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Chromatographic immunoassays: strategies and recent developments in the analysis of drugs and biological agents

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Abstract

A chromatographic immunoassay is a technique in which an antibody or antibodyrelated agent is used as part of a chromatographic system for the isolation or measurement of a specific target. Various binding agents, detection methods, supports and assay formats have been developed for this group of methods, and applications have been reported that range from drugs, hormones and herbicides to peptides, proteins and bacteria. This review discusses the general principles and applications of chromatographic immunoassays, with an emphasis being given to methods and formats that have been developed for the analysis of drugs and biological agents. The relative advantages or limitations of each format are discussed. Recent developments and research in this field, as well as possible future directions, are also considered.

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Key terms

Chromatographic immunoassay: A technique in which an antibody or antibody-related agent is attached to a support and used as part of a chromatographic system for the isolation or measurement of a specific target; also known as an immunochromatographic assay, a flow immunoassay or a flow-injection immunoassay.

Direct detection chromatographic immunoassay: A chromatographic immunoassay that is used for direct detection of a target.

Immunoextraction: The use of immobilized antibodies to isolate a given target prior to its analysis or measurement by a second method.

Simultaneous injection method: A type of competitive binding chromatographic immunoassay in which a sample is mixed with a fixed amount of the label and applied together to a column containing a limited amount of an immobilized antibody or related binding agent for the target.

Sequential injection method: A type of competitive binding chromatographic immunoassay in which the sample is applied first to a column containing an immobilized antibody or related binding agent, followed later by application of the label.

Displacement immunoassay: A type of chromatographic immunoassay in which a target displaces a label from a column containing an immobilized antibody or related binding agent.

One-site immunometric assay: A type of chromatographic immunoassay in which a sample containing the target is incubated with an excess of labeled antibodies (or related binding agents) that can bind to this target, followed by injection of this mixture onto a column that contains an immobilized analog of the target.

Chromatographic sandwich immunoassay: A type of chromatographic immunoassay that employs two types of antibodies or binding agents that can bind simultaneously to the same target; one type of binding agent is immobilized to a support, and the second binding agent is labeled and mixed with the sample before these components are applied to the column or applied to the column after the sample has been injected.

The analysis of drugs and biological compounds is an essential component in clinical chemistry, therapeutic drug monitoring, proteomics and the screening or development of new pharmaceutical agents [1,2]. Common challenges that are often faced in these fields are the complexity of the samples that are being examined and the low concentrations of the desired compounds that must be measured. One way to overcome these challenges is to utilize immunoassays, which can often provide sensitive and specific measurements

for drugs and other compounds in complex samples [1–5]. An immunoassay can be defined as an analytical technique that utilizes antibodies or antibody-related agents to selectively bind a given target compound [3–5]. Antibodies are glycoproteins that are produced by the immune system in response to a foreign agent, or antigen [2,3]. Antibodies can be generated against a wide range of compounds and can have both specific and strong binding, with many antibody–target interactions having association equilibrium constants in the range of 10^5 to 10^{12} M^{-1}. These interactions are reversible and usually involve a combination of steric effects and noncovalent forces such as dipole-related interactions, ionic forces, nonpolar interactions and hydrogen bonding [6].

Antibodies have been used in many formats to carry out immunoassays. One method that has been of interest in recent years is a chromatographic immunoassay or immunochromatographic assay (also sometimes called a 'flow immunoassay' or 'flowinjection immunoassay') [5–13]. In this technique, an antibody or antibody-related agent (e.g., an antibody fragment) is attached to a chromatographic support or used as part of a chromatographic system for the isolation or measurement of a specific target. A simple example of such a method is shown in Figure 1 [14]. Various binding agents, detection methods, supports and assay formats have been reported for this group of methods. In addition, this set of techniques has been used in applications ranging from low mass compounds such as drugs, hormones and herbicides to higher mass targets such as peptides, proteins and bacteria [6–14].

This review will discuss the general principles, components and applications of chromatographic immunoassays. Particular emphasis will be given to methods that have been developed for the analysis of drugs and biological agents. This discussion will include an overview of the types of binding agents, detection methods, supports and elution conditions that can be used in this approach. This will be followed by a description of the various measurement formats that have been reported for chromatographic immunoassays. Representative applications for each format will be presented, and the relative advantages or limitations of each format will be examined. Recent developments and research in this field, as well as possible future directions in this area, will also be considered.

General components of chromatographic immunoassays

Antibodies & related binding agents

The antibody or binding agent that is used in a chromatographic immunoassay is usually the key factor in determining the specificity for this type of method [6,7]. A typical IgG class antibody, as used within the scheme shown in Figure 1A, has a 'Y'-shaped structure that is composed of four polypeptide chains (i.e., two identical sets of heavy and light chains) which are linked by disulfide bonds [3,6]. The F_c region (i.e., the 'crystallizable fragment' or 'constant region') is located in the lower stem region of this structure and is highly conserved from one antibody to the next in the same class. Two identical F_{ab} regions (or 'antigen binding' regions) are found in the upper portion of the antibody and contain the sites at which a given target, or 'antigen,' can bind to the antibody. A change in the amino acid sequence within the F_{ab} regions from one type of antibody to the next is what makes it possible for the body to produce antibodies against a wide variety of foreign agents [6,8].

Many chromatographic immunoassays utilize intact polyclonal antibodies or monoclonal antibodies. Polyclonal antibodies are the typical antibodies that are produced by the immune system. This type of preparation often consists of a heterogeneous population of antibodies with a range of affinities and binding regions on a particular antigen or target. This type of antibody is often used when strong binding is required in an immunoassay and when some cross-reactivity of the antibodies with agents that are closely related to the target is either desired or acceptable [6,7]. Monoclonal antibodies are produced from single hybrid cell lines, or 'hybridomas,' and are used when antibodies with a high level of specificity and good batch-to-batch reproducibility are needed [6–8]. Other types of antibodies that have been used occasionally in chromatographic immunoassays are autoantibodies, which are naturally occurring antibodies produced against components of the original host, and anti-idiotypic antibodies, which bind to receptors or their substrates in the body [6]. In addition, antibody fragments, such as those consisting of the F_{ab} portion of an antibody, can be used as binding agents in some types of chromatographic immunoassays [6,7].

