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4-24-2010

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Bioanalysis. Author manuscript; available in PMC 2011 February 1.

Published in final edited form as:
Bioanalysis. 2010 April ; 2(4): 769–790. doi:10.4155/bio.10.31.

Immunoaffinity chromatography: an introduction to applications and recent developments

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Abstract

Immunoaffinity chromatography (IAC) combines the use of LC with the specific binding of antibodies or related agents. The resulting method can be used in assays for a particular target or for purification and concentration of analytes prior to further examination by another technique. This review discusses the history and principles of IAC and the various formats that can be used with this method. An overview is given of the general properties of antibodies and of antibody-production methods. The supports and immobilization methods used with antibodies in IAC and the selection of application and elution conditions for IAC are also discussed. Several applications of IAC are considered, including its use in purification, immunodepletion, direct sample analysis, chromatographic immunoassays and combined analysis methods. Recent developments include the use of IAC with CE or MS, ultrafast immunoextraction methods and the use of immunoaffinity columns in microanalytical systems.

Immunoaffinity chromatography (IAC) is a type of LC in which the stationary phase consists of an antibody or antibody-related reagent. This technique represents a special sub category of affinity chromatography, in which a biologically related binding agent is used for the selective purification or analysis of a target compound. The selective and strong binding of antibodies for their given targets has made these agents of great interest for many years as immobilized ligands in affinity chromatography [1–4]. Other methods sometimes included under the heading of IAC are those that use immobilized targets for antibody purification. However, in this review, the emphasis will be placed on methods that use antibodies as the immobilized ligands.

The earliest use of IAC and related methods was in the selective purification of compounds from biological samples. For instance, target compounds immobilized onto kaolin and charcoal were used as early as 1935 to isolate antibodies associated with syphilis and TB [5]. A total of 1 year later, Karl Landsteiner and co-workers isolated antibodies by using

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Financial & competing interests disclosure
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in this manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, or royalties. Part of the work described in this review was supported by the NIH under grants R01 GM044931 and R01 DK069629. Some of the work described in the areas of the chromatographic immunoassays and ultrafast immunoextraction are the subjects of US Patents 6 ,720,193 and 6 ,727,104.
No writing assistance was utilized in the production of this manuscript.

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targets immobilized onto chicken erthyrocyte stroma by a diazo coupling method [6]. The first modern use of IAC is generally attributed to Campbell *et al.*, who immobilized serum albumin to *p*-aminobenzylcellulose in 1951 for use in antibody purification [7]. Since that time, there have been thousands of reports using IAC for both the isolation of chemicals or biochemicals [1,3,4,8–12] and analytical applications [2,4,8,11–20].

This review will first examine the main components and underlying principles of IAC. This will include an overview of the general properties of antibodies and of antibody-production methods. The supports and immobilization methods used with antibodies in IAC and the selection of application and elution conditions for IAC will also be discussed. Several applications of IAC will then be considered, including the use of this method in purification, immunodepletion, direct sample analysis and chromatographic immunoassays. The use of IAC in combination with other methods for compound analysis will also be examined. Recent developments in this field will then be presented, including the combined use of IAC with CE or MS, the creation of ultrafast immunoextraction methods and the use of immunoaffinity columns in micro-analytical systems. A variety of applications will be listed in this review but an emphasis will be given to those that are related to bioanalysis.

Basic components of IAC

Structure & properties of antibodies

The basis for IAC relies on the selective binding of antibodies. This binding is a result of a large variety of noncovalent interactions that can occur between an antibody and an antigen and can result in association equilibrium constants in the range of 10^5–10^{12} M^{-1} [20]. It has been estimated that the human body is able to produce between 10^7 and 10^8 different types of antibodies, with each capable of binding to a separate antigen [1]. The typical structure of an antibody, using IgG as an example, consists of four polypeptide chains. These four chains consist of two identical heavy chains and two identical light chains that are linked by disulfide bonds to from a Y-shaped structure (FIGURE 1). The lower stem region of an antibody is referred to as the F_c *region* and is highly conserved from one antibody class to the next. The upper arms of the antibody are called the F_{ab} *regions*. The amino acid sequences in the F_{ab} regions are identical within a single type of antibody but are highly variable between different antibodies. It is this variability that allows antibodies to bind a wide range of antigens.

A foreign agent that is capable of initiating antibody production is called an antigen. Common antigens include viruses, bacteria and foreign proteins from animals and plants that are capable of producing an immune response [1]. Due to the large size of naturally occurring antigens, antibodies that bind to several different regions of the antigen with a range of binding affinities are often generated. Each individual location on an antigen that can bind to a given antibody is called an epitope. In order for a substance to be recognized by the body’s immune system and to lead to the production of antibodies, this substance must have a size that corresponds to a mass of several thousand Daltons [1]. Antibodies can also be produced against smaller substances, but these substances first must be coupled to a larger species (e.g., a carrier protein) before antibody production can occur. A small substance that is used to produce antibodies after being linked to a carrier agent is known as a hapten.

The two main types of antibodies that are used in IAC are polyclonal antibodies and monoclonal antibodies. Polyclonal antibodies are produced from multiple cell lines within the body and as a population can bind a variety of epitopes on a single antigen with a range of binding strengths. Monoclonal antibodies are produced by the fusion of a myeloma cell line with spleen cells obtained from an animal that has been immunized with the desired

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antigen. Because monoclonal antibodies are generated from a single cell line, they bind to a single epitope with identical binding affinities. Two other types of antibodies that can be used in IAC are autoantibodies, which are polyclonal antibodies obtained from patients with auto immune diseases, and anti-idiotypic antibodies, which are antibodies that can mimic the interactions of antigens, hormones or substrates for cell receptors [1].

Recent advances have led to the production of artificial antibodies that have high binding affinities [21]. These artificial antibodies can be produced by combining two or more ligands with moderate affinity on a synthetic tether or polymer. One method of producing these so-called synbodies utilizes a small library of short, unstructured polypeptides that are capable of binding independent sites on a protein target. These polypeptides can easily be linked to bivalent reagents at different distances and orientations [21]. More information on antibody libraries, synbodies and the related topic of recombinant antibodies can be found elsewhere [22–24].

Production of antibodies

Polyclonal antibodies can be produced against a given target by injecting the antigen or a hapten–carrier conjugate into a laboratory animal (e.g., a mouse, rabbit, goat or sheep) [1]. Often this solution of the antigen or hapten–carrier conjugate contains an enhancing agent called an adjuvant. After this initial injection, blood samples from the animal are collected after approximately a month (e.g., 3–4 weeks, although the exact times used in this sequence may vary) and tested for the presence of antibodies that are specific for the desired target. Another injection of the antigen or hapten conjugate (called a ‘booster’) is then made into the animal. The animal’s blood is then retested later (e.g., after 10 days) for the presence of antibodies. Based on the antibody levels that are detected, the animal can be allowed to rest for a period of time (e.g., a few weeks) before being administered another booster injection. This booster/bleed routine can be repeated several times until the antibody concentrations for the required antigen reach the desired level (i.e., as determined by an assay of the blood). At this point, antibody-containing serum can be collected from the animal and stored for later use (FIGURE 2) [1].

The antibodies that are produced upon the first exposure of an animal to a foreign agent are typically IgM class antibodies. After repeated exposure, IgG class antibodies will also be produced. It is this secondary immune response that produces antibodies that are best suited for IAC applications. Antibodies that are produced through the normal immune response are polyclonal antibodies that can bind with various strengths and to a variety of epitopes on the antigen. Before these antibodies can be used in IAC, some further purification is often required. This purification may involve the use of ion-exchange chromatography [25], precipitation with ammonium or dextran sulfate [26] or isolation on a protein A or protein G column [27]. The isolated products from each of these techniques will contain some antibodies that do not bind to the desired antigen, but these other antibodies can later be removed by using an immobilized antigen column [28].

Monoclonal antibodies can be produced by isolating a single antibody-producing cell and combining this cell with a carcinoma or myeloma cell (FIGURE 3). The resulting hybrid cell line, called a hybridoma, is relatively easy to culture and grow for long-term antibody production [1]. This approach for monoclonal antibody production was first reported by Köhler and Milstein in 1975 [29]. In this method, a solution of the antigen or the hapten–carrier conjugate is mixed with adjuvant and injected subcutaneously into an animal. The animal is later given a booster shot, killed and the spleen harvested. The lymphocyte cells are mixed with myeloma cells in the presence of polyethylene glycol, which is added to promote cell fusion. The cells are then grown in the presence of drugs that kill myeloma cells and unfused lymphocytes, but permit the growth of hybridoma cells. Individual

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cultures of hybridomas are examined for the production of specific antibodies and those that make the desired antibody are cloned to produce a homogenous culture of cells making a monoclonal antibody. Although this process can be tedious and difficult, it has the advantage of producing antibodies in relatively large quantities that have well-defined specificity [1].

Supports for IAC

There are several types of supports that can be utilized to place antibodies within columns for use in IAC. Traditional immunoaffinity supports have been based on low-performance materials such as carbohydrate-related media (e.g., agarose and cellulose) or synthetic organic supports (e.g., acrylamide polymers, copolymers or derivatives, polymethacrylate derivatives and polyethersulfone matrixes). TABLE 1 lists several commercial supports in these categories that can be used to immobilize antibodies for IAC. The low cost of these materials has made these supports popular for IAC applications involving target purification or offline immunoextraction. However, most of these materials can be used at only relatively low back pressures and are best suited for work under gravity flow or with a peristaltic pump. These supports also can have slow mass-transfer properties.

These disadvantages have limited the use of the low-performance immunoaffinity supports in applications requiring their direct use with HPLC. When antibodies are used within an HPLC column, the resulting method is referred to as HPIAC. Immunoaffinity supports that are used for HPIAC must be rigid and have higher efficiency than typical low-performance supports [30]. Examples of materials that have been used in HPIAC include derivatized silica [31], glass [32], azalactone beads [33], methacrylate polymeric supports [34] and polystyrene-based perfusion media [35].

In addition to good efficiency and mechanical stability, the ideal support for IAC should have low nonspecific binding and be easily modified for antibody attachment. Another item to consider for a porous material is the size of its pores for immobilization of antibodies and binding by antibodies to the target. Supports with small pores have the largest amount of total surface area, but much of this area may not be accessible for antibody immobilization [30]. By contrast, supports with larger pores do not have accessibility problems, but their lower surface area can result in small amounts of immobilized antibodies. As a result, the maximum coverage for antibodies is often observed for supports with pore sizes of 300–500 Å, which is approximately three- to five-times the diameter of an antibody [36]. This size range is also suitable for the binding of immobilized antibodies to many small- or medium-sized targets (i.e., agents with sizes less than about 100–150 kDa), although larger pore sizes may be needed for larger targets. Other support materials that have recently been used in IAC are disks, fibers and monolithic rods [30,37]. Unique features offered by these newer support materials include their good mass-transfer and flow properties [18]. A general procedure for immobilizing antibodies onto monoliths can be seen in FIGURE 4.

