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Review

Survey of recent advances in analytical applications of immunoaffinity chromatography

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Abstract

Methods that use immunoaffinity chromatography (IAC) for sample preparation or detection are becoming increasingly popular as tools in the analysis of biological and nonbiological compounds. This paper presents an overview of immunoaffinity chromatography and examines some recent developments of this technique in analytical applications. The emphasis is placed on HPLC-based IAC methods or those that combine IAC with other instrumental techniques; however, novel approaches that employ low-performance IAC columns for chemical quantitation are also considered. Particular applications that are examined include (1) the use of IAC in the direct detection of analytes, (2) the extraction of samples by IAC prior to on- or off-line detection by other methods, (3) the use of IAC in chromatographic-based immunoassays, and (4) the development of postcolumn reactors based on IAC for the detection of analytes as they elute from other types of chromatographic columns. The advantages and limitations for each approach are considered. In addition, a summary is provided of reports in the literature that have used IAC for these various formats.

Keywords: Reviews; Immunoaffinity chromatography; Antibodies

Contents

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1. Introduction

Immunoaffinity chromatography (IAC) refers to any chromatographic method in which the stationary phase consists of antibodies or antibody-related reagents. The high selectivity of antibodies in their interactions with other molecules, and the ability to produce antibodies against a wide range of solutes, has already made IAC a popular tool for the purification of biological compounds. The first known preparative use of IAC was reported by Campbell et al. in 1951, who used an antigen immobilized to *p*-aminobenzyl cellulose for antibody purification [1]; there are now hundreds to thousands of similar IAC methods that have been developed for the isolation of antibodies, hormones, peptides, enzymes, recombinant proteins, receptors, viruses, and subcellular components [2–9]. In recent years, the high selectivity of IAC has also made it appealing as a means for the development of a variety of specific analytical methods. The purpose of this review will be to discuss the basic principles of IAC and show how it has been used in such analytical applications.

1.1. Antibody structure and production

The key component of any IAC method is the antibody preparation that is used as the stationary phase. An antibody is simply a type of glycoprotein that is produced by the body in response to a foreign agent, or antigen. It has been estimated that each body has the capability of producing between 10^6–10^8 types of antibodies, each with the ability to bind to a different foreign agent. The basic structure of a typical antibody (i.e. immunoglobulin G, or IgG) consists of four polypeptides (two identical heavy chains and two identical light chains) that are linked by disulfide bonds. These polypeptide chains form a Y- or T-shaped structure. The amino acid composition in the lower, stem region is highly conserved within the same general group of antibodies (e.g., IgG-class antibodies). The stem region also contains a number of carbohydrate residues that are covalently linked to the antibody's polypeptide chains. Two equivalent antigen binding sites are present at the two upper ends of the antibody. Located within or near these binding sites are areas in which the amino acid composition is highly variable from one type of antibody to the next; it is this difference in composition that allows the body to produce antibodies with a variety of different binding affinities and specificities to foreign agents which enter the body [10].

A foreign agent that is capable on its own of giving rise to antibody production once it has entered the body is referred to as an antigen. Examples of common antigens include bacteria, viral particles and foreign proteins (e.g., proteins from animals, plants or food that produce an allergic response). Since all of these agents are rather large when compared to the size of typical antibody binding site, each is capable of binding many different types of antibodies at a variety of locations on the agent; the individual locations on an antigen that can potentially bind to antibodies are called the epitopes. In order for a foreign agent to be an antigen, it must be large enough to be recognized and processed by the body's immune system. This generally requires that the agent have a molecular mass of at least several thousand daltons. However, smaller solutes (e.g., many drugs or pesticides) can also give rise to antibody production if they are first coupled to a larger species, such as a carrier protein; the agent that is coupled to the carrier is then called the hapten.