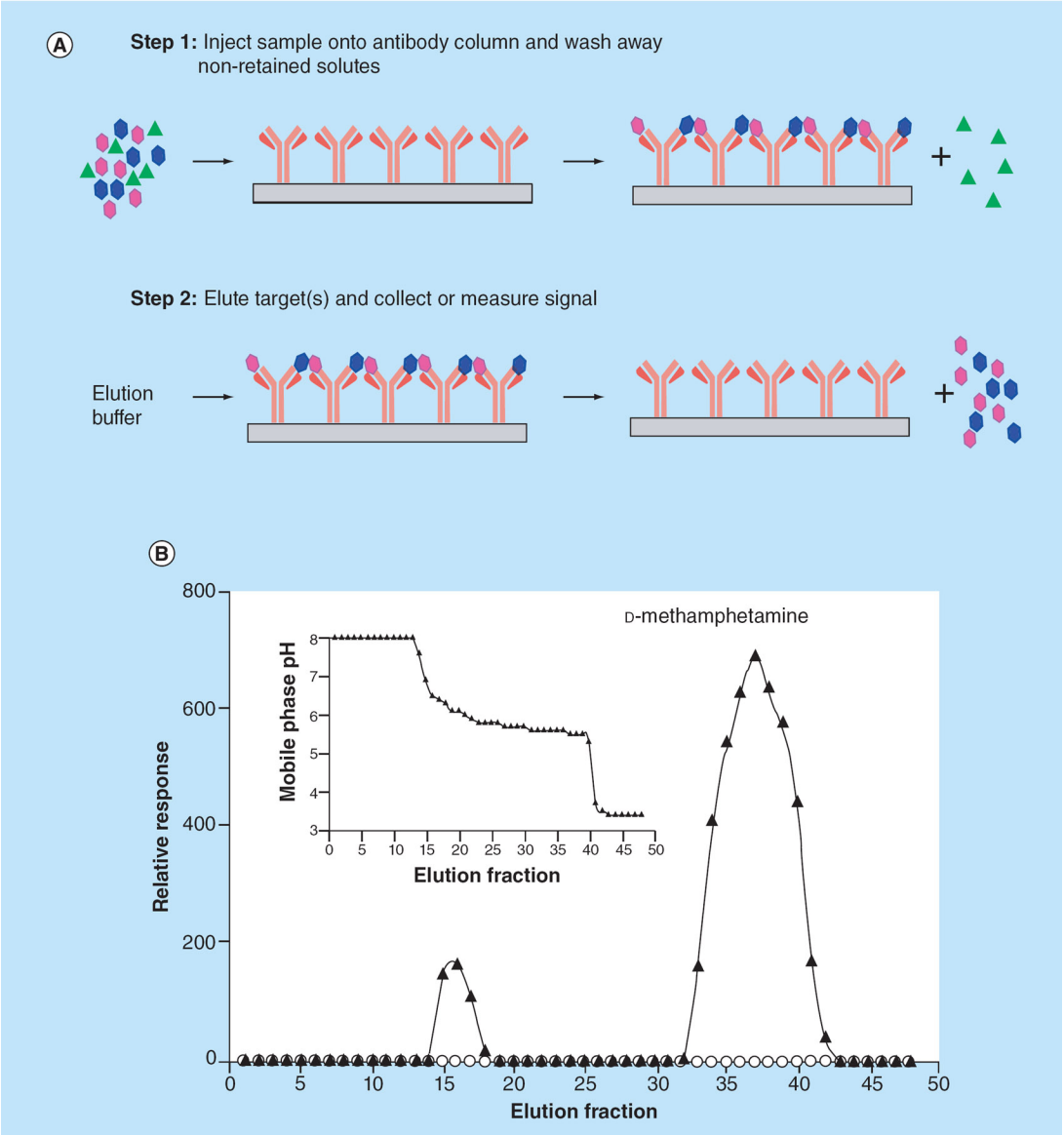

Figure 1. Direct detection format for a chromatographic immunoassay**. (A)** General scheme for a chromatographic immunoassay with direct detection of its target(s) and **(B)** use of a low-performance 150 mm × 10.0 mm id antibody column for the immunoextraction of d-methamphetamine and related compounds from urine, followed by analysis of these extracted solutes by GC–MS. The fractions in **(B)** each a volume of 0.6 ml and were collected at 0.4 ml/min; the contents of these fractions were then derivatized with trichloroanhydride and placed into ethyl acetate prior to injection onto a GC–MS system. The results in **(B)** are for the analysis of normal urine (circles) or urine (triangles) from a methamphetamine addict; the inset shows the pH gradient that was used for elution of the retained target from the antibody column. **(B)** Adapted with permission from [14].

There has been additional work involving the use of genetically engineered antibodies or antibody-like molecules in chromatographic immunoassays [6]. The use of these binding agents can provide greater versatility than traditional antibodies in the categories of compounds that can be analyzed by a chromatographic immunoassay and may even lead to an improvement in the binding strength and specificity for a target [6,15]. In some cases, these agents can be created to be 'bifunctional' or 'bispecific,' which allows their interaction with two different antigens or targets at the same time [6]. A few studies have also explored the use of recombinant single-chain antibody fragments for use in the chromatographic purification of proteins from cell cultures and cell lysates [6,16].

Aptamers have been used as 'antibody mimics' or alternatives to antibodies in some chromatographic binding assays [17–20]. These binding agents consist of single-stranded DNA or RNA sequences that are often 10 to 100 nucleotides in length and that have the ability to form highly ordered 3D structures [19]. Aptamers are prepared through a selection and amplification process known as SELEX (i.e., the 'systematic evolution of ligands by exponential enrichment'), which can be used to generate specific binding agents with relatively high affinities for a particular target [17,18]. These agents have been used to bind targets that have included drugs, peptides, proteins and cells [17–20]. It is possible to easily modify the structure of an aptamer to include a label or to immobilize the aptamer to a support [18,19]. In addition to being used in biosensors [17,18], aptamers have been employed in chromatography for both the purification of proteins [20] and in various forms of chromatographic binding assays [19].

A molecularly imprinted polymer (MIP) is another type of binding agent that can be used as an alternative to antibodies [21–25]. A MIP is a synthetic polymer that is prepared to have recognition sites for a given target. This type of material is produced through a polymerization reaction which makes use of one or more functional monomers, a cross-linking agent and a template, with the latter being the target of interest or a compound that is related in structure to the target [21–23]. A common scheme for preparing a MIP, or for 'molecular imprinting,' is shown in Figure 2. During this procedure, the functional monomers are allowed to interact with complementary regions on the template. These monomers are then set in place by reacting them with a cross-linking agent. The result is a highly ordered

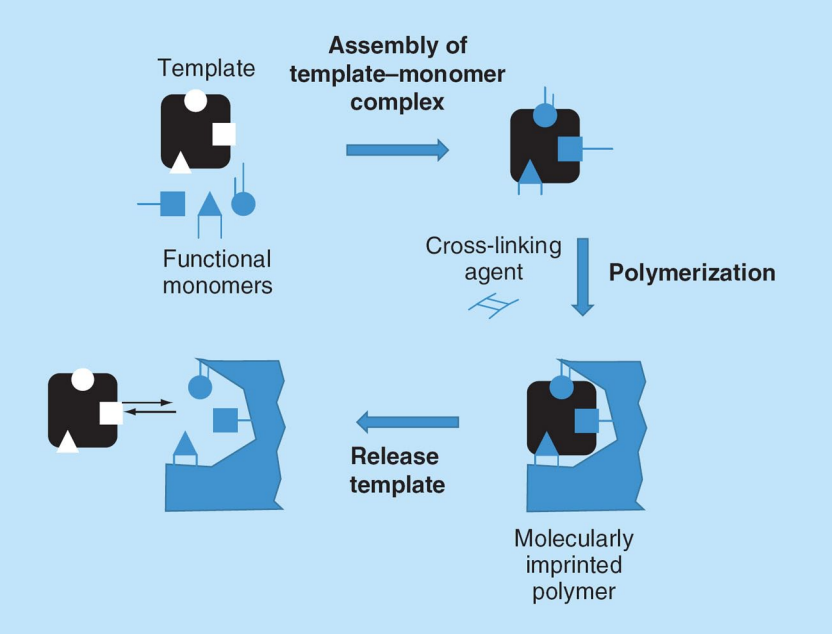

Figure 2. An example of a procedure for the creation of a MIP against a given target [21–23]. MIP: Molecularly imprinted polymer.

polymer that contains cavities and functional groups that are complementary to the size, shape and structure of the template. The template is then removed, leaving behind a specific binding site that can later be used to extract or retain the same template or desired target from a sample [21–24]. Although MIPs are based on a polymeric network rather than a biological agent, it has been found that MIPs can be generated with specificities and affinities that are similar to those of antibodies [25]. These features have made MIPs of interest as binding agents in biosensors, traditional immunoassays and extractions [21–25], as well as in some types of chromatographic methods [21,25].

Detection methods in chromatographic immunoassays

Chromatographic immunoassays can be used with a variety of detection methods. Common examples are absorbance and fluorescence detection, with chemiluminescence, electrochemical detection, radiometric detection, thermal measurements and MS also having been employed [7,8,10]. Many of these detection methods require the use of an antibody or target analog that contains a chemical label or

enzymatic tag. However, some of these methods make use of the inherent properties of the target for its direct detection [7,10].

This latter situation often occurs in the case of absorbance detection. This method works best for targets that can absorb light in the UV or visible range and that have moderate or relatively high concentrations in the sample. Typical detection limits for this approach are in the 10^{-8} to 10^{-7} M range when used for direct target detection, with some applications even reaching levels of 10^{-9} M [8]. Lower detection limits can also be obtained with absorbance detection when this is combined with enzyme labels that can generate products which will absorb UV or visible light [8,10,12].

Fluorescence detection can be used with analytes that are naturally fluorescent or with analogs of these targets that contain a fluorescent tag. Examples of fluorescent tags that have been used in chromatographic immunoassays are fluorescein, Texas red, Cascade blue and Lucifer yellow [8]. A major advantage of fluorescence over absorbance is that it is capable of providing much more selective detection and lower detection limits. The limit of detection when using traditional fluorescent tags on a target or labeled compound is often in the range of 10^{-9} to 10^{-11} M [7,8]. Even lower detection limits are again possible when fluorescence detection is combined with an enzyme label [8]. Near infrared fluorescent tags have also been employed in chromatographic immunoassays [26–28], resulting in detection limits as low as 10^{-10} to 10^{-13} M [26,27].