Antibody-immobilization methods

Antibodies can be immobilized onto supports by using a variety of techniques that range from covalent attachment to adsorption-based methods. Of these techniques, those that make use of covalent attachment are the most common, but even these methods can range from the use of random attachment via amino or carboxyl groups to more site-selective immobilization approaches that make use of modified carbohydrate residues or thiol groups. The ideal situation in any of these immobilization methods is to have antibodies attached to the support in a way that does not affect the activity of the binding sites or the accessibility of these sites to their target compound [38].

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Antibodies can be immobilized through free amine groups by using supports that have been activated with agents such as *N,N′*-carbonyldiimidazole, cyanogen bromide, *N*-hydroxysuccinimide or tresyl chloride/tosyl chloride. Antibodies can also be immobilized through amine groups using a support that has been treated to produce reactive epoxy or aldehyde groups on its surface [33,39,40]. The use of amine groups is one of the easiest ways to immobilize antibodies but can cause a decrease in activity if the antibodies have some of these amine groups in their antigen-binding sites [41]. In addition, this approach can cause the antibodies to be immobilized in a random orientation, leading to steric hindrance and a decrease in binding efficiency [42,43].

Antibodies or F_{ab} fragments can be covalently linked to IAC supports through more site-selective methods. This can be achieved by utilizing the free sulfhydryl groups that are created when F_{ab} fragments are generated. These groups can be used for immobilization by using techniques such as the divinylsulfone, epoxy, iodoacetyl/bromoacetyl, maleimide or tresyl chloride/tosyl chloride methods [33,39,40]. In addition, site-selective immobilization can be accomplished by coupling antibodies through the carbohydrate residues that are located in their F_c regions. This process is carried out by first oxidizing the carbohydrate residues under mild conditions to generate aldehyde residues. These aldehyde groups are then reacted with a hydrazide- [44] or amine-containing support [33,39].

A third way antibodies can be immobilized onto supports is by using a secondary ligand to adsorb these antibodies. This can be accomplished by using antibodies that have been reacted with biotin or biotinylated, and then adsorbed to a support that contains immobilized avidin or streptavidin. The most popular biotinylation technique is to incubate antibodies with *N*-hydroxysuccinimide-*D*-biotin at pH 9. The strong noncovalent linkage of biotin to strepavidin or avidin can then be used to immobilize these antibodies. These linkages have association equilibrium constants in the range of 10^{13}–10^{15} M^{-1} [45,46] and can resist many types of elution conditions without dissociation. In addition, a modified version of avidin called neutravidin (from Pierce) can be used for the immobilization of biotinylated antibodies. If amine-based coupling of biotin to the antibodies is employed, the biotin can attach at or near the antibody’s binding sites and cause a decrease in binding. There will also be random orientiation of the resulting biotinylated antibodies on the streptavidin support. These problems can instead be minimized by using hydrazide-biotin [47], which is reacted with the carbohydrate residues of antibodies after these regions have been oxidized under mild conditions to produce some aldehyde groups, as discussed in the previous paragraph.

Another approach for antibody immobilization utilizes the strong binding of protein A or protein G to the F_c regions of many antibodies. Protein A and protein G are bacterial cell wall proteins that have strong binding for many types of antibodies under physiological conditions. However, if the pH of the surrounding solution is decreased to approximately pH 2–3, this binding will be weakened and the retained antibodies can be eluted [4,33,39]. Protein A and protein G supports are useful when high antibody activity is needed and it is desirable to have frequent antibody replacement in an IAC column. Long-term reproducibility of the IAC binding capacity is possible when using protein A or protein G supports, but a much larger amount of antibodies will be needed than is required for traditional immobilization methods if the antibodies are eluted and replaced on a regular basis. If desired, protein A or protein G supports can be prepared with a permanent coating of antibodies by cross-linking the antibodies to the immobilized protein A or protein G by using carbodiimide [48] or dimethyl pimelimidate [49,50].

Application & elution conditions

The application and elution conditions are another important set of factors to consider in the design and use of an IAC method. The application buffer used in IAC is generally chosen

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for its ability to promote fast and efficient binding of the desired analyte or target compound to the immobilized antibodies. Optimum binding for antibodies typically occurs under physiological conditions, so IAC generally makes use of a neutral pH buffer (i.e., pH 7.0–7.4). Under these conditions, the equilibrium constants for antibody binding is usually in the range of 10^6–10^{12} M^{-1}. Due to this strong binding between the antibody and its target, isocratic elution is often not feasible unless the IAC method is using low-affinity antibodies (i.e., those with association equilibrium constants of less than 10^6 M^{-1}) [51–53].

The elution conditions for IAC need to allow for fast elution of the analyte while still allowing later regeneration of the immobilized antibodies. The need for fast but reversible binding and regeneration is especially important when an IAC column is to be used for a large number of samples. Elution is often carried out by temporarily lowering the effective strength of antibody binding to the target. The most-common approaches for elution in IAC include changing the mobile phase pH or adding a chaotropic agent to the mobile phase. Other, less-common IAC elution methods include adding a competing agent, organic modifier or denaturing agent to the mobile phase, or changing the temperature of the column during elution [54]. Usually the elution buffer is applied in a step gradient, but gradual or nonlinear gradients can be used as well [1,2,4]. FIGURE 5 shows a common scheme by which an IAC column can be used to selectively bind and elute analytes from a sample.

Changing the mobile phase pH is the most popular method for eluting retained compounds from IAC columns. This approach is usually conducted by applying an acidic buffer (pH 1–3) to the column. Alkaline elution conditions have been used in conjunction with low-performance IAC supports [55,56], but cannot be used with common HPIAC supports, such as silica or glass beads, due to the instability of these supports at a pH greater than 8.0 [2]. One difficulty with changing the pH of the mobile phase is the possibility of denaturing the immobilized antibodies or any retained compounds that are susceptible to variations in pH. However, many IAC columns have been shown to be quite stable when moderate pH changes in the range of 7.0–7.5 to 2.5–3.0 are used for elution [1,2].

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To avoid denaturing effects that are caused by lowering the pH, the elution of retained compounds in IAC can alternatively be performed by adding chaotropic agents such as thiocyanate (SCN^-), trifluoroacetate (CF_3COO^-), perchlorate (ClO_4^-), iodide (I^-) or chloride (Cl^-) to the elution buffer. The elution strength of these agents follows the approximate order $SCN^- > CF_3COO^- > ClO_4^- > I^- > Cl^-$. These agents are typically used at concentrations of 1.5–8 M and have been shown to be effective in dissociating high-affinity antibody–antigen complexes [57]. When an organic modifier is used in the mobile phase, care must be taken to ensure that the concentration of the organic additive does not permanently denature the antibodies. When methanol or other organic modifiers are used, the capacity of the immunoaffinity column has been shown to decrease. This denaturation is not irreversible, but kinetic regeneration can take a few days [54]. In one study, an anticlenbuterol immunoaffinity column was regenerated 20-times after offline elution with 2 ml of ethanol 80% in water [58]. However, this column was shown to lose half its activity over these 20 regenerations. Therefore, if harsh elution conditions are required, the capacity of the immunoaffinity column should be much larger than the actual amounts of analyte that are to be measured or isolated.

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Formats & applications of IAC

Immunoaffinity chromatography is a powerful technique that can selectively isolate a given compound from complex samples. As a result, many formats utilizing IAC have involved preparative applications or selective analyses. IAC has also been used with both direct and

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indirect detection methods and has been coupled with other methods such as HPLC, GC, MS and CE. Each of these formats will be discussed in more detail in the following sections.

On/off elution & direct detection methods

Preparative applications generally use the on/off mode of IAC, as shown in FIGURE 5. This format can also be used with direct detection for chemical analysis. In the on/off mode, a sample is applied to the IAC column in the presence of an application buffer. As the sample is applied to the column, only the analyte and closely-related compounds are retained by the column, while other sample components pass through non-retained. After the analyte has been bound to the column and other materials have been washed away, an elution buffer is applied and the analyte is eluted. After this elution step, the application buffer is reapplied to the column to allow for regeneration of the antibodies prior to another sample application.

The on/off mode of IAC is commonly used in biochemistry and other fields for the selective purification of target compounds from complex samples. Compounds that have been isolated by this approach include proteins, glycoproteins, carbohydrates, lipids, bacteria, viral particles, drugs and environmental agents [1,3,4,8–12]. This mode can also be used for the direct detection of an analyte by placing a suitable detector after the IAC column [1,2]. For this type of application, the analyte must be present at relatively high concentration and be eluted in a sharp, well-defined peak that allows a good detection limit. Depending on the desired level of detection, UV/visible absorbance, fluorescence and MS have all been used for detecting analytes in the on/off mode of IAC. Specific examples of analytes that have been measured by this approach include human serum albumin [59,60], recombinant tissue-type plasminogen activator [61], recombinant antithrombin III [61], IgG [59,62], *Escherichia coli* [63], isoproturon [64], phenylurea herbicides [64,65], benzidine [66], dichlorobenzidine [66], aminoazobenzene [66], azo dyes [66], triazine [65], diethylstilbestrol [67], acetylcholinesterase [68], transferrin [69] and insulin [70].

Immunoextraction & immunodepletion

When IAC is used to remove a specific analyte or group of analytes from a sample prior to analysis by a second analytical method, this approach is referred to as immunoextraction [2,15,71]. In contrast to the on/off method discussed in the previous section, immunoextraction is coupled with a second analytical method such as LC. Immunoextraction can be carried out either offline or online with the second analytical method. In the offline mode, antibodies are typically immobilized onto a low-performance support and packed into a small disposable syringe or SPE cartridge. Samples are then applied through the affinity support, which binds the analytes of interest while other sample components are washed away. An elution buffer is then passed through the affinity support to elute the extracted analytes. In addition to removing undesirable sample components, immunoextraction can allow for analyte concentration. In fact, by applying a larger sample volume to the immunoaffinity support (as long as column capacity is not exceeded), more analyte can be made available for detection by the second analytical method due to the essentially irreversible binding of the analyte to the antibodies under typical application conditions [72–74]. Just as in traditional SPE methods, in offline IAC the eluted fraction can be collected, dried down, and dissolved in a solvent more suitable for analysis. If necessary, the sample can also be derivatized prior to analysis. Offline immunoextraction coupled with other methods has been used in the analysis of urine, food, water and soil extracts. Examples of analytes that have been examined by this approach include α_1-anti-trypsin, atrazine, benzylpenicilloyl-peptides, bovine serum albumin, carbendazim, chloramphenicol, cortisol, clenbuterol and phenytoin, among others [1,2].