One way to produce antibodies that will bind a given test agent is to inject a solution of that agent or an agent/carrier conjugate into a laboratory animal like a mouse or rabbit. Samples of the animal's blood are then collected at specified intervals (typically a few weeks or months after injection of the

test solute) to collect any antibodies that have been generated against the foreign agent. This method generally results in a heterogeneous mixture of antibodies that bind with a variety of strengths and to various epitopes on the antigen or hapten/carrier. Since these antibodies are actually produced by several different cell (or clonal) lines within the body, they are known as polyclonal antibodies. In the last twenty years techniques have also been developed that allow for the isolation of single antibody-producing cells and the combination of these cells with carcinoma cells to produce cell lines that are relatively easy to culture and grow for long-term antibody production. These combination antibody-producing/long-lived cell lines are known as hybridomas and their product is a single type of well-defined antibody known as a monoclonal antibody. In addition to traditional polyclonal and monoclonal antibodies, there has also been recent work aimed at using genetic engineering and bacteria or bacteriophages for the generation of antibodies or antibody-like molecules [11–13]. Although these types of ligands have currently been used in only a few IAC applications, they do represent an attractive alternative for the large scale production of immunoaffinity supports [14].

1.2. Immunoaffinity supports and antibody immobilization

The support material and method used for antibody immobilization are two other items that are necessary to consider in the development of a successful IAC method. Traditionally, most IAC applications have been based on low-performance supports. These are generally carbohydrate-related materials (i.e. agarose or cellulose) or synthetic organic supports (e.g., acrylamide polymers, copolymers or derivatives, polymethacrylate derivatives and polyethersulfone matrixes). There are many commercial supports that fit into this category, such as Affinica Agarose/Polymeric Supports (from Schleicher and Schuell), AvidGel (BioProbe), Bio-Gel/Affi-Gel (BioRad), Fractogel (EM Separations), HEMA-AFC (Alltech), Reacti-Gel (Pierce), Sepharose/Superose/Sephacryl (Pharmacia), Trisacryl/Ultrogel (IBF) and TSK Gel Toyopearl (TosoHaas) [15,16]. The low back-pressure of these supports means that they can be operated under gravity flow, a slight applied vacuum or peristaltic flow. This makes these gels relatively simple and inexpensive to use for IAC, particularly in off-line immunoextraction methods or techniques that involve the use of IAC with flow injection analysis systems (see Sections 3.1 and 4). The main disadvantage of these materials are their slow mass transfer properties and their limited stability at high flow rates and pressures. These factors limit the usefulness of such supports when performing IAC in standard HPLC systems.

IAC can be used as an HPLC method if more rigid and higher efficiency materials are employed. Supports that have been developed for this purpose include derivatized silica, glass and certain organic matrixes such as azalactone beads or polystyrene-based perfusion media. Trade names (and suppliers) of such materials include AvidGel CPG (BioProbe), HiPAC (ChromatoChem), Protein-Pak Affinity Packing (Waters) and Ultraaffinity-EP (Bodman) for the silica or glass supports; Emphaze (3M Corp./Pierce) for the azalactone beads; and POROS (PerSeptive Biosystems) for the perfusion media. The use of these supports along with an antibody or related ligand is referred to as high-performance immunoaffinity chromatography (HPIAC) [4,16]. The mechanical stability and efficiency of these materials makes them attractive for use with standard HPLC equipment, which in turn helps to provide improved speed and precision for analytical applications of IAC.

There are many different methods that can be used for antibody immobilization to both low- and high-performance supports. One common approach involves direct, covalent attachment of the antibodies. This is often done by reacting free amine groups on the antibodies with supports that are activated with agents such as *N*,*N*′-carbonyl diimidazole, cyanogen bromide, *N*-hydroxysuccinimide and tresyl chloride/tosyl chloride, or with supports that have been treated to produce reactive epoxide or aldehyde groups on their surface [15–17]. The use of antibody amine groups is probably the easiest route to immobilization but can give rise to less than optimum activity because of random orientation or denaturation of the immobilized antibodies.