Two other detection methods that usually require the use of a label are those based on chemiluminescence or radioisotopes. Chemiluminescence makes use of a label or reagent that is capable of producing light as a result of a chemical reaction [7,8,29,30]. Two examples of chemiluminescent tags are acridinium ester and derivatives of luminol [8]. Advantages of this detection method are the low background response and ease with which small amounts of light can be measured, which can provide detection limits down to 10^{-12} to 10^{-13} M [7,29,30]. Radiolabels such as iodine-125 have been used for detection in a few chromatographic immunoassays for targets such as methotrexate and 2,4-dinitrophenol, with detection limits ranging from 10^{-7} to 10^{-9} M [8,31]. However, this detection approach has several potential disadvantages. These disadvantages include safety issues that are associated with the handling of radiolabels and the limited stability of radiolabels over extended periods of time [8,10].

MS, thermometric analysis and electrochemical detection have also been employed in chromatographic immunoassays. MS has been combined with both on-line and off-line chromatographic immunoassays and has been used for the analysis of a variety of targets [6,9,32]. Thermometric detection has been used with enzyme labels to measure the heat that is produced by an enzymatic reaction [7,8]. The limits of detection that have been obtained in chromatographic immunoassays based on thermometric detection have ranged from 10^{-8} to 10^{-10} M [7,8,33]. Electrochemical detection has been used with enzyme labels and chromatographic immunoassays to measure the change in current that occurs upon the oxidation or reduction reaction of an electrochemically active product. This combination has provided limits of detection ranging from 10^{-7} to 10^{-11} M [7,8].

In chromatographic immunoassays that use enzyme labels, the enzyme tag is combined with a substrate to produce an enzymatic product, which is then measured [8,10]. A major advantage of using an enzyme label is its ability to act as a catalyst and produce many copies of the product, which results in signal amplification [8]. The enzymes that are most often utilized as labels in chromatographic immunoassays are β-galactosidase, alkaline phosphatase and horseradish peroxidase. Other enzymes that have been used in these assays are catalase, glucose oxidase and adenosine deaminase. The products of these enzymes have been measured through the use of light absorption, fluorescence, chemiluminescence, electrochemical detection and thermometric analysis [8].

Liposomes have also been employed as labels in chromatographic immunoassays [7,8]. In this case, the liposome label is composed of phospholipids that have been coupled with either an antibody or antigen; the liposome also contains many copies of a water soluble marker (e.g., a fluorescent compound). During the detection phase of the chromatographic immunoassay, the structure of the liposome is disrupted through the addition of a detergent or by using shear force to release markers within the liposome for detection. This process can lead to a large signal, as each liposome may contain up to 10^3 copies of the marker compound. Liposome labels have been used in chromatographic immunoassays for the detection of various targets, such as caffeine, theophylline, antitheophylline, imazethapyr and 17-estradiol [8].

Supports, immobilization methods & elution conditions

Another important component of a chromatographic immunoassay is the support that contains the immobilized antibody or antibody-related agent. The type of support that is used will be determined by the overall goal of the assay. For example, supports that are to be employed in offline or manual methods and that do not require high efficiency are often based on large and relatively nonrigid materials such as agarose or cellulose. Other supports that are sometimes used in these methods are organic polymers such as polyacrylamide, polymethacrylate, polyethersulfone and related derivatives [6,7]. These materials are often inexpensive and easy to use at low back pressures; however, they tend to have slow mass transfer properties and can be difficult to use in automated systems, such as those utilized in HPLC. Most of these materials are used in packed columns for chromatographic immunoassays [6–8,10,13], but it is also possible to use low-performance supports to carry out chromatographic immunoassays in planar systems, as occurs in lateral flow immunoassays [19]. A typical system for this latter method is shown in Figure 3A. Nitrocellulose is a common support that is employed in these planar methods [19].

Supports are also available for chromatographic immunoassays that can be used in HPLC systems. An example of such a system is provided in Figure 3B. The supports that are used in these systems are often more expensive, smaller in size and have greater mechanical stability than those that are used in low-performance or planar systems. Examples of supports that can be used with HPLC include small diameter silica or porous glass beads, perfusion supports and some types of monolithic materials [6–8]. The advantages of using these materials with HPLC to carry out chromatographic immunoassays include the ease with which such systems can be automated, the ability to couple these methods on-line with other analytical techniques (e.g., MS or other forms of HPLC), and the speed and precision of the resulting assays [6–8,10,12,13].

The method of attachment for the immobilized agent in a chromatographic immunoassay is another important factor to consider [6,8]. This process often involves covalent immobilization. This type of immobilization can be conducted by reacting free amine groups on an antibody or related binding agent with a support that has been activated with chemicals such as *N,N*′-carbonyl diimidazole, cyanogen

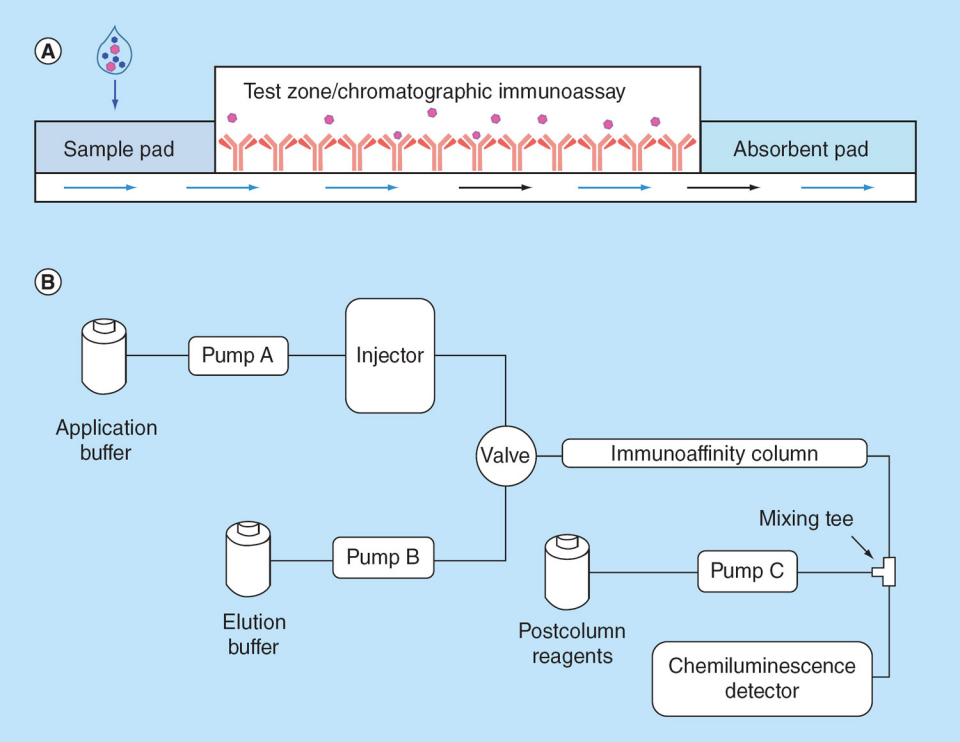

Figure 3. Examples of chromatographic immunoassays using low- or high-performance supports. **(A)** General scheme for the use of a chromatographic immunoassay in a planar system, such as a lateral flow immunoassay [19] and **(B)** an example of an HPLC system for conducting a chromatographic sandwich immunoassay, as based on the use of a postcolumn reactor for chemiluminescent detection. The system in **(B)** is based on a method that was reported in [29].

bromide, *N*-hydroxy succinimide or tresyl chloride/tosyl chloride [6,8,34,35]. Activated supports with oxidized functional groups, such as aldehydes or epoxy groups, can also be utilized to immobilize these binding agents through their free amine groups [6,7]. In an alternative approach, carbohydrate groups found in the F_c regions of antibodies can be oxidized and used for the site directed immobilization of antibodies onto supports that contain hydrazide or amine groups [34,35]. In addition, F_{ab} fragments can be coupled through their free sulfhydryl groups with thiol-reactive supports [6,34,35].

Antibodies or binding agents such as aptamers can also be immobilized through the use of secondary binding agents [6,7,19]. For instance, this may involve the biotinylation of antibodies or aptamers, followed by the biospecific coupling of these agents to supports that contain immobilized streptavidin or avidin [19,35]. Another option

for antibodies is to adsorb them to supports that contain immobilized immunoglobulin binding agents such as protein A or protein G. These latter agents bind to the F_c regions in many types of antibodies and can allow for the site-specific but reversible coupling of an antibody to a chromatographic support [6–8,13,35]. If desired, the adsorbed antibodies can later be released from the protein A or protein G by lowering the pH of the mobile phase. The protein A or G support can then be regenerated prior to the application of a fresh portion of antibodies [7,10,13].