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A related method that uses IAC is immunodepletion. In immunodepletion, an antibody column is used to remove abundant analytes from a complex sample prior to using a second method of analysis for the minor sample components. Typically, this method is used to remove high- and mid-abundance proteins from serum samples prior to the analysis of low-abundance proteins, as is often required in proteomics. In contrast to other methods that can be used to remove high- and mid-abundance proteins (e.g., precipitation, SPE, ultracentrifugation, molecular-weight separation and pI separation), immunodepletion can provide highly selective depletion of multiple high-abundance proteins simultaneously [75–80].

Hybrid IAC methods

The on/off mode of IAC can also be used for online analytical applications. In this format, IAC is directly coupled to a second analytical technique for analysis. By directly combining immunoextraction with a second analytical method, such as HPLC, sample pretreatment can be automated. IAC is often coupled with reversed-phase (RP)LC [2,13,15], but methods in which IAC has been coupled with size exclusion, ion-exchange chromatography [81], CE [82–86], MS [87], GC [88,89] and microfluidic devices [14,90] have also been reported.

One reason immunoextraction is commonly coupled with RPLC is due to the widespread use of RPLC in chemical separations. In addition, the elution buffer used in IAC is an aqueous solution, which acts as a weak mobile phase for RP columns. Online immunoextraction coupled with RPLC has been used to measure compounds in food extracts [91], bodily fluids [92–99], cell extracts [100] and environmental samples [74,101]. An example of an HPLC system which allows for the combination of immunoextraction with RPLC is shown in FIGURE 6.

Competitive binding assays

Another way in which IAC can be used as an analytical tool is in an immunoassay format. This approach is known as a chromatographic immunoassay or flow-injection immunoassay. One general type of chromatographic immunoassay is a competitive binding assay, in which a signal is generated as the analyte competes with some labeled species for antibody binding sites.

The most common type of competitive binding immunoassay in IAC is the simultaneous injection immunoassay. A schematic diagram of this format is shown in FIGURE 7. In this method, a sample is mixed with a labeled analog of the analyte and applied to a column that contains a limited amount of immobilized antibodies. The limited amount of antibodies causes the labeled analog (A^*) and the analyte (A) to compete for binding sites. Due to the presence of this competition, the amount of labeled analog that is detected in the bound and/or retained fractions is affected by the presence of analyte [20]. Typical calibration curves are prepared by plotting the relative response of the labeled analog, B/B_o, versus the concentration of analyte in the sample, where B is the amount of labeled analog bound in the presence of a given amount of analyte and B_o is the amount of labeled analog bound in the absence of any analyte. This type of calibration curve will have a maximum value of 1 when no analyte is present and should approach 0 at high analyte concentration, assuming there is no non-specific binding between the labeled analog and the column. A large variety of analytes have been measured by using simultaneous injection competitive binding immunoassays (TABLE 2) [102–131]. Factors that affect the response of these methods include the relative amount of analyte applied to the column, the flow rate and the amount of labeled analog used in the experiment [132,133].

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A second type of competitive binding immunoassay is the sequential injection immunoassay [20]. As shown in Figure 8, this method differs from the simultaneous injection immunoassay format in that the sample is injected onto the column followed by a later injection of label analog. A calibration curve is again generated by plotting the relative response (B/B_o) versus the analyte concentration. The sequential injection binding assay has one important advantage over the simultaneous injection format in that the labeled analog never comes into contact with the analyte, which means sample matrix effects during the detection of the labeled analog can be eliminated. This feature allows for improved reproducibility, a wider range of detection formats and lower background signals. Several applications of sequential injection immunoassay applications can be seen in TABLE 3 [134–144]. The sequential injection format tends to give lower LOD than the simultaneous format, because the analyte has a better chance of binding to the column in this format. However, the simultaneous injection format has higher upper LOD and a wider dynamic range [145]; this wider dynamic range is a result of the increased ability of the analyte to compete with the labeled analog when they are applied to the immunoaffinity column at the same time.

A third type of competitive binding immunoassay is the displacement immunoassay, as illustrated in FIGURE 9 [20]. In this method, an IAC column is first saturated with a labeled analog of the desired analyte. Sample is then injected onto this column and displaces any labeled analog that is momentarily free in solution. This displaced analog is then eluted from the column and gives a response that is proportional to the amount of analyte in the sample. As long as enough labeled analog remains bound to the column to give a consistent and measurable signal, several samples can be injected onto the IAC column before this column must be regenerated [146]. To help ensure enough labeled analog is present, IAC columns with large capacities are often employed for this format. Additionally, the stability of the signal depends on the rate of dissociation of the labeled analog from the immobilized antibodies [146,147]. When performing displacement immunoassays, slow flow rates tend to increase the displacement effect because longer times of contact between the sample and column allow for more labeled analog to dissociate from the immobilized antibodies. Several applications for displacement assays can be seen in TABLE 4 [146–152].

Noncompetitive immunoassays

Noncompetitive immunoassays are another group of immunoassays that use indirect detection. These assays are often referred to as immunometric methods. In these methods, there is no competition between the analyte and other substances. Two types of non-competitive immunoassays have been used in IAC: sandwich immunoassays and one-site immunometric assays.

Sandwich immunoassays utilize two different antibodies that bind the same analyte. One of the antibodies is immobilized onto a solid support and is used to extract the analyte from samples. A second, labeled, antibody is either mixed with the sample prior to application or applied to the column directly after the sample is applied to the IAC column. Once the analyte is ‘sandwiched’ between the two antibodies, an elution buffer is applied to elute the analyte and labeled antibodies. The labeled antibodies can then be detected and give a response that is directly proportional to the amount of retained analyte. A typical scheme for this type of assay is given in FIGURE 10. When the sample and labeled antibodies are mixed prior to application onto the column, better detection limits can be obtained than when sequential injection of the sample and labeled antibodies is employed, because there is more effective binding between the labeled antibody and analyte. A calibration curve for sandwich immunoassays is constructed by plotting the relative response of the eluted labeled antibody against the amount of analyte in a sample and can give a linear response over a broad range of analyte concentrations [2,20].

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Due to the direct relationship between the amount of analyte and the response in a sandwich immunoassay, plus the ability to use an excess of labeled antibodies to help promote good detection, this approach allows for better signal-to-noise ratios and lower limits of detection than competitive binding immunoassays [20]. Sandwich immunoassays also tend to be more selective than competitive binding immunoassays, because two types of antibodies are used for analyte binding and detection instead of one [153]. In general, low flow rates are desirable to allow sufficient time for analyte–antibody complexes to bind to an IAC column in this format. In addition, columns that can effectively capture the antibody–analyte complex with low nonspecific binding are best for sandwich immunoassay development. The main disadvantage of the sandwich immunoassay is that only large analytes (e.g., large peptides, proteins and biomacromolecules) can be quantified by this approach because the analyte must be able to bind two antibodies simultaneously. One other disadvantage of this approach is the added cost of performing a sandwich immunoassay due to the need for two different antibodies per analyte. Examples of sandwich immunoassays that have been carried out by using IAC can be found in TABLE 5 [60,110,154–161].

The format for a one-site immunometric assay is provided in FIGURE 11 [20]. In this method, the sample is incubated with a known excess of labeled antibodies or F_{ab} fragments that can bind to the analyte. This mixture is then applied to a column with an immobilized analog of the analyte, which is used to remove the excess and unbound labeled antibodies/ antibody fragments. The bound analyte-labeled antibodies elute in the nonretained fraction with the analyte and give a signal that is proportional to the amount of analyte in the original sample. Typically, calibration curves for one-site immunometric assays are created by plotting the relative response of the labeled antibody–analyte complex against the amount of analyte in the sample. To obtain a maximum signal in a one-site immunometric assay, the binding between the labeled antibodies and the analyte must reach equilibrium and the flow rate must allow for the capture of all the excess binding agents on the IAC column. The column should also have a binding capacity that exceeds the amount of binding agent that is applied between regeneration steps. Some advantages of one-site immunometric assays are that they can detect both large and small analytes, their signal is directly proportional to the amount of analyte and a multitude of elution conditions can be used with the immobilized analog columns. The main disadvantage of one-site immunometric assays is obtaining affinity ligands with the required activity and purity to create an assay in which all the excess agent binds to the affinity column and gives a low background signal [162]. Examples of reports that have utilized the one-site immunometric format in IAC are listed in TABLE 6 [163–178].

Postcolumn immunodetection

An IAC column can also be used to monitor the presence of a specific analyte as it elutes from another chromatographic column. This use of IAC is referred to as immunodetection. Immunodetection can be performed using either direct detection or indirect detection. Typically, a postcolumn reactor and an IAC column are attached to the exit of an analytical HPLC column as shown in FIGURE 12. As the analyte elutes, it is mixed with excess labeled antibody. The excess labeled antibodies are removed using an immobilized analog of the analyte column. Bound, labeled antibodies pass through the analog column and give a signal proportional to analyte concentration. Reviews of this technique can be found elsewhere [2,164]. When performing postcolumn immunodetection, it is especially important to ensure the eluant from the HPLC column is properly adjusted to allow for maximum antibody binding in the postcolumn reactor (pH, ionic strength and removal/ minimization of organic modifiers).

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Recent developments in IAC

The development of IAC is ongoing and continues to be integrated with other analytical techniques, including CE and MS. Other new developments include ultrafast immunoaffinity CE and the use of antibodies in microanalytical systems.

When immobilized ligands are used in CE, the method is referred to as affinity electrophoresis [179]. Affinity ligands, such as antibodies, can be immobilized in CE capillaries by several methods, including physical entanglement in gels and covalently binding the ligand to the capillary wall, or polymers, frits or beads inside the capillary [82]. CE can also be used with antibodies/antibody fragments to quantitatively measure analytes by allowing CE to separate free analytes from analyte–antibody complexes [180]. These formats can be either competitive or noncompetitive, with noncompetitive formats giving better LOD [82]. CE immunoassays are utilized due to their ease of automation and their relatively fast separation of antibodies, analytes and/or antibody–analyte complexes [86]. Another advantage of CE immunoassays is that only small amounts of sample and reagents are used while still maintaining good LOD [181]. The best LOD in CE immunoassays are generally achieved when using laser-induced fluorescence detection or MS.

By combining IAC with MS (IAC–MS), a technique is produced that utilizes the selectively of antigen–antibody interactions and the sensitivity of MS. When performing online IAC–MS, the IAC elution buffer should contain only volatile buffer salts (e.g., ammonium acetate and ammonium formate) to avoid lowering ionization efficiency. In addition, methanol or acetonitrile is often added to the eluant prior to ionization to increase sensitivity. MALDI can use immobilized antibodies on the target to help extract desired compounds from a sample prior to analysis by MS [87].