Antibodies, or antibody fragments, can also be covalently immobilized through more site-selective methods. For example, free sulfhydryl groups that are generated during the production of antibody Fab fragments can be used to couple these fragments to supports by using techniques such as the divinylsulfone, epoxy, iodoacetyl/bromoacetyl, maleimide, TNB-thiol or tresyl chloride/tosyl chloride methods [15–17]. Another route for site-selective immobilization involves the coupling of antibodies through their carbohydrate residues. This is done by mild oxidation of these residues with periodate or enzymatic systems to produce aldehyde residues. These aldehyde groups can then be reacted with a hydrazide or amine-containing support for antibody immobilization [15]. The advantage of both the sulfhydryl and carbohydrate-based approaches is that they are believed to produce immobilized antibodies or antibody fragments that have fairly well-defined points of attachment and greater accessibility of the antibody binding regions to antigens in solution. This, in turn, results in IAC columns that have higher relative binding activities than comparable columns made by amine-coupling methods.

Noncovalent immobilization can also be used for the site-selective coupling of antibodies to IAC supports. For example, antibodies that have been oxidized to produce aldehyde groups in their carbohydrate residues can be reacted with biotin-hydrazide, which in turn can be used to bind the antibodies noncovalently to an immobilized streptavidin support [15]. Another common approach for indirect immobilization involves adsorbing the antibody to a secondary ligand such as protein A or protein G. Both protein A and G are bacterial cell wall proteins that have the ability to bind to the stem (or Fc) region of many types of antibodies. This binding is quite strong under physiological conditions but can be easily disrupted by decreasing the pH of the surrounding solution [4,15]. This particular method is appealing in cases where high antibody activity is needed and it is desirable to have frequent replacement of the antibodies in the IAC column. This allows good long-term reproducibility for the IAC column binding capacity but does require the use of much larger amounts of antibody than direct immobilization methods.

1.3. Antibody–antigen interactions and elution conditions

The general process of binding between an immobilized antibody (Ab) and a solution-phase antigen (Ag) in an IAC column can be described by the following equations:

$$\mathrm{Ab} + \mathrm{Ag} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{Ab-Ag} \tag{1}$$

$$K_a = k_a/k_d \tag{2}$$

$$= \{\mathrm{Ab-Ag}\}/\{\mathrm{Ab}\}[\mathrm{Ag}] \tag{3}$$

where K_a is the association equilibrium constant for the binding of Ab with Ag, Ab–Ag is the resulting antibody–antigen complex, and [] or {} represents the molar concentration or surface concentration of each species at equilibrium. The term k_a is the second-order association rate constant for antibody–antigen binding and k_d is the first-order dissociation rate constant for the antibody–antigen complex.

The value of the association equilibrium constant for a typical antibody–antigen interaction under physiological conditions is in the range of 10^8 to 10^{12} M^{-1}. This results in extremely strong binding between analytes and immunoaffinity supports under standard sample application conditions (i.e. a neutral pH application buffer with low-to-moderate ionic strength). For example, in a typical HPIAC column such values for K_a would lead to solute capacity factors of 10^3–10^7 and mean retention times of 1 day to several decades when using the sample application buffer for isocratic elution [16]. The result is a chromatographic system that essentially has irreversible binding to the analyte under common sample injection conditions.

Although it is possible in IAC to perform isocratic elution on a reasonable time scale by using a competing agent in the mobile phase and low affinity antibodies (see the discussion on weak affinity chromatography under Section 2), this does not work for the high or moderate affinity antibodies that are used in the vast majority of IAC columns. The only way that solutes can usually be eluted from these antibodies is to change the column conditions in order to lower the effective value of K_a (i.e. increase

the relative value of k_d versus k_a) for antibody–analyte binding. The use of an acidic buffer (i.e. pH 1–3) and a step elution scheme is often used for this purpose. Another common approach is to perform gradient elution by gradually increasing the amount of a chaotropic agent (e.g., sodium thiocyanate or sodium iodide) that is present in the elution buffer. In some cases an organic modifier or denaturing agent (e.g., urea) might also be employed in step or gradient elution schemes [4]. The proper choice of an elution buffer is important in analytical applications of IAC since it is usually desirable to elute the analyte as quickly as possible while avoiding any irreversible damage to the immobilized antibody support. This currently needs to be addressed on a case-by-case basis and is essential to consider if the same IAC column is to be reused for a large number of samples.