The application of a sample or assay components in a chromatographic immunoassay is usually conducted through the use of a buffer that allows for strong and efficient binding. In most cases, physiological conditions (e.g., a pH of 7.0–7.4) are utilized to allow optimum binding to occur between the antibody or binding agent and its target [6–8]. Typical elution conditions that are used to later dissociate an antibody from its target might involve lowering the pH of the mobile phase or changing the composition of this mobile phase, such as through the addition of a chaotropic agent (e.g., thiocyanate, trifluoroacetate, iodide or chloride) [6–8,12]. If the support or column is to be used for multiple assays, as is usually the case in HPLC based applications, care must be taken to select elution conditions that do not cause irreversible damage to the immobilized antibodies or binding agents within the column [6,36]. It is also necessary in this situation to allow sufficient time for the column to regenerate prior to application of the next sample [36].

Chromatographic immunoassays with direct target detection

For some types of targets and samples it is possible to use chromatographic immunoassays with a 'nonlabel' format and direct detection of the target [6–8]. This type of direct detection chromatographic immunoassay is usually carried out by using step or gradient elution, as is represented in Figure 1 [6,7,11]. In this method, a sample is first applied to a column containing immobilized antibodies or related agents that can bind to some components in the sample. This application is carried out under conditions in which the column can selectively retain its target, while other components of the sample tend to wash through the column. An elution buffer is later used

to release the target from the column for its detection or for use in a second analytical method. Once the target has been removed, the column may then be regenerated before the application of the next sample [6–8,10].

The specificity and response of a chromatographic immunoassay with direct detection will depend on the nature of the target and its interaction with the immobilized antibodies or binding agents [8]. In this format, it is usually desirable to have relatively strong binding of the target with the immobilized binding agents so that this compound can be effectively retained by the column during the sample application step. However, it should also be possible to later release the target, such as by changing the pH, polarity or composition of the buffer that is passing through the column [6,8]. The immobilized binding agents should also be sufficiently selective for the target to avoid giving a response that might be due to other sample components [7,8].

The relative response of a chromatographic immunoassay with direct detection will tend to increase as the amount of target in the sample increases [7,8,10,37,38]. One factor that will determine the usable range of this response is the amount of active binding agent that is present in the column [8]. For instance, as the amount of the applied target is increased, the binding sites in the column will eventually become saturated and will no longer provide a change in response as the amount of the target is increased further. The application flow rate can also affect this response, where low flow rates will tend to provide a better capture efficiency and lower detection limit for the target, while higher flow rates will give a lower capture efficiency but a broader dynamic range [8]. Another interesting feature for this type of assay is that the response is generally dependent on the moles of the applied target rather than on its initial concentration [8,37]. A practical consequence of this feature is that adjustments can be made to the volume of sample that is applied to the column in order to adjust the calibration range for the target to lower concentration-based limits of detection. This effect has been demonstrated with a multicolumn chromatographic immunoassay system for atrazine and its degradation products, where an increase in the applied sample volume from 0.4 to 3.0 ml resulted in a steeper slope for the calibration plot and a decrease in the concentration- based detection limit of atrazine from 170 to 20 ng/l [37].

There have been several reports that have made use of direct detection in chromatographic immunoassays. These applications have utilized detection based on light absorption, fluorescence, chemiluminescence or MS [7,10,38–47]. This method has been used for large targets such as proteins, peptides and carbohydrates [7]. For instance, one report utilized this format in a dual-column HPLC system for the simultaneous analysis of human serum albumin (HSA) and IgG in serum [38]. Other applications have utilized the direct detection format in clinical tests for bacteria such as *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* [39,40]. This format has also been employed for the analysis of various low mass targets, including drugs, herbicides, toxins and synthetic dyes [7,9,41–45]. A recent report used chemiluminescent detection to directly measure bovine serum albumin that had been selectively adsorbed to columns packed with molecularly imprinted microspheres [46]. A related system has been utilized with magnetic oil-based surface molecularly imprinted nanoparticles for the analysis of bisphenol A [47].

Although the use of direct detection in a chromatographic immunoassay has the advantages of speed and simplicity, there are several possible disadvantages for this format. First, the target must have some inherent signal that can be measured (e.g., absorbance or fluorescence), and the target needs to be present in a sample at a concentration that will provide a measurable signal [8,10]. This requirement tends to limit the use of direct detection in chromatographic immunoassays to major sample components or those with moderate concentrations. As a result, this method is usually not suitable for trace analysis unless it is used with large sample volumes [8,37]. In these situations, an alternative would be to use a chromatographic immunoassay based on indirect detection (e.g., a competitive binding immunoassay or immunometric assay), as will be discussed later in this review.

Another way to enhance the capabilities of a chromatographic immunoassay with direct detection is to couple this format with other analytical techniques. When immobilized antibodies are used to isolate a target prior to its measurement or analysis by a second method, this approach is often referred to as immunoextraction [7,8]. This may be carried out by using columns that are prepared in-house, or by using one of the growing number of columns that are available commercially for this type of work [48–52]. In many cases, the column that is used for immunoextraction can be placed on-line with the second

analysis method, as has been used with various forms of HPLC, MS and CE [8,9,12,32]. Analytical methods can also be combined off-line with immunoextraction, as is commonly used in coupling this technique with GC or GC–MS [7]. For instance, the results in Figure 1B were obtained by using the off-line immunoextraction of d-methamphetamine to aid in the measurement of this drug in urine by GC–MS [14]. A related approach to immunoextraction is 'immunodepletion,' in which an immobilized antibody column is used to selectively extract major components from a sample (e.g., major proteins), while other components (e.g., trace proteins) are allowed to pass through the column for further analysis [7,53].

There are many examples of hybrid methods that have made use of immunoextraction [7–10,12,13]. For instance, immmunoextraction has been coupled with LC–MS for the measurement of *S*-methamphetamine in urine [45]. Immunoextraction columns have been used off-line with ultra-high performance LC (UHPLC) and MS/MS for the determination of aflatoxin M_1 in ice cream [49], and immunoaffinity columns were utilized off-line with LC–MS/MS for the analysis of multiple mycotoxins in food [50,51]. In addition, immunoextraction has been coupled both on-line and off-line with HPLC and fluorescence detection for the measurement of aflatoxins and mycotoxins in food [48,52,54], and used off-line with HPLC for the measurement of phenylethanolamine in animal feed and meat or liver samples [55]. Immobilized antibody fragments have been used to analyze the enantiomers of diarylalkyltriazole [56], and an immunoaffinity column has been used to separate ginsenoside epimers [57]. A chromatographic immunoassay with direct detection was combined with reversed-phase LC for measuring the free fractions of warfarin enantiomers in mixtures of this drug with HSA [58]. Immunoextraction has also been coupled with biointeraction analysis, as has been used to isolate and examine the binding of *in vivo* glycated HSA with sulfonylurea drugs [59].

Several applications have been reported for MIPs in the extraction of target compounds prior to their analysis by a second method. Examples include approaches that have been developed for the analysis of various herbicides and drugs in serum, urine, food or tissue samples [21,25]. A nano-sized MIP was prepared with multiple templates and used to extract various metabolites of polycyclic aromatic hydrocarbons from urine, followed by the separation and measurement of

these compounds by UHPLC with fluorescence detection [60]. Microspheres based on MIPs were created and used for isolating clenbuterol and other β-agonists from pork tissue, followed by their analysis using UHPLC coupled with MS/MS [61].

Competitive binding chromatographic immunoassays

A second group of chromatographic immunoassays are those that employ a competitive binding format. These methods are based on the indirect detection of a target through its competition with a labeled analog of the target (known as the 'label') for sites that are available in a column containing an antibody or related binding agent [8,10]. There are various types of competitive binding chromatographic immunoassays. The three most common approaches are the simultaneous injection method, the sequential injection method and the displacement immunoassay [7,8,10].

Simultaneous injection methods

The simultaneous injection method is a type of competitive binding chromatographic immunoassay in which a sample is mixed with a fixed amount of the label and both are applied together to a column containing a limited amount of an immobilized antibody or related binding agent for the target (see Figure 4) [7,8,62,63]. The target and label compete for this binding agent as the sample/label mixture is passed through the column. Once the nonretained portion of the mixture has been washed from the column, an elution buffer can be used to release the captured target and label. The amount of label that elutes either in the nonretained fraction or the retained peak can then be used for the indirect measurement of the amount of target that was in the original sample [7,8].