Due to the speed and specificity of antibody–antigen interactions, IAC can also be used for very fast immunoextractions that often take less than a second to perform. Ultrafast immunoextraction has been used to quantify the free fraction for drugs and hormones in clinically related samples [182–184]. Measuring the free fractions of drugs and hormones in serum is often difficult for other methods, because any removal of the free fraction can perturb protein binding and cause additional drug or hormone to dissociate in the sample. In recent work, ultrafast immunoextraction conducted in less than a few hundred milliseconds has been used with both direct detection based on fluorescence and chromatographic immunoassays using a displacement format for detection of warfarin, phenytoin and thyroxine in protein and serum samples [182–184].

Owing to recent advances in microfabrication technologies, micro and ultramicro (nano) analytical systems can also be developed to utilize the specificity of antibodies [185]. These micrototal analysis systems can be engineered in a variety of formats, including the construction of microarrays which utilize several channels that can each separate multiple analytes all at once. Much like CE immunoassays, these systems need to use quite sensitive detectors, due to the small amounts of analyte that must be measured. One example of a micrototal analysis systems utilizing laser-induced fluorescence detection detected α-fetoprotein [186,187]. More information on the combined use of IAC with microanalytical systems can be found in a review by Phillips [90].

Conclusion

This review has discussed how immunoaffinity chromatography can be used to extract compounds of interest from samples either for purification or quantification. The general parameters of antibodies and antibody production were discussed, along with various immobilization methods. Support materials and the characteristics needed for various types

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of analyses were also discussed. Multiple applications of IAC were presented including immunoextraction, immunodepletion and chromatographic immunoassays. In addition, combining other analysis methods with IAC was discussed including CE, MS and microanalytical systems.

Future perspective

Given the continued importance of antibodies as tools in examining biological samples, it is expected that the use of IAC and related methods will continue to grow in the field of bio-analysis. In the next 5–10 years it is anticipated that further work will continue in the creation of improved columns for IAC based on materials such as monolithic media or new chromatographic supports. The combined use of these IAC columns with MS, CE and other methods is also expected in fields such as proteomics, metabolomics and the high-throughput screening of biomarkers. It is also anticipated that applications of rapid immunoaffinity methods, chromatographic immunoassays and IAC columns in microanalytical systems will increase in such fields as the need increases for selective and fast methods for the examination of complex biological samples.

Executive summary

- Immunoaffinity chromatography (IAC) combines the use of LC with the specific binding of antibodies or related agents.
- IAC can be used in specific assays for a particular target or for purification and concentration of analytes prior to further examination by another technique.
- Factors to consider in the development of an IAC method include the supports and immobilization methods used for antibodies and the selection of application and elution conditions for the resulting columns.
- Applications of IAC include its use in purification, immunodepletion, direct sample analysis, chromatographic immunoassays and combined analysis methods.
- Recent developments of IAC include its use with CE or MS, ultrafast immunoextraction methods and microanalytical systems.

KEY TERMS

Immunoaffinity chromatography	Type of LC in which the stationary phase consists of an antibody or antibody-related reagent, which is used for the selective purification or analysis of a target compound
Immunodepletion	Use of an antibody column to remove abundant analytes from a complex sample prior to using a second method of analysis for minor sample components
Chromatographic immunoassay	Use of immunoaffinity chromatography as an analytical tool is in an immunoassay format
Ultrafast immunoextraction	Type of immunoextraction that is designed to operate on a time scale that is typically less than a second
Microanalytical system	System produced by using microfabrication or other techniques to provide a miniaturized device for chemical analysis

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Displacement immunoassay	Type of competitive binding immunoassay in immunoaffinity chromatography in which an antibody column is first saturated with a labeled analog of the desired analyte, with the sample then being injected to displace any labeled analog that is momentarily free in solution
Immunometric method	Type of immunoassay that uses indirect detection of the analyte through labeling with a binding agent rather than through competition with a labeled analyte analog
Immunodetection	Use of an immunoaffinity column to monitor the presence of a specific analyte as it elutes from another chromatographic column

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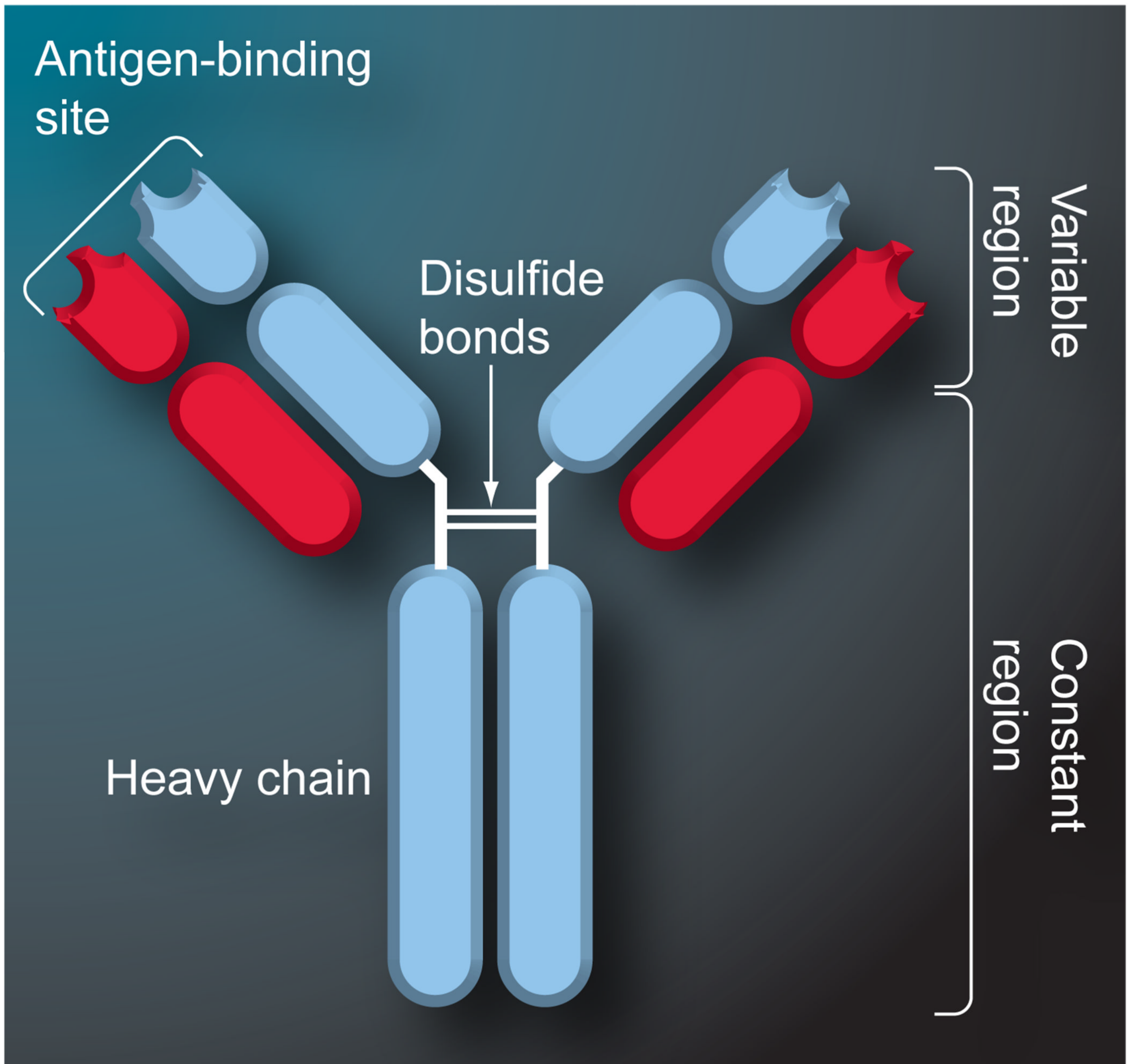

Figure 1. Typical structure of an IgG-class antibody

IgG-class antibodies consist of four polypeptide chains that are linked by disulfide bonds to create a 'Y'-shaped structure with two identical binding sites.

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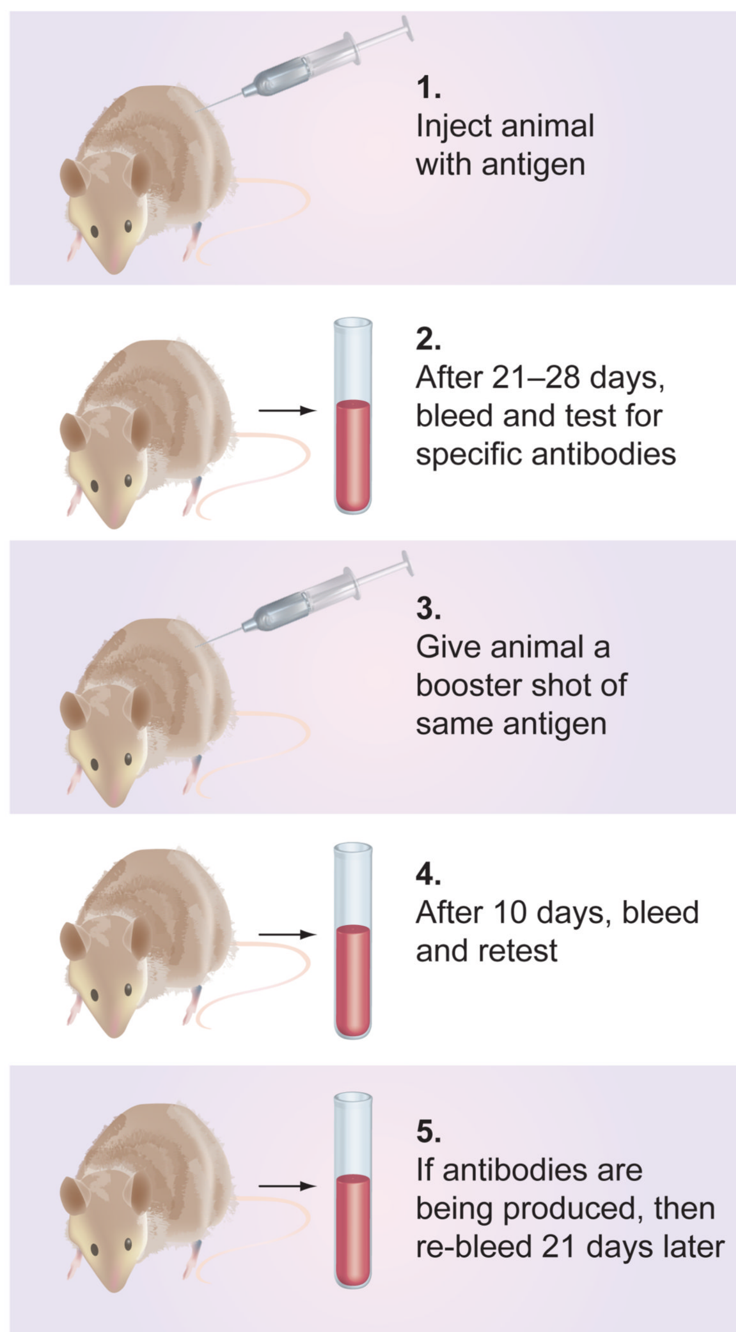