2. Immunoaffinity chromatography with direct detection

The simplest format for IAC in analytical applications involves the adsorption of test solutes by an immobilized antibody column, followed by the later release and detection of these solutes. This is referred to as the traditional ''on/off'' or direct detection mode of IAC. Fig. 1 illustrates the elution scheme that is typically used in this type of analysis. In this scheme the sample of interest is first injected onto the IAC column under mobile phase conditions in which the analyte will have strong binding to the immobilized antibodies in the column. Due to the specificity of the antibody–analyte interaction, other solutes present in the sample are nonretained and washed through the column by the application buffer. After these nonretained solutes have been removed, a second buffer that causes dissociation of the retained analyte is applied. The analyte is then detected as it elutes from the column in this buffer. Once the test analyte has been fully eluted, the initial application buffer is reapplied to the system, the immobilized antibodies are allowed to regenerate (i.e. to change back to their initial conformational states) and the next sample is injected.

The scheme shown in Fig. 1 has been used for

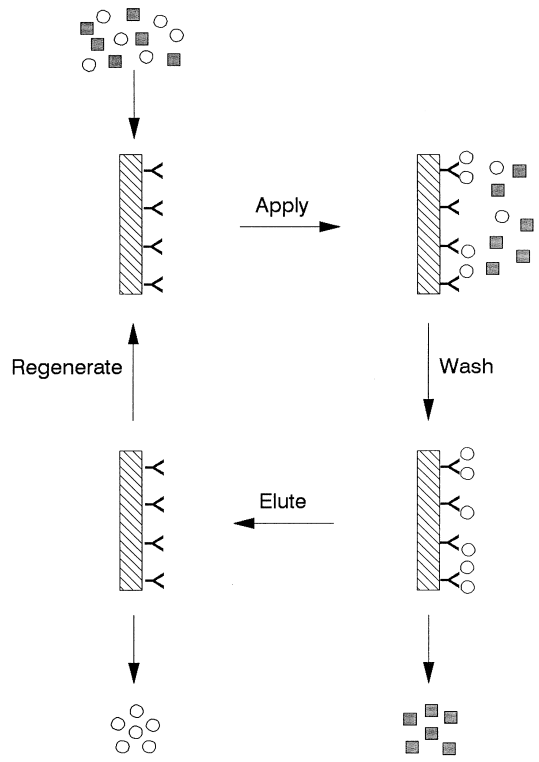

Fig. 1. General operating scheme for immunoaffinity chromatography with direct analyte detection (i.e. the on/off of IAC). The open circles represent the analyte and the squares represent other, nonretained sample components.

many years in the purification of various biological agents by IAC [2–9]. The advantages of using this approach in analytical applications, particularly when performed by HPIAC, include its relative simplicity, good precision, and potential for fast sample analysis. Examples of specific applications based on the direct detection mode of HPIAC are provided in Table 1 [18–39]. These examples represent a large range of biological and clinical samples that include various body fluids, cell or tissue extracts and aliquots from bioreactors. The HPIAC methods used for these applications have been shown in many studies to have good correlation versus reference techniques, such as immunoassays or electrophoresis, but typically take much less time to perform. The precision of these methods are generally in the range of 1–5% [22,29,30,33] and, when used with appropriate elution schemes, the HPIAC columns are often

Table 1
Examples of direct analyte detection by high-performance immunoaffinity chromatography[a]

