 1995;112C:257–66.
230. Nakano NI, Shimamori Y, Yamaguchi S. Binding capacities of human serum albumin monomer and dimer by continuous frontal affinity chromatography. J Chromatogr 1982;237:225–32.
231. Nakano NI, Shimamori Y, Yamaguchi S. Mutual displacement interactions in the binding of two drugs to human serum albumin by frontal affinity chromatography. J Chromatogr 1980;188: 347–56.
232. Chattopadhyay A, Tian T, Kortum L, Hage DS. Development of tryptophan-modified human serum albumin columns for site-specific studies of drug-protein interactions by high-performance affinity chromatography. J Chromatogr B 1998;715:183–90.
233. Jones K. A review of biotechnology and large scale affinity chromatography. Chromatographia 1991;32:469–80.
234. Scawen MD. Dye affinity chromatography. Anal Proc 1991;28: 143–4.
235. Lopatin SA, Varlamov VP. New trends in immobilized metal affinity chromatography of proteins. Appl Biochem Microbiol 1995;31:221–7.
236. Winzerling JJ, Berna P, Porath J. How to use immobilized metal ion affinity chromatography. Methods 1992;4:4–13.
237. Porath J. Immobilized metal ion affinity chromatography. Protein Expr Purif 1992;3:263–81.
238. McGown LB, Joseph MJ, Pitner JB, Vonk JB, Linn CP. The nucleic acid ligand: a new tool for molecular recognition. Anal Chem 1995;67:663A–8A.
239. Turek C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 1990;249:505–10.
240. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. Nature 1990;346:818–22.
241. Kriz D, Ramstrom O, Mosbach K. Molecular imprinting: new possibilities for sensor technology. Anal Chem 1997;69:345A–9A.
242. Sellergren B. Noncovalent molecular imprinting: antibody-like molecular recognition in polymeric network materials. Trends Anal Chem 1997;16:310–9.
243. Creaser CS, Feely SJ, Houghton E, Seymour M, Teale P. Online immunoaffinity chromatography-high-performance liquid chromatography–mass spectrometry for the determination of dexamethasone. Anal Commun 1996;33:5–8.