Figure 2. Process for polyclonal antibody production

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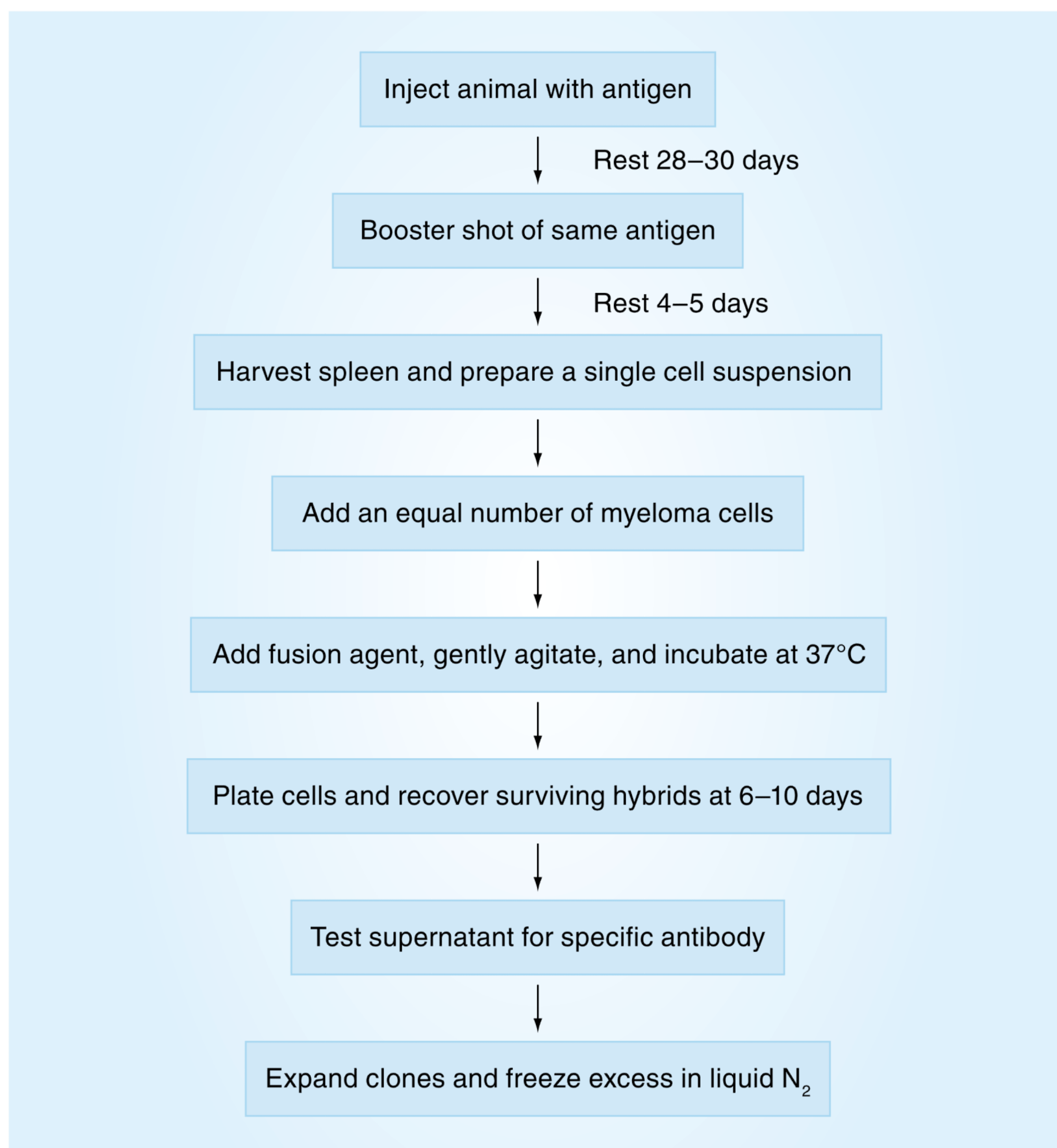

Figure 3. Process for monoclonal antibody production

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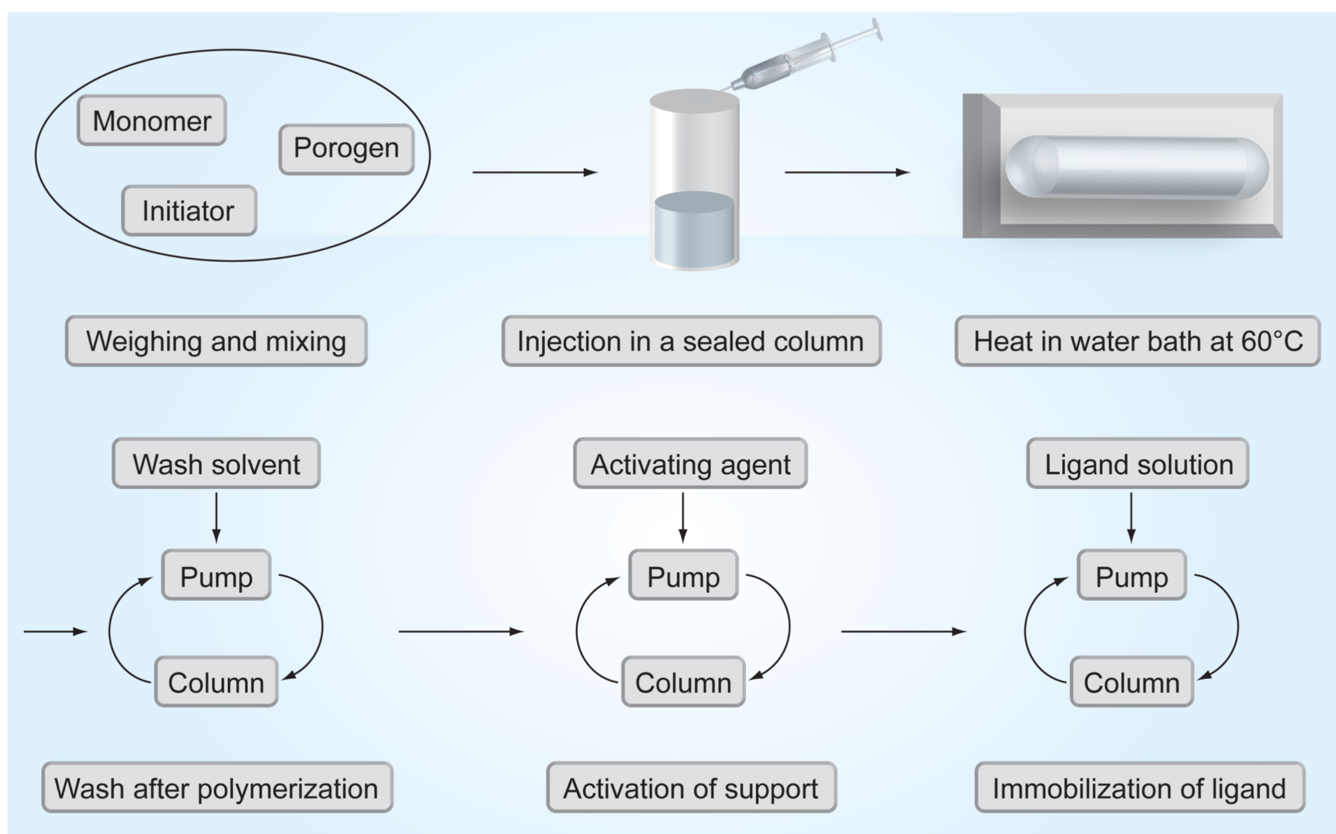

Figure 4. General procedure for immobilizing antibodies within a monolithic column

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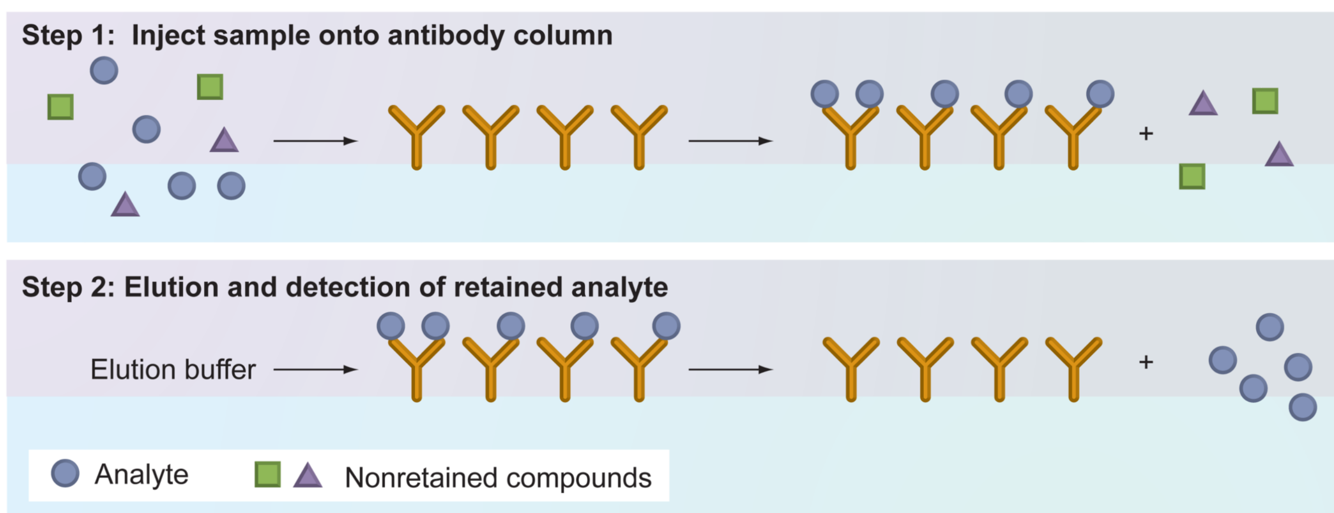

Figure 5. Typical format in which a sample containing the analyte is applied to an immunoaffinity column and nonretained sample components are allowed to pass through

The analyte is later eluted by disrupting the antibody–antigen interactions with an appropriate elution buffer. This on/off mode can be used for direct detection and/or purification of the analyte.