Analyte	Detection method	Sample [Refs.]
Anti-idiotypic antibodies	UV absorbance	Serum [18,19]
Antithrombin III	Fluorescence	Cell culture [20]
Bovine growth hormone	UV absorbance	Aqueous standards [21]
Fibrinogen	UV absorbance	Plasma [22]
Fungal carbohydrate antigens	PAD	Fungal isolates [23]
Glucose tetrasaccharide	PAD or radiolabels	Serum, urine [24,25]
Glutamine synthetase	UV absorbance/enzyme assay	Bacterial extracts [26]
Granulocyte colony stimulating factor	Fluorescence[b]	Plasma, CSF, BMAF [27]
Group A-active oligosaccharides	Radiolabels	Aqueous standards [25,28]
Human serum albumin	UV absorbance	Serum [29], urine [30]
Immunoglobulin G antibodies	UV absorbance	CSF [31]
Immunoglobulin E antibodies	UV absorbance/ELISA	Plasma, serum [32]
Interferon	UV absorbance	Plasma/serum, urine, saliva, CSF [33], fermentation broth [34], bacterial extract [35]
Interleukin-2	Fluorescence[b]/receptor assay	Tissue samples [36]
Lymphocyte receptors	UV absorbance	Cell extracts [37]
β_2-Microglobulin	UV absorbance/EIA	Plasma [38]
Tissue-type plasminogen activator	Fluorescence	Cell cultures [20]
Transferrin	UV absorbance	Serum [39]

[a] Abbreviations: BMAF, bone marrow aspirate fluid; CSF, cerebrospinal fluid; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; PAD, pulsed amperometric detection.
[b] Fluorescence measured on-line after precolumn sample derivatization with *o*-phthaldialdehyde [27] or fluorescein isothiocyanate [36].

stable for several hundred sample applications [22,30,36,39].

Fig. 2a shows an example of a typical chromatogram generated by HPIAC in the direct detection mode. This approach ideally uses antibodies in the IAC column that are selective for only the analyte(s) of interest and that have little or no binding to other sample components. Because of this, the resulting chromatograms generally contain only two peaks, with the first peak representing all nonretained sample components that elute during the application/wash step and the second peak representing solutes that were retained by the IAC column and later dissociated when the elution buffer was passed through the column. It is usually quite easy to obtain baseline resolution between the nonretained and retained peaks in this mode of HPIAC, since the retention time of the second peak is controlled by simply changing the time at which the elution buffer is applied. This factor, along with the purity of the retained solute peak and the relative speed of this method, are some of the advantages of using HPIAC and direct detection for sample analysis.

One requirement of the direct detection mode is that there must be some way to monitor the analyte as it leaves the column. The various detection methods that have been reported for use in HPIAC are shown in Table 1. For carbohydrate analytes, pulsed-amperometric detection has been employed in several cases for direct detection; for protein solutes, UV absorbance measurements at 210–215 nm or 280 nm are most commonly used. All of these approaches need enough analyte to give a measurable signal. Because of the strong binding and slow dissociation of most antibody–analyte interactions (see Section 1.3), IAC columns tend to produce a response that is related to the moles of applied solute rather than to the solute's initial sample concentration [40–42]. This means that only small sample volumes are needed to provide a detectable signal if the analyte is present at intermediate or high concentrations. For more dilute solutions, the IAC column can be used to concentrate the desired test substance from large sample volumes prior to detection [42]; however, some caution must be used in this latter approach since there is also an increased chance of other, undesired sample components adsorbing nonspecifically to the column, thus giving