The response of this method is dependent on many factors. These factors include the moles of label that are combined with the sample, the moles of the immobilized binding agent that are present, the injection flow rate and the adsorption kinetics of the column [8,62]. It is also important to use a label which can produce a signal that will not be significantly affected by the presence of the sample matrix, if

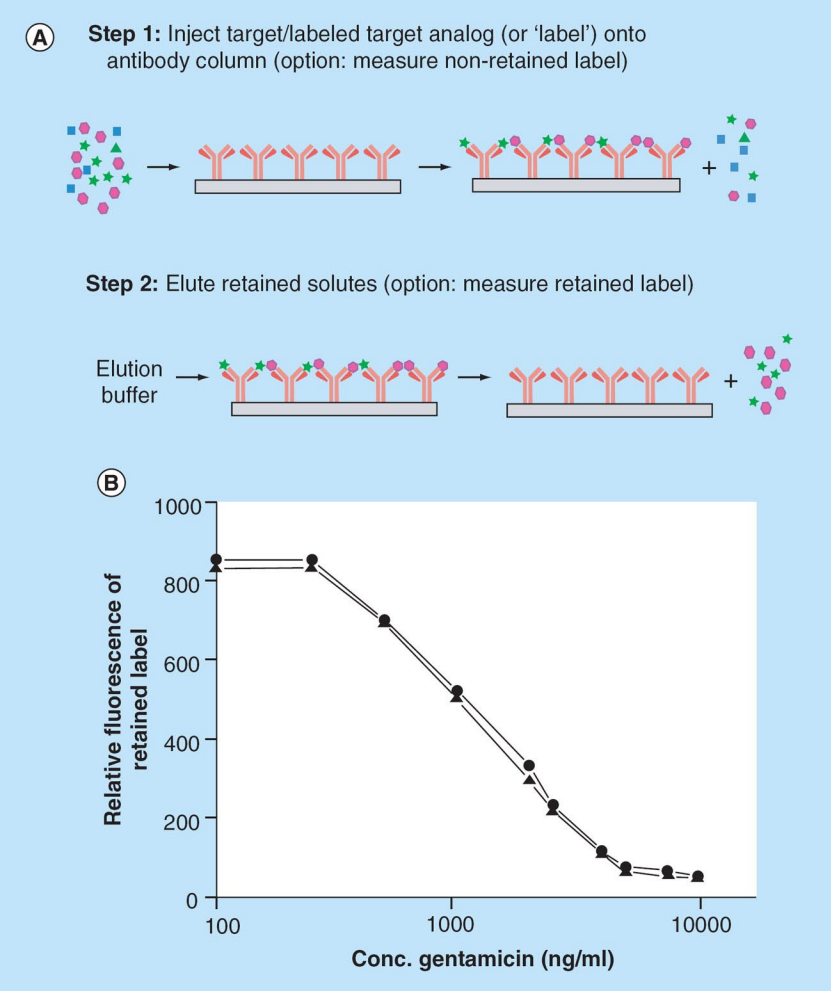

Figure 4. Chromatographic competitive binding immunoassay. **(A)** General scheme for a chromatographic-based competitive binding immunoassay using the simultaneous injection format and **(B)** a calibration curve obtained for gentamicin in this type of assay based on fluorescence detection and a 10 cm × 3 mm id antibody column. The labeled target analog, or 'label,' in **(A)** is represented by the stars and the target is represented by the hexagons. The plots in **(B)** were obtained from a set of two trials that were conducted after the initial development of the column (circles) and after 2 months (triangles). **(B)** Adapted with permission from [66].

the nonretained fraction is measured, or the elution buffer, if the retained fraction is used for the analysis [8]. The lowest detection limits in this method tend to occur at low-to-moderate flow rates and when using columns where the amount of active binding sites is in the same general range as the moles of target that are to be detected in the samples [8,62,63]. The fact that a labeled target analog is used in

this method generally provides simultaneous injection methods with lower detection limits than can be obtained with chromatographic immunoassays that employ direct target detection [8].

The simultaneous injection method has been used to measure many compounds, including both low and high mass targets, in complex samples [7,8,62–66]. This format has been used with absorbance measurements or fluorescent tags for the determination of proteins such as transferrin, IgG and HSA [8,62,63]. Other reports have used the simultaneous injection method with fluorescent labels for analysis of the hormones testosterone, adrenocorticotropic hormone and cortisol [7,8]. This method has been employed for the measurement of cephalexin in milk [64], and the measurement of atrazine in water and citrus fruits [65]. The simultaneous injection method has also been used with a fluorescent label for measuring gentamicin in serum, as is illustrated in Figure 4B. In this last example, results were obtained within 10 min, and the assay gave good agreement with an HPLC reference method [66]. A variation of the simultaneous injection method was reported for the analysis of Hg^{2+}, in which an immobilized analog of the target (i.e., a protein–Hg^{2+} conjugate) was combined with both samples and antibodies that could bind to this metal ion [67].

Sequential injection methods

The sequential injection method is similar to the simultaneous injection method but has the sample being applied first to a column containing an immobilized antibody or related binding agent, followed later by application of the label [8,68]. The amount of label that elutes in either the nonretained or retained fractions is again used as an indirect measure of the amount of target that was in the sample [8]. The use of separate steps for the injection of the sample and label allows the target to have the first opportunity to bind to the immobilized binding agent; this feature tends to result in lower limits of detection than can be obtained with the simultaneous injection format [62]. The use of separate application steps also creates a situation in which the label is never in contact with the sample. This approach helps to minimize or eliminate any effects the sample matrix may have on the measurement of the label [7,8,68].

Many of the same factors that affect the response of the simultaneous injection format affect the sequential injection method. For instance, the moles of the label and binding agent are important, in addition to the flow rate that is used for injection and the adsorption kinetics within the column [8,68]. The best limits of detection again tend to occur at low-to-moderate flow rates, but separate flow rates can now be utilized for the application of the sample and label [68]. Another difference between the simultaneous and sequential injection methods is in the effect of varying the amount of the label. In the simultaneous injection method, changing the amount of the label can have a relatively large effect on the limit of detection [8,62,63], while this effect is not as large in the sequential injection method [62,68].

There have been many applications of the sequential injection method for measuring drugs, proteins and other targets [8,62,68–72]. For instance, nonlabeled HSA was used as both the target and 'label' in an HPLC based sequential injection method that could measure this protein in samples in less than 10 min [68]. This general approach was used with an enzyme label and an antibody capillary column to measure hyaluronan in serum as a potential biomarker for liver disease and cancer [68]. The sequential injection method has also been utilized in bioprocess monitoring for the analysis of proteins such as IgG and HSA [8]. This method has been used with liposome labels and fluorescence detection for the measurement of IgE in serum [71]. It has further been employed with a label based on liposome-encapsulated quantum dots in a multiplex assay for a variety of sulfonamides and quinoline residues in milk [72].

Displacement immunoassays

A displacement immunoassay is another form of a competitive binding chromatographic immunoassay [7,8,73]. In the most common format for this approach (see Figure 5), the label is first applied to a column containing an immobilized antibody or related agent and allowed to occupy most or all of the binding sites in the column. The excess label is then washed away and the sample is injected. As the sample passes through the column, the target can bind to any sites that are momentarily unoccupied by the label. This competition results