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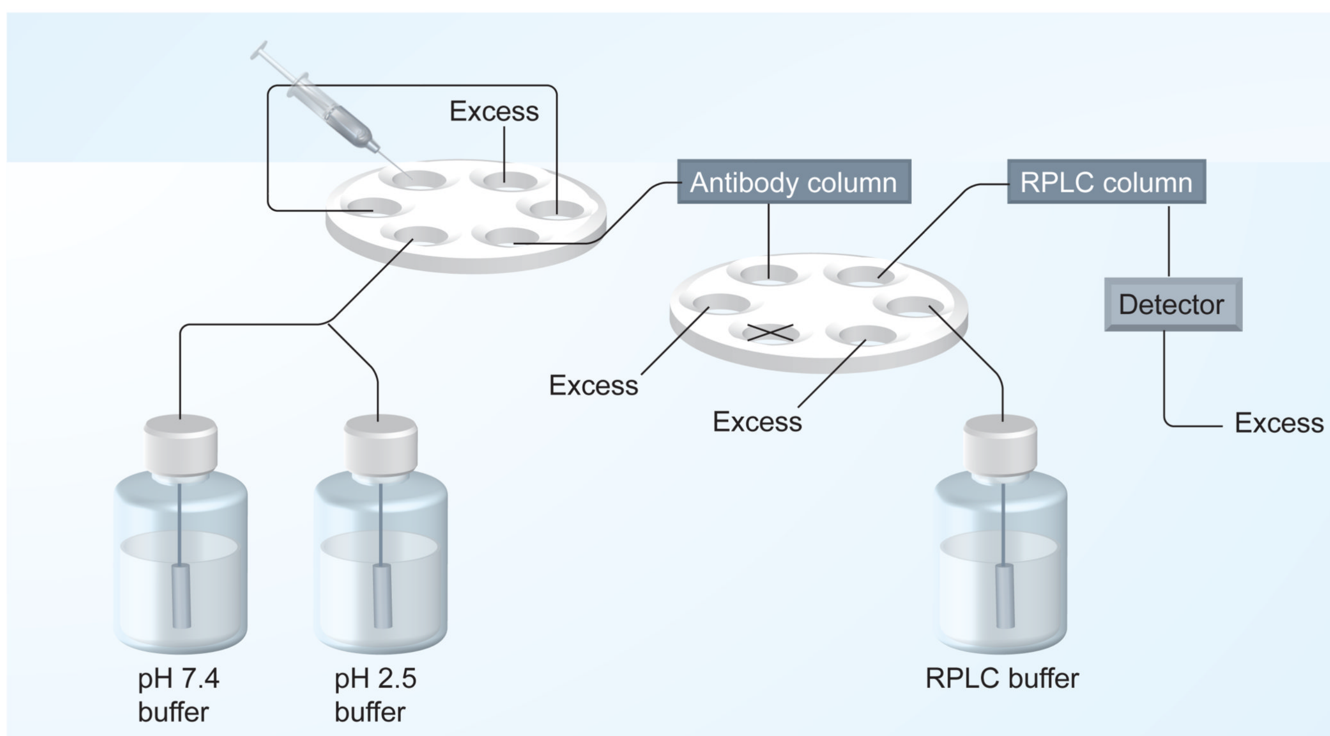

Figure 6. System for combining immunoaffinity chromatography with reversed-phase chromatography

An antibody column is used to extract or preconcentrate analytes from a sample prior to separation of these analytes using a reversed-phase column. This particular system has been used to measure virginiamycin in water samples with a LOD of 1 ppb.

RP: Reversed-phase.

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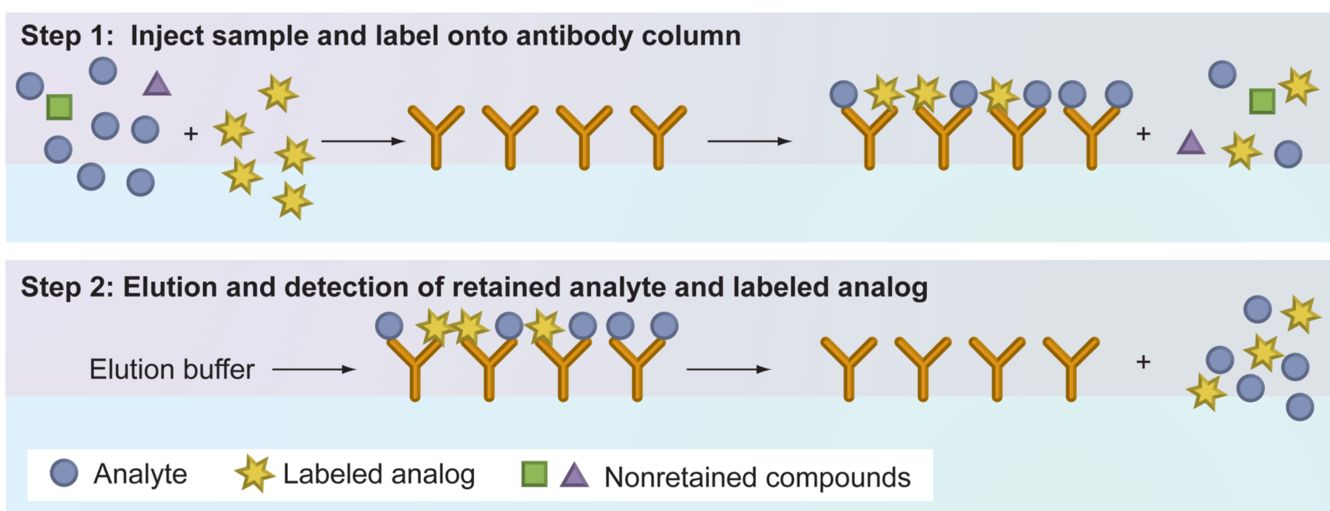

Figure 7. Simultaneous injection immunoassay format

In this type of competitive immunoassay, the sample and a labeled analog of the analyte are mixed and injected onto an immunoaffinity column. The labeled analog and analyte can bind to this column while other sample components pass through nonretained. The analyte and labeled analog are then eluted with an appropriate mobile phase/elution buffer. In this method the analyte concentration in the sample is inversely related to the amount of retained labeled analog that is detected.

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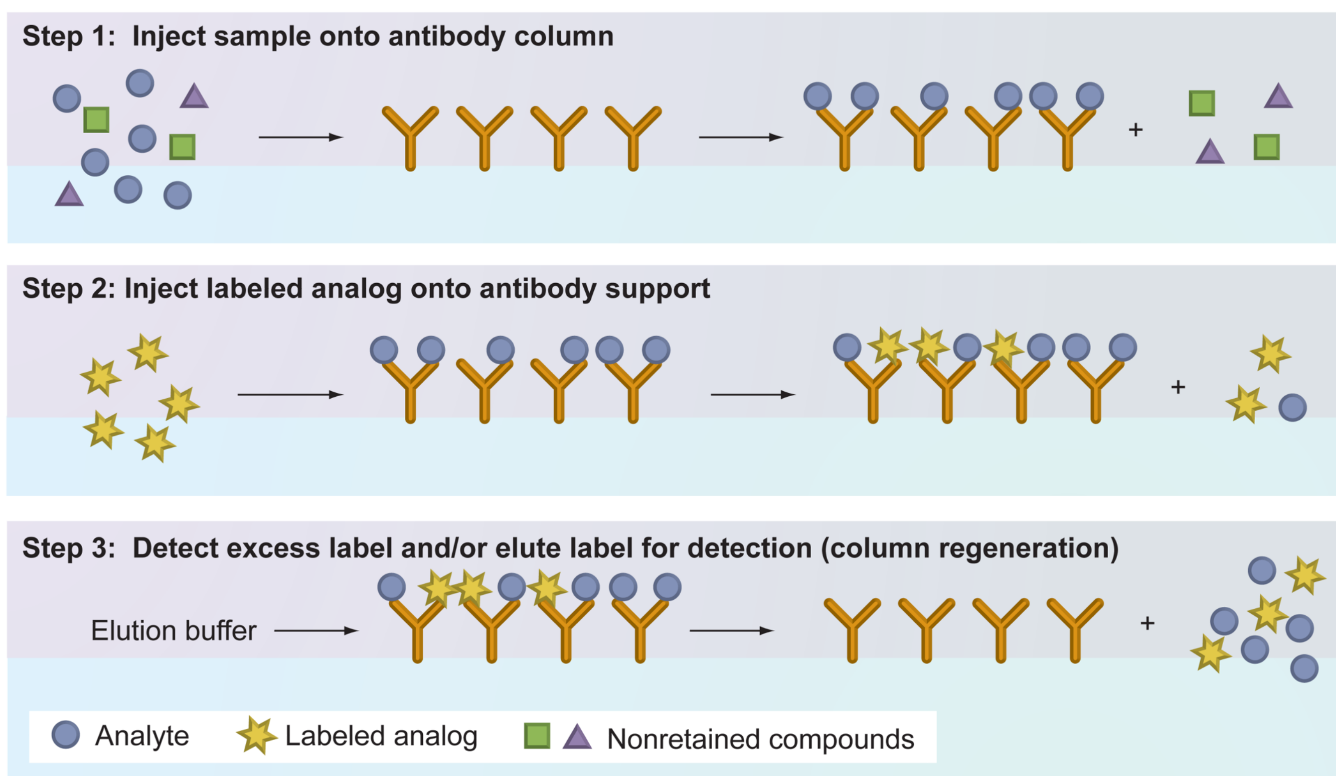

Figure 8. Sequential injection immunoassay format

In this format, the sample is applied onto an immunoaffinity column and the analyte is allowed to bind. A labeled analog of the analyte is then injected onto the same column and also allowed to bind to any remaining free antibody sites. An elution buffer is used to later remove both the retained analyte and labeled analog for the column. The amount of retained labeled analog will be inversely related to the amount of analyte that was in the original sample.