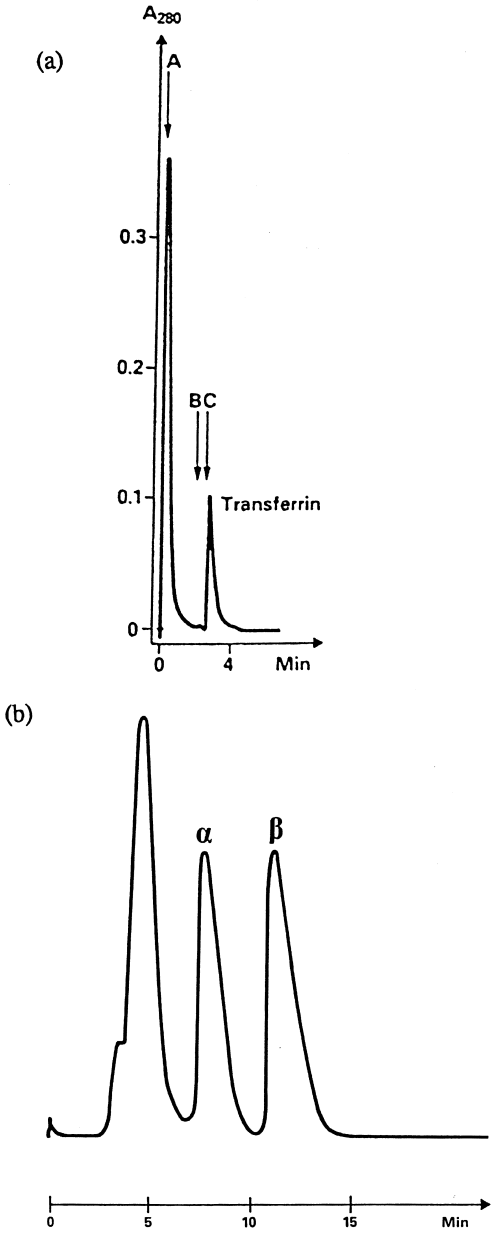

Fig. 2. Examples of HPIAC with direct detection involving (a) step gradient elution and (b) isocratic elution. The chromatogram in (a) is for the analysis of a 50 μl serum sample applied to an immobilized antitransferrin antibody column; the arrows indicate the point at which the application buffer (A), a separate wash buffer (B) and an elution buffer (C) were applied to the column. The chromatogram in (b) shows the isocratic separation on an immobilized IgG monoclonal antibody column for a 20 μl sample of fetal bovine serum spiked with the α- and β-*p*-nitrophenyl glycosides of maltose. (Reproduced with permission from Refs. [39] and [43]).

rise to an increase in the background signal. In some cases minor sample components have been monitored by using precolumn derivatization to place easy-to-detect labels, such as fluorescent tags [27,36] or radiolabels [25,28], onto sample solutes prior to injection. Alternatively, fractions of the column eluent can be collected and later analyzed by a separate technique such as an immunoassay [32,38] or an assay for biological activity (e.g., when the eluting solute is an enzyme [26] or a substance with a known receptor [36]).

The method of antibody immobilization used in on/off methods is another item to consider in HPIAC assay design, especially when working with protein analytes and UV absorbance detection. In this particular situation, it is necessary that the antibodies remain on the IAC column during the elution step so that they do not contribute to measurements of the retained solute peak. Such a problem can potentially occur when the antibodies are adsorbed to a protein A or protein G support, since antibodies will dissociate from these materials under many of the same conditions that are often used for analyte elution in IAC [23,29,31]. This situation can be avoided by covalently immobilizing the antibodies to the IAC support [21,22,34,38] or by cross-linking the antibodies to an immobilized protein A or protein G matrix [23,29,31]. Alternatively, biotinylated antibodies that are noncovalently adsorbed to immobilized streptavidin supports can be used, as this interaction appears to be strong enough to prevent significant antibody release under typical IAC elution conditions [18,19,27,36,37].