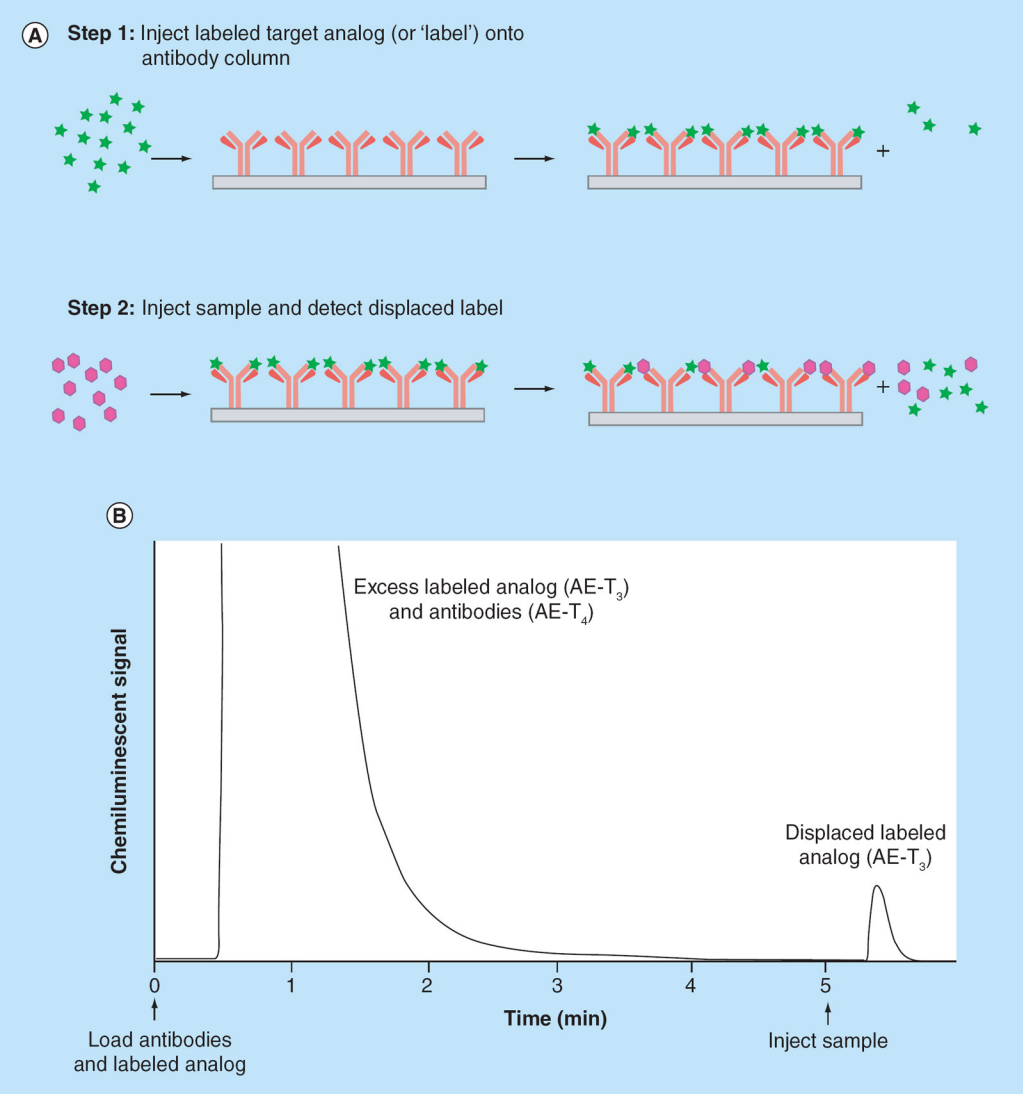

Figure 5. Chromatographic displacement immunoassay**. (A)** General scheme for a chromatographic-based displacement immunoassay and **(B)** a typical chromatogram obtained by this method for thyroxine (T_4) when using acridinium ester-labeled triiodothyronine (AE-T_3, which was the 'label' in this method) and anti-T4 antibodies that were adsorbed to a 10 mm × 2.1 mm id protein G column. The labeled target analog, or 'label,' in **(A)** is represented by the stars and the target is represented by the hexagons. In **(B)**, the antibodies and label were combined and applied simultaneously to the protein G column; the displacement peak was monitored through the use of a postcolumn reactor to detect the chemiluminescence of the label. **(B)** Reproduced with permission from [30].

in the displacement of some label and produces a peak for the displaced label that is related to the amount of target that was in the sample [7,8].

There are several potential advantages to this approach when compared with other types of competitive binding immunoassays. First, the response due to the displaced label increases as the amount of the target is increased, and often does so in a linear manner at low-to-moderate target concentrations [7,8]. Second, the displacement peak often appears with or just after the nonretained sample components, which can lead to a method with short analysis times [8,27,30]. Third, this method can be utilized for relatively high throughput measurements, especially if the amount of label that is present in the column is sufficient for use with multiple sample injections before the column must be regenerated and reloaded with the label [7,8,27,30,73]. Like the simultaneous and sequential injection methods, a displacement immunoassay can often be used to obtain lower limits of detection than can be acquired with chromatographic immunoassays that employ direct detection. The main disadvantage of all these competitive binding immunoassays when compared with direct detection is that they require a labeled analog of the target [7,8].

Several factors can affect the behavior of a displacement assay. These factors include the amount and type of label that is used, the injection flow rate and the application conditions that are employed for the label and the sample, the amount of binding agent that is in the column, and the association/dissociation rates of the binding agent for the target and label [8]. For instance, the injection flow rate must be slow enough to provide sufficient time for the target to bind to the column and displace enough label to give a measurable response. This method also requires that the label dissociates from the antibodies at a rate that is neither too fast nor too slow. A fast rate of label dissociation will result in a high signal for the first few sample injections, but this signal will quickly decrease for later sample injections as the label leaves the column. On the other hand, a slow rate of label dissociation will lead to broad displacement peaks that may be difficult to detect [8]. Another consideration is the binding constant for the label with the immobilized antibodies or binding agents. Best displacement results tend to be obtained when the label has a binding constant that is not as strong as it is for the target [8,30].

A variety of applications have been reported for displacement immunoassays [7,27,30,74,75]. One recent application has been the use of this format in combination with ultrafast immunoaffinity

extraction for the analysis of free drug or free hormone fractions in serum or in mixtures of these agents with transport proteins [27,30]. An example of such an application, which was combined with chemiluminescent detection to measure the free fraction of thyroxine in serum, is illustrated in Figure 5B. This technique gave a result within 30 s of sample injection and had a detection limit of 6 pM [30]. Displacement immunoassays have also been used to detect drugs such as cocaine or benzoylecgonine and targets such as coumaphos or ochratoxins [7,74,75].

Immunometric assays

Chromatographic immunoassays can also be carried out by using various immunometric formats [7,8,10–12]. These methods are noncompetitive immunoassays that utilize indirect detection and a labeled antibody or labeled binding agent to measure a target [5]. The two primary formats for these assays, when used in chromatographic systems, are sandwich immunoassays (i.e., two-site immunometric assays) and one-site immunometric assays [7,8].

Sandwich immunoassays

A chromatographic sandwich immunoassay employs two types of antibodies or related binding agents that can bind simultaneously to the same target [3,8,10]. Figure 6 shows one way in which this type of assay can be carried out in a chromatographic system. In this example, one type of antibody or binding agent is immobilized to a support and placed within the column. The other antibody/binding agent is labeled and mixed with the sample before both of these components are applied to the column or applied to the column after the sample has been injected [29]. The result is the formation of a sandwich complex in which the target is bound to both a labeled binding agent and an immobilized binding agent. The retained target and its associated labeled binding agent can later be eluted from the column. The amount of retained labeled binding agent is then measured and used to provide a response that is related to the amount of target that was in the original sample. If desired, the column or support may then