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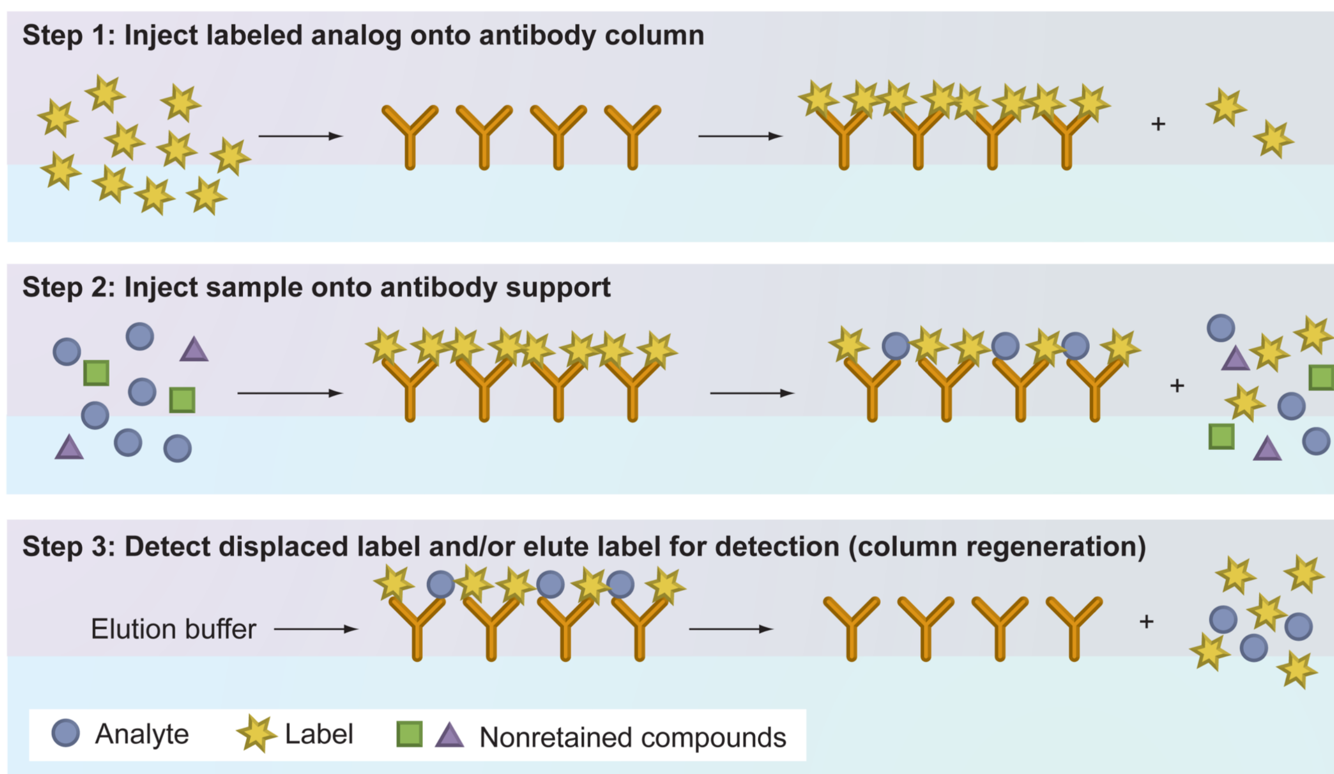

Figure 9. Displacement immunoassay format

In this type of competitive binding immunoassay, a labeled analog of the analyte is injected onto an immunoaffinity column. A sample is then injected onto the column and the analyte is allowed to displace some of the labeled analog. The size of the peak for the displaced label is directly related to the amount of analyte that was in the sample.

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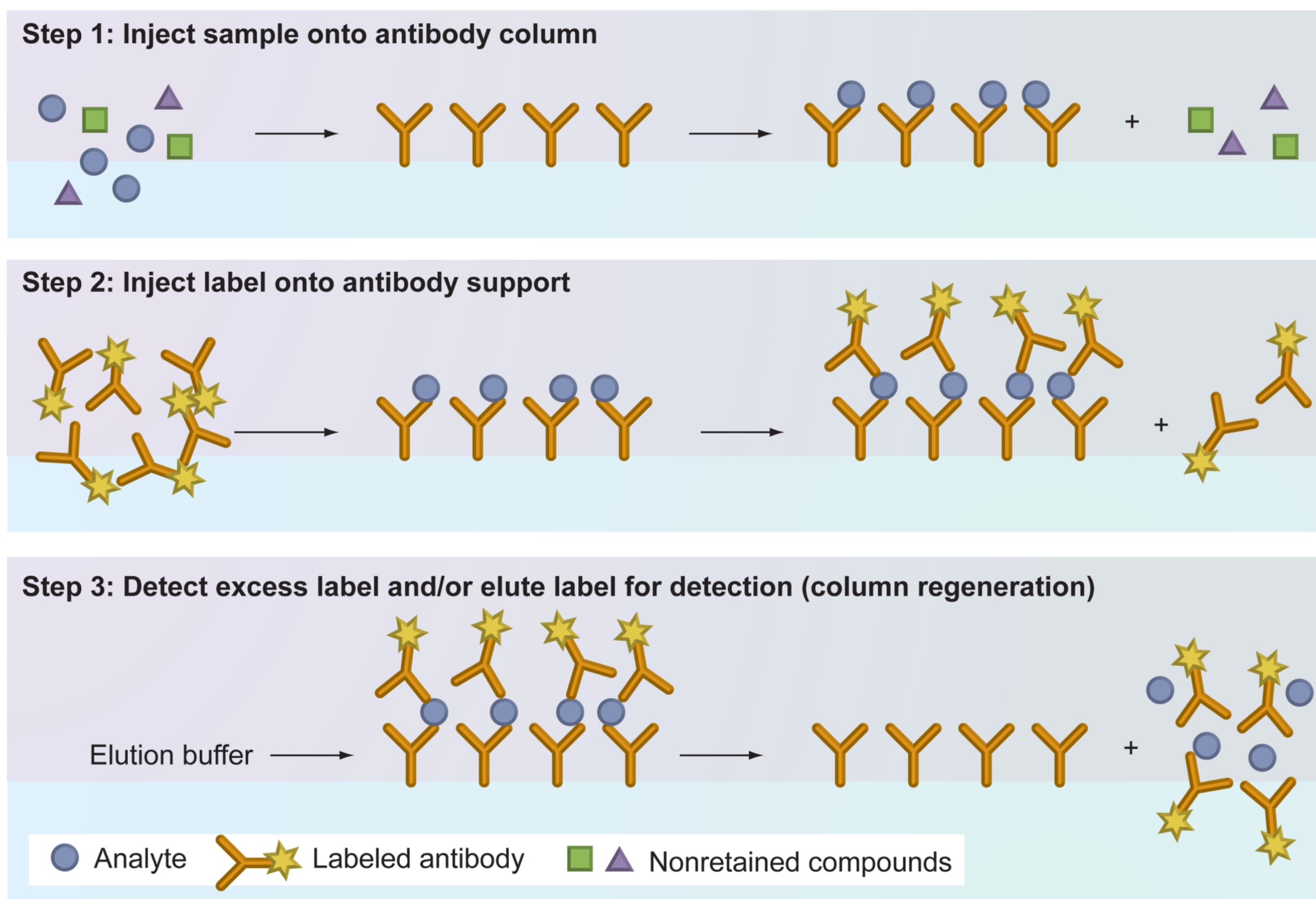

Figure 10. Sandwich immunoassay format

In this type of noncompetitive binding immunoassay, the sample is injected onto an immunoaffinity column and the analyte allowed to bind to the immobilized antibodies. A labeled antibody that is specific for the analyte is then injected onto the same column and also allowed to bind, creating a sandwich immune complex for the analyte. An elution buffer is applied to disrupt the antigen–antibody binding and regenerate the column. The amount of retained, labeled antibody that is eluted during this step is directly proportional to the amount of analyte that was present in the original sample.

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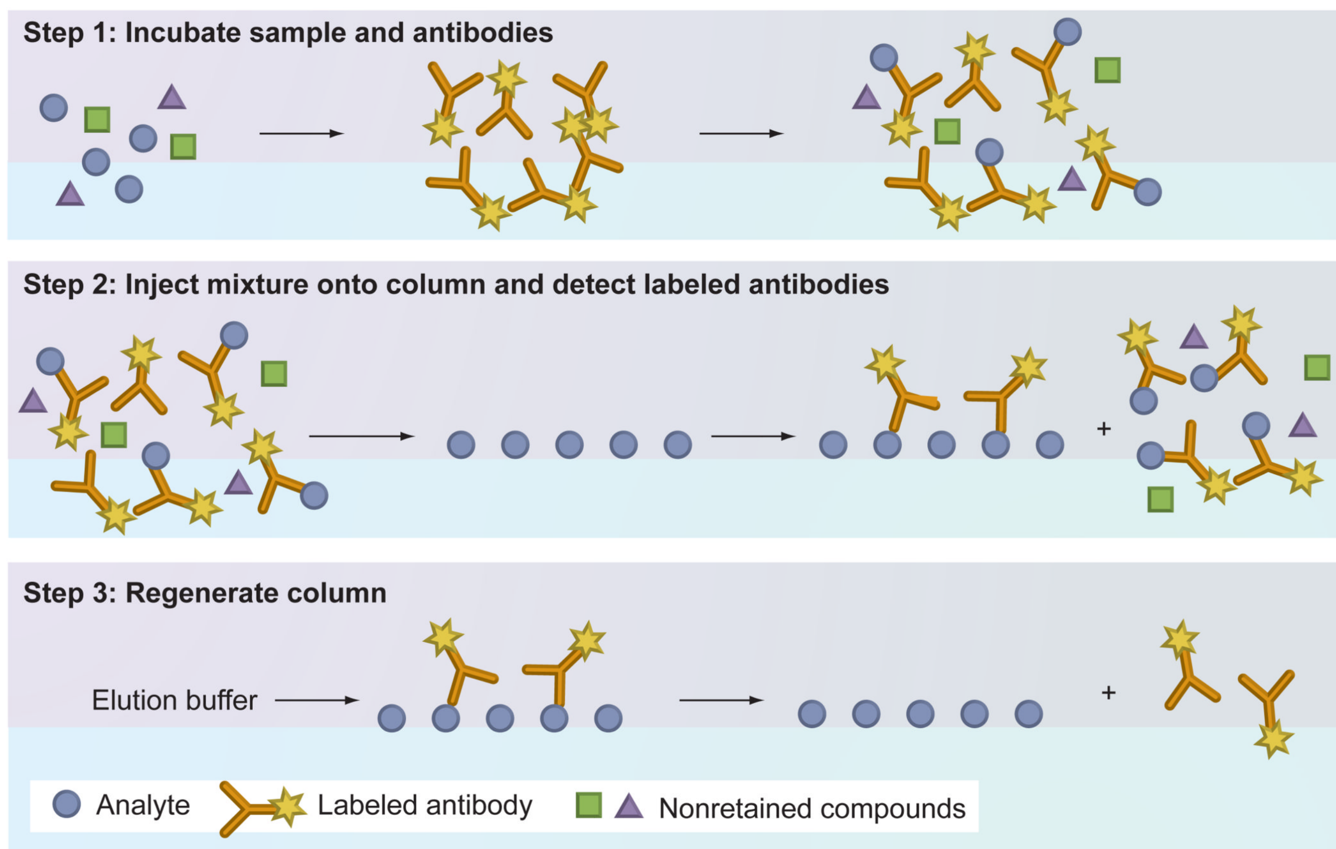

Figure 11. One-site immunometric assay

In this noncompetitive immunoassay format, the analyte and labeled antibodies are mixed and incubated prior to injection onto an immobilized analyte column. The analyte column binds any excess antibodies and the analyte-bound antibodies are eluted in the nonretained fraction, providing a signal that is directly related to the analyte's original concentration. The column is later regenerated by eluting off the excess labeled antibodies.