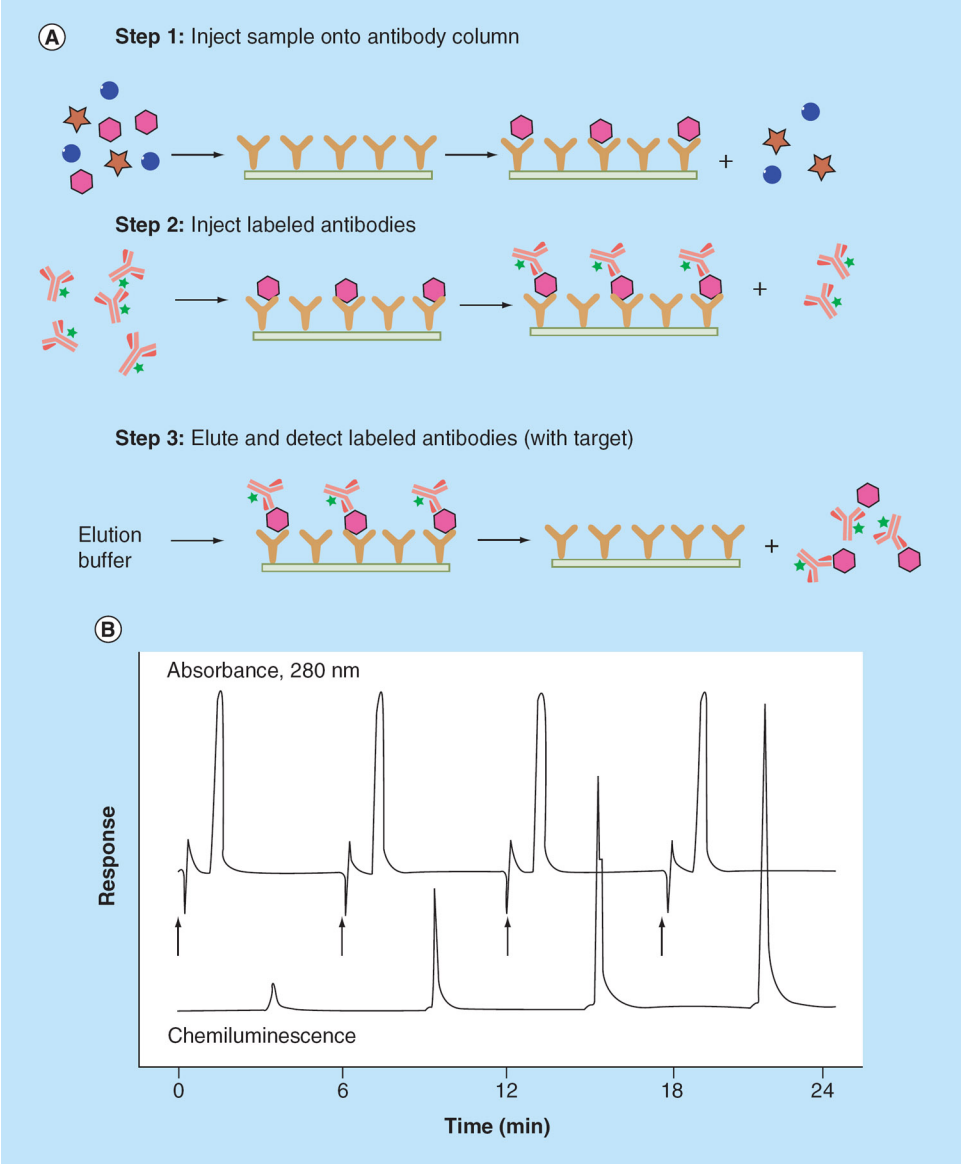

Figure 6. Chromatographic sandwich immunoassay. **(A)** General scheme for a chromatographic-based sandwich immunoassay and **(B)** a set of chromatograms obtained for the detection of PTH in plasma by using a 20 mm × 4 mm id column containing immobilized antibodies that could bind to one region of PTH, along with soluble acridinium ester-labeled antibodies against a different region of PTH to form a sandwich complex with this target. The tag on the soluble and labeled antibodies in **(A)** is represented by the stars and the target is represented by the hexagons. A postcolumn reactor was used in **(B)** to detect the chemiluminescence of the labeled antibodies that were retained by the column; sample injections were made every 6 min, as indicated by the arrows. Both the response due to the nonretained sample components, as measured at 280 nm, and the chemiluminescent response measured for samples containing (from left-to-right) 0, 25, 50 or 75 pM PTH are shown in **(B)**. PTH: Parathyroid hormone. **(B)** Adapted with permission from [29].

be regenerated and used for the next application of the sample and labeled binding agent [8,10].

One advantage of using a chromatographic sandwich immunoassay, especially when compared with simultaneous and sequential injection competitive binding immunoassays, is that it can give a linear response over a relatively broad range of target concentrations [8]. This response, plus the selectivity that is provided by using two types of binding agents for the same target, can result in better selectivity and lower limits of detection than can usually be obtained in competitive binding assays or direct detection methods [3,7,8,12]. A limitation of sandwich immunoassays is that they can only be used with relatively high mass targets such as proteins, large peptides or other macromolecules that can interact with two antibodies/binding agents simultaneously. The need for two types of binding agents can also make this approach more expensive than a competitive binding assay [8].

Several types of targets have been examined by using chromatographic-based sandwich immunoassays [7,8]. For instance, chromatographic sandwich immunoassays using chemiluminescent tags or enzyme labels have been developed for human and mouse IgG [7,8,76,77]. Related methods using fluorescence or amperometric detection have been utilized for the measurement of HSA [7,78]. A system based on this format has been developed for analysis of parathyroid hormone in plasma by using chemiluminescent labeled antibodies, with results being obtained within 6 min of sample injection [29], as shown in Figure 6B. Another method combined chemiluminescent detection with traditional antibodies or nanobodies that were attached to magnetic beads and silica nanoparticles for use in flow-based sandwich immunoassays that could measure microcystin-LR or prealbumin [79,80]. Other chromatographic sandwich immunoassays have been described for IL-5, anti-HCV antibodies, potato virus X, cardiac troponin and myoglobin [81–84]. Sandwich immunoassays have also been applied to the determination of allergens, bacteria and toxins in food [85–89].

Chromatographic sandwich immunoassays have been developed for other applications. This type of assay was utilized for examining a lipopolysaccharide antigen and cells of *Brucella abortus* [90]. A method for measuring hepatitis B virus surface antigen in serum was created by using a chromatographic sandwich immunoassay with

fluorescence detection [91]. A test strip utilizing this general approach was developed for the detection of rabies in canine serum [92]. Quantum dots were employed in a sandwich immunoassay format for the analysis of C-reactive protein [93], and chromatographic sandwich immunoassays have been made for detecting lily mottle virus and lily symptomless virus [94,95]. In addition, sandwich immunoassays in a chromatographic format have been employed with gold nanoparticle labels for the diagnosis of malaria and for the detection of *S. aureus* [96,97]. Aptamers have also been utilized in sandwich immunoassays on planar supports [98–100]. For instance, a flow-based sensor for thrombin was created that used immobilized aptamers and aptamers that were conjugated to gold nanoparticles for detection [98]. Similar approaches using aptamers have been reported for Ramos cells [99] and *E. coli* [100].

One-site immunometric assays

One-site immunometric assays can also be carried out in a chromatographic format [8]. This method first involves the incubation of a sample containing the target with an excess of labeled antibodies, or related binding agents like antibody fragments that can bind this target (see Figure 7). This mixture is then injected onto a column that contains an immobilized analog of the target. The target and its complex with the labeled antibody/binding agent will tend to pass through the column, while the immobilized analog will capture any excess of the labeled binding agent that is still present. The size of the nonretained peak due to the complex of the target with the labeled agent provides a response that is related to the amount of target that was in the sample. An elution buffer can later be passed through the column to release the captured and labeled binding agent; the size of this retained peak can also be used to determine how much target was present in the sample. If desired, the column can be regenerated before making another series of injections of the target and labeled binding agent [101,102].

A linear increase in response over a relatively broad range of target levels can be obtained when using the nonretained peak due to the labeled binding agent and its complex with the target [8,102].

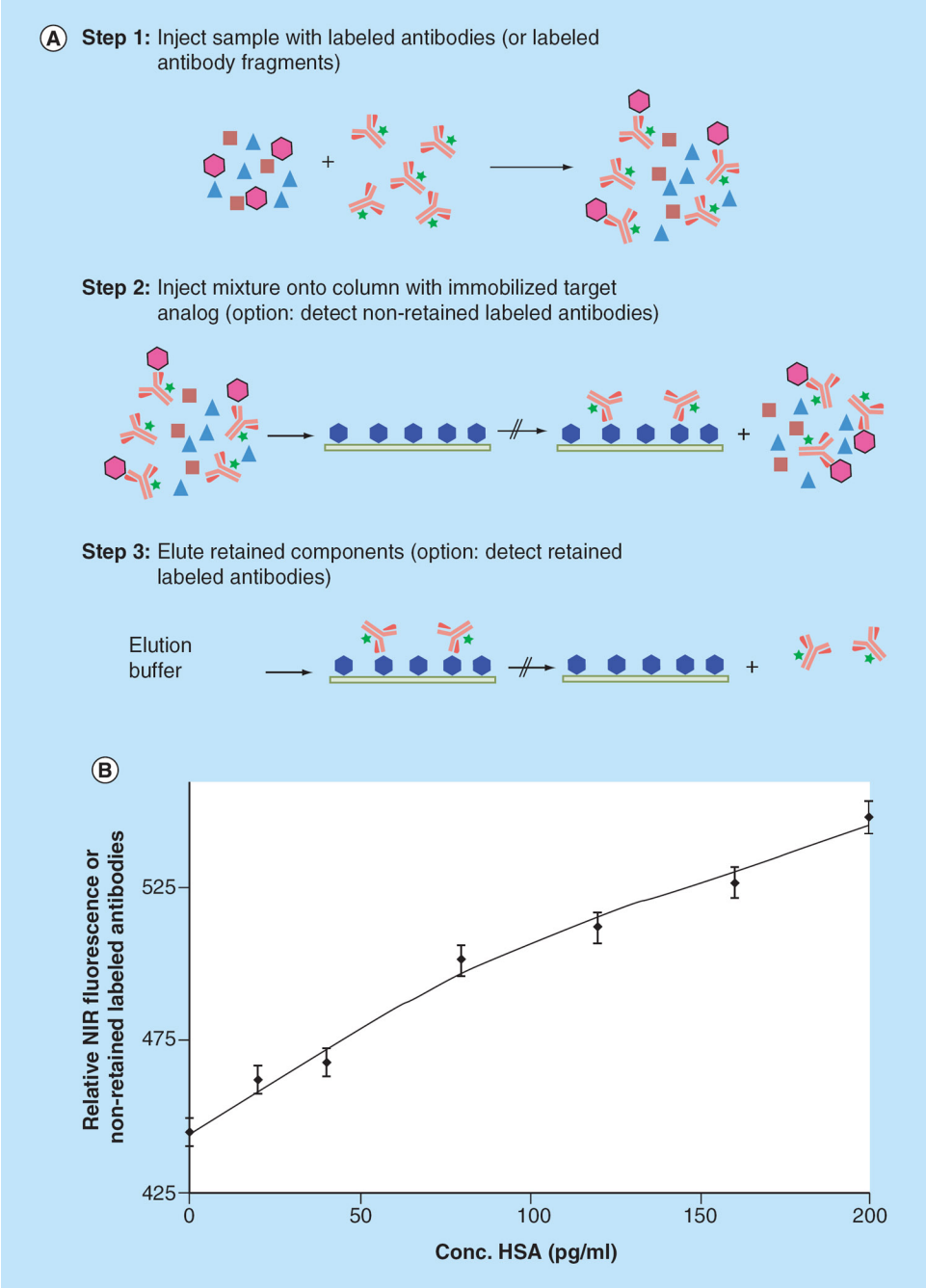

Figure 7. Chromatographic one-site immunometric assay. **(A)** General scheme for a chromatographic-based onesite immunometric assay and **(B)** calibration plots obtained in this method by using anti-HSA antibodies that were labeled with a NIR fluorescent tag and injected with samples onto a 5 mm × 2.1 mm id microcolumn containing immobilized HSA. The tag on the soluble and labeled antibodies in **(A)** is represented by the stars, the target is represented by the hexagons in solution and the immobilized target analog is represented by the hexagons at the surface of the support. The error bars in **(B)** represent a range of ± 1 standard error of the mean (n = 3). HSA: Human serum albumin; NIR: Near-infrared. **(B)** Reproduced with permission from [26].

Alternatively, a decrease in response with the target's concentration is seen if the retained fraction of the labeled binding agent is used [8]. The response of this method will be affected by the amount of the labeled binding agent that is combined with the sample, the time that is allowed for the incubation step, the binding capacity of the immobilized analog column and the capture efficiency of the column for the labeled binding agent [8,102]. One advantage of this method when compared with other chromatographic immunoassays is that if the column binding capacity is sufficiently high, multiple sample/labeled binding agent mixtures can be injected between elution/regeneration cycles. Another advantage of using an immobilized analog column is that elution conditions can be used that may not be feasible in techniques that instead employ immobilized antibodies or antibody fragments (e.g., competitive binding immunoassays or sandwich immunoassays) [8,102]. The main limitation of this format is the need for a different immobilized analog column for each target or group of targets that are to be analyzed [8].

One-site immunometric assays have been described for drugs, hormones, proteins and other targets [26,101– 106]. An example is provided in Figure 7B, in which this format was used with affinity microcolumns and fluorescence or near-infrared fluorescence detection for the trace analysis of a protein (HSA), giving results within 35 s to 2.8 min of sample injection [26]. This format has also been used with fluorescence detection for the analysis of granulocyte-colony stimulating factor, modified with poly(ethylene glycol), in rat serum [103] and IL-10 in cell media [104]. This approach has been utilized with enzyme labels to detect digoxin or digoxigenin [105], with chemiluminescence detection for the measurement of thyroxine [102], and with a liposome label to monitor 17-beta-estradiol [106]. Several of these methods have further been used in postcolumn reactors for the detection of targets as they are separated by other chromatographic methods, such as reversed-phase LC [7,101,103].

Conclusion

This review examined the general principles of chromatographic immunoassays and the use of these methods for the analysis of drugs, biological compounds and other targets in complex samples. The

main components of a chromatographic immunoassay were discussed, including the types of binding agents, detection methods and supports that have been used in these techniques. The various formats that can be used in a chromatographic immunoassay were then examined. This included a brief discussion of the advantages or limitations of each method and the factors that affect their response. Recent research and developments in this area were discussed, and a variety of applications for chromatographic immunoassays were presented. These applications ranged from small targets such as drugs, hormones and environmental agents to larger targets such as peptides, proteins, viral particles, bacteria and cells.

Future perspective

The current field of chromatographic immunoassays already encompasses a relatively broad range of assay formats and applications. It is expected that the types of components that can be used in these methods and the formats in which they can be employed will continue to undergo development. One possible area of future research is in the types of binding agents that can be used in chromatographic immunoassays and related methods. This is expected to include the more extensive use of aptamers [17–20] and MIPs [21–25,46,47] as alternatives to antibodies. It is also anticipated that there will be more applications in chromatographic immunoassays that make use of binding agents such as bispecific antibodies, single-chain antibody fragments and 'nanobodies' [6,16,80,107]. The use of other synthetic mimics of antibodies (or 'synbodies') [108] is another area for possible growth in this field.

Other work that is expected to continue in chromatographic immunoassays is in the types of detection methods, supports and assay formats that can be used in these techniques. For example, the use of nanomaterials, such as gold or silver nanoparticles, and quantum dots should continue to lead to new or enhanced detection methods and formats for these methods [19,85–88,93– 100,109]. The utilization of alternative supports, such as affinity monoliths, is also expected to continue [110–116]. In addition, further research is anticipated in the development of miniaturized chromatographic immunoassays that can be employed in microfluidic devices and portable

or disposable devices [19,26–28,30,82–88,100,113,116]. These developments, in turn, should increase the range of applications for which chromatographic immunoassays can be used in areas that include clinical chemistry, pharmaceutical testing, drug development and biomedical research [7,8].

Executive summary

Background

- Chromatographic immunoassays use an antibody or related binding agent as part of a chromatographic system for the isolation or measurement of a specific target.

General components of chromatographic immunoassays

- Polyclonal or monoclonal antibodies are often used in these methods, along with antibody fragments; other binding agents that have been employed include autoantibodies, anti-idiotypic antibodies, genetically engineered antibodies or antibody fragments, aptamers and molecularly imprinted polymers.
- A wide range of detection methods have been used in chromatographic immunoassays, such as absorbance or fluorescence detection, chemiluminescence, electrochemical detection, radiometric detection, thermal measurements and MS, as well as methods that use enzymes or liposomes as labels.
- Various support materials and immobilization schemes can be used in chromatographic immunoassays, along with formats based on columns or planar supports.

Chromatographic immunoassays with direct target detection

- Some chromatographic immunoassays can be used for the direct detection of a target, as may involve detection based on light absorption, fluorescence, chemiluminescence or MS.
- It is also possible to use the immunoextraction of a target prior to its measurement or analysis by a second method, such as HPLC, ultra-high performance LC, LC–MS or LC–MS/MS.

Competitive binding chromatographic immunoassays

- Competitive binding immunoassays can be carried out in a chromatographic immunoassay by using simultaneous or sequential injection of the sample and a labeled analog of the target.
- A displacement immunoassay format is another form of a competitive binding immunoassay that can be carried out in a flow-based system.

Immunometric assays

- Labeled antibodies or binding agents can also be used in chromatographic immunoassays, resulting in methods that are known as immunometric assays.

- The two common forms of chromatographic-based immunometric assays are sandwich immunoassays and one-site immunometric assays.

Conclusion

- Chromatographic immunoassays have been used for the selective analysis of drugs, hormones, peptides, proteins and other agents in areas that include clinical chemistry, pharmaceutical analysis, biomedical research and food testing.

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