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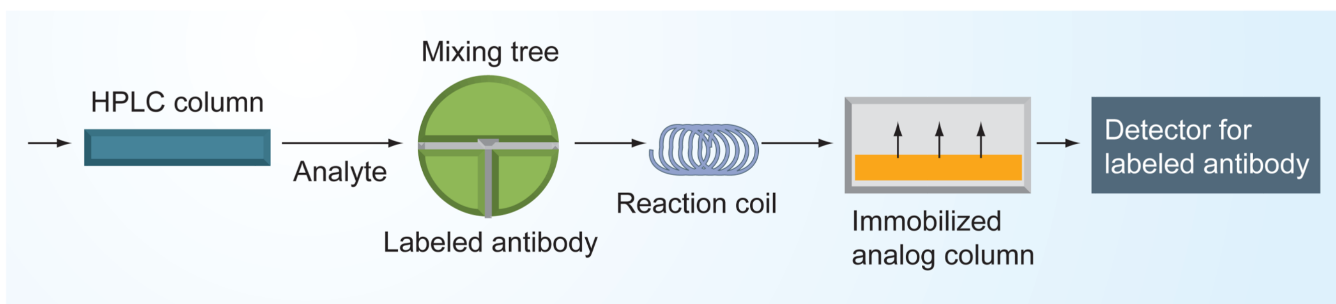

Figure 12. General scheme for postcolumn immunodetection

In this detection method, eluent from an HPLC column is directed into a reaction coil along with an excess of labeled antibodies that are able to bind the desired analyte. The excess antibodies bind to an immobilized analog column and the labeled analyte–antibody complexes are monitored as they elute from the analog column, providing a signal that is directly related to the original amount of eluting analyte. Reproduced with permission from [15]. © American Chemical Society (2001).

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Table 1

Commercially available supports that can be used for immunoaffinity chromatography.

Support type	Supplier
Low- or medium-performance supports	
Affi-Gel	BioRad
Affinica Agarose/Polymeric Supports	Schleicher and Schuell
AvidGel	BioProbe
Bio-Gel	BioRad
Fractogel	EM Separations
HEMA-AFC	Alltech
Reacti-Gel	Pierce
Sephacryl	Pharmacia
Sepharose	Pharmacia
Superose	Pharmacia
Trisacryl	IBF
TSK Gel Toyopearl	TosoHaas
Ultragel	IBF
High-performance supports	
AvidGel CPG	BioProbe
HiPAC	ChromatoChem
Protein-Pak Affinity Packing	Waters
Ultraafinity-EP	Bodman
Emphaze	3M Corp./Pierce
POROS	ABI/PerSeptive Biosystems
Data from [1].	

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Table 2

Examples of simultaneous injection immunoassays.

Analyte	Detection method	Assay characteristics	Ref.
Methods based on antibodies adsorbed on protein A or protein G			
Human transferrin	Fluorescence	Range: <500 μg/ml	[103]
Transferrin	Fluorescence	LOD: 25 μg/ml	[104]
Adrenocorticotropic hormone	Fluorescence	Range: 0.2–10 mg/l	[105]
Testosterone	Fluorescence	LOD: 0.5 μg/ml	[106]
Theophylline	Fluorescence	LOD: 0.3 ng/ml; range: <500 μg/l	[107]
Atrazine	Fluorescence	LOD: 2.1 μg/l; range: 2.1–50 μg/l	[108]
Cephalexin	Electrochemical	LOD: 1 μg/l	[109]
IgG	Absorbance	LOD: 333 zmol	[110]
Theophylline	Electrochemical	LOD: 25 ng/ml	[111]
Anti-BSA	Fluorescence	LOD: 0.2 nM; linear range: 0.4–0.8 nM	[112]
Methods based on covalently immobilized antibodies			
HSA	Thermometric	LOD: 10^{-10} M	[113]
Theophylline	Fluorescence	LOD: 3 μg/l; range: 3–75 μg/l	[102]
Pullulanase, IgG, antithrombin	Fluorescence	Range: μg/ml	[114]
IgG	Fluorescence	LOD: 155 ng/ml	[115]
IgG	Fluorescence	LOD: 4×10^{-9} M	[116]
Gentamicin	Fluorescence	LOD: 200 ng/ml; working range: 250–5000 ng/ml	[117]
IgG	Fluorescence	Linear range: 1–5 μg/ml	[118]
Insulin	Thermometric	LOD: 0.025 μg/ml; working range: 0.05–2 μg/ml	[119]
Carbaryl	Fluorescence	LOD: 20 ng/l	[120]
Atrazine	Fluorescence	LOD: 75 ng/l	[121]
Isoproturon	Absorbance	LOD: 0.09 μg/l	[122]
Gentamicin	Thermometric	Range: 10–400 μg/l	[123]
IgG	Electrochemical		[124]
Digoxin	Chemiluminescence	LOD: 0.2 ng/ml	[125]
IgG	Chemiluminescence	LOD: 7 fmol	[126]
Theophylline	Fluorescence		[127]
Theophylline	Fluorescence	Range: 0.025–0.4 mg/l	[128]
Theophylline, caffeine	Fluorescence	Range: 3×10^{-5}–3×10^{-8} M	[188]
Antitheophylline	Fluorescence	Range: 4×10^{-7}–6×10^{-9} M	[188]
Methotrexate	Radioactivity	Range: 1–100 μg/l	[129]
Thyroxine	Electrochemical	LOD: 25 μg/l; linear range: 25–50 μg/l	[130]
Cortisol	Fluorescence	Range: 1–60 μg/dl	[131]

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Table 3

Examples of sequential injection immunoassays.

Analyte	Detection method	Assay characteristics	Ref.
Imazethapyr	Fluorescence	LOD: 500 ppb	[134]
Imazethapyr	Fluorescence	LOD: 0.5 ppb	[134]
IgG	Thermometric	Range: 10–400 μg/ml	[135]
IgG	Thermometric	LOD: 33 pmol	[136]
Digoxin	Electrochemical	LOD: 10 pg/ml; range: 10–1000 pg/ml	[137]
Atrazine	Fluorescence	Range: 0.02–0.3 μg/l	[138]
Atrazine	Fluorescence	Range: 0.03–0.5 μg/l	[139]
HSA	Electrochemical		[140]
α-amylase	Absorbance		[141
Anti-IgG	Chemiluminescence	LOD: 1 fmol	[142]
Imazethapyr	Fluorescence	LOD: 0.1 ppb	[143]
Imazethapyr	Fluorescence	Range: 0.1–100 ng/ml	[144]

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Table 4

Examples of displacement immunoassays.

Analyte	Detection method	Assay characteristics	Ref.
Cocaine, benzoylecgonine	Fluorescence		[149]
TNT, DNT	Fluorescence	LOD: 2.5 ng/ml; range: 20–1200 ng/ml	[148]
Cocaine, benzoylecgonine	Fluorescence		[150]
2,4-dinitrophenol	Radioactivity	LOD: 140 nM; linear range: 570–4600 nM	[147]
2,4-dinitrophenol	Fluorescence	Linear range: 290–2300 nM	[147]
Cortisol	Fluorescence	Dynamic range: 12.5–1250 pmol	[151]
Polychlorinated biphenyls	Fluorescence	LOD: 4 ppm; linear range: 4–20 µg/ml	[152]
Transferrin, HSA	Absorbance		[153]

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Table 5

Examples of sandwich immunoassays.

Analyte	Detection method	Assay characteristics	Ref.
Thyroid-stimulating hormone	Absorbance	Range: 0–0.29 nM	[154]
hCG	Fluorescence	Range: 0–66.6 ng/ml	[154]
HSA	Fluorescence	LOD: 0.001 mg/ml	[59]
PTH, interleukin-5	Fluorescence	LOD: 10 nM; linear range: <250 μM	[155]
IgG	Absorbance	LOD: 3 fmol; range: 3.33–130 fmol	[110]
IgG	Fluorescence	Linear range: 0.5–50 pmol/l	[156]
HSA	Electrochemical	Range: 1–10 mg/ml	[157]
Anti-IgG	Electrochemical	Range: 3–225 fmol	[158]
IgG	Electrochemical	Range: 5–400 ng/ml	[159]
IgG	Chemiluminescence	Range: 0.2–20 fmol	[160]
Parathyroid hormone	Chemiluminescence	LOD: 0.24 pM (16 amol); linear range: 0.24–67 pM	[161]

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Table 6

Examples of one-site immunometric assays.

Analyte	Detection method	Assay characteristics	Ref.
Granulocyte colony-stimulating factor	Fluorescence	LOD: 1.5 ng/120 µl	[163]
Digoxin and metabolites	Fluorescence	LOD: 2×10^{-10} M	[164]
Digoxin and metabolites	Fluorescence	LOD: 160 pg/ml; linear range: 0.2–2 nmol/l	[165]
Digoxin	Fluorescence	LOD: 200 fmol	[166]
Digoxigenin	Fluorescence	LOD: 50 fmol; linear range 50–1000 fmol	[167]
Digoxin	Fluorescence	LOD: 0.025 nM	[167]
Digoxigenin	Fluorescence	LOD: 0.01 nM	[167]
Interleukin-10	Fluorescence	LOD: 40 fmol	[168]
Digoxin	Absorbance	LOD: 0.2 µg/l	[169]
2,4-D	Electrochemical	LOD: 0.25 µg/l	[170]
Digoxigenin	Electrochemical	LOD: 0.5 amol; linear range: 0.38–7.7 fmol	[171]
α-(difuoromethyl)-ornithine	Fluorescence	LOD: 200 amol; linear range: 5×10^{-11}–2.5×10^{-9} M	[172]
Fatty acid-binding protein	Absorbance	Range: 1–12 µg/l and 12–2000 µg/l	[173]
Thyroxine	Chemiluminescence	LOD: 10^{-11} M	[174]
4-amino-L- and D-phenylalanine	Chemiluminescence	LOD: 1.76 pmol/ml	[175]
17-estradiol	Fluorescence		[176]
α-fetoprotein	Fluorescence	LOD: 0.1 ng/ml; linear range: 0.5–60 ng/ml	[177]
Terbutryn	Grating coupler	LOD: 15 µg/l; linear range: 20–200 µg/l	[178]