The choice of elution conditions is important in determining both the detectability of the analyte and the overall speed of the IAC method. Step elution is commonly used in the on/off mode of HPIAC because of its speed and sharp analyte peaks; but gradient elution is also employed for cases where there is a need for gentler elution conditions, a more gradual change in background signal or better resolution between the eluting solutes. When using either step or gradient elution, changes in buffer pH or the addition of chaotropic salts to the mobile phase are by far the most common approaches for solute dissociation in the on/off mode, but the use of organic modifiers has been reported as well [26]. With step elution, it has been shown in a number of

studies that total cycle times of only 5–6 min per sample can be obtained in the on/off mode of HPIAC [29,39]. Somewhat longer times are required when using gradient elution, but even here cycle times of less than 12–15 min can be obtained [27,33].

With some analytes, particularly low molecular mass solutes, it can sometimes be hard to find a set of step or gradient elution conditions that allow for the rapid dissociation of analyte from the column [40]. The result is a broad peak for the retained analyte that can be difficult to detect and/or that elutes over an extended period of time (i.e. 5–10 min or longer). One solution to this problem is to use a stronger elution buffer, but care needs to be taken in doing this in order to avoid irreversible damage to the immobilized antibodies or support in the IAC column. Another, gentler solution is to capture and reconcentrate the analytes during their elution from the IAC column by using a second on-line column, such as a reversed-phase or ion-exchange column. A more detailed description of this technique is provided in Section 3.2 concerning on-line immunoextraction/HPLC methods.

The step and gradient elution methods are usually needed in the on/off mode of HPIAC in order to disrupt the strong analyte–antibody interactions that occur under typical sample application conditions. But there are some cases in which isocratic elution of solutes in the presence of a competitive displacing agent can instead be employed. In particular, this has been reported for systems that use antibodies with weak affinities (i.e. $K_a < 10^4\ M^{-1}$) and fast solute association/dissociation kinetics. An example of such a separation is shown in Fig. 2b. This approach, known as weak affinity chromatography [43,44], has been used for the analysis of various carbohydrates [23–25,28] and is similar to traditional types of HPLC in its operation. One advantage of using isocratic elution and weak affinity antibodies is that several related solutes can be separated in a single run by HPIAC. Isocratic methods are also attractive since they potentially allow less harsh conditions to be used for analyte elution, thereby increasing IAC column lifetimes. The main disadvantage of this approach is the increased time required for solute analysis versus step or gradient elution methods.

Along with the basic format shown in Fig. 1, there have been a number of reports in which the on/off mode of IAC has been combined with other analytical methods. Several examples in which this mode has been used for either solute extraction or detection are provided in Sections 3 and 5. One example of a hybrid method in which the on/off mode was used for both extraction and detection is work by Hage and Walters, in which IAC and protein A columns were coupled and used for the simultaneous analysis of human serum albumin (HSA) and immunoglobulin G (IgG) in serum samples [29]. A column switching valve and single set of application and elution buffers were used in this system, with the retained HSA and IgG being eluted sequentially through the same UV absorbance detector. A second example is work by Ruhn et al., who used HPIAC along with flow injection analysis for the determination of urinary albumin [30]. This was done by attaching the FIA system to the outlet of the HPIAC column during the sample application step to quantitate the amount of creatinine in the nonretained peak (see Fig. 3); the creatinine levels were then used to correct for fluctuations in the concentration of HSA due to normal variations in urine output and volume. A third example is a report by Phillips, in which an HPIAC column followed by an immobilized receptor cartridge was used for the measurement of both total and bioactive interleukin-2 in tissue samples [36].

3. Immunoextraction methods

The technique of immunoextraction refers to the use of IAC for the removal of a specific solute or group of solutes from a sample prior to determination by a second analytical method. This employs the same general operating scheme as other types of IAC (see Fig. 1), but involves combining the immunoaffinity column either off-line or on-line with some other method for the actual quantitation of analytes. Based on the large number of papers that have appeared on this subject, immunoextraction currently represents one of the most commonly employed uses of IAC in chemical analysis. This section will examine some applications of immunoextraction, including both off-line methods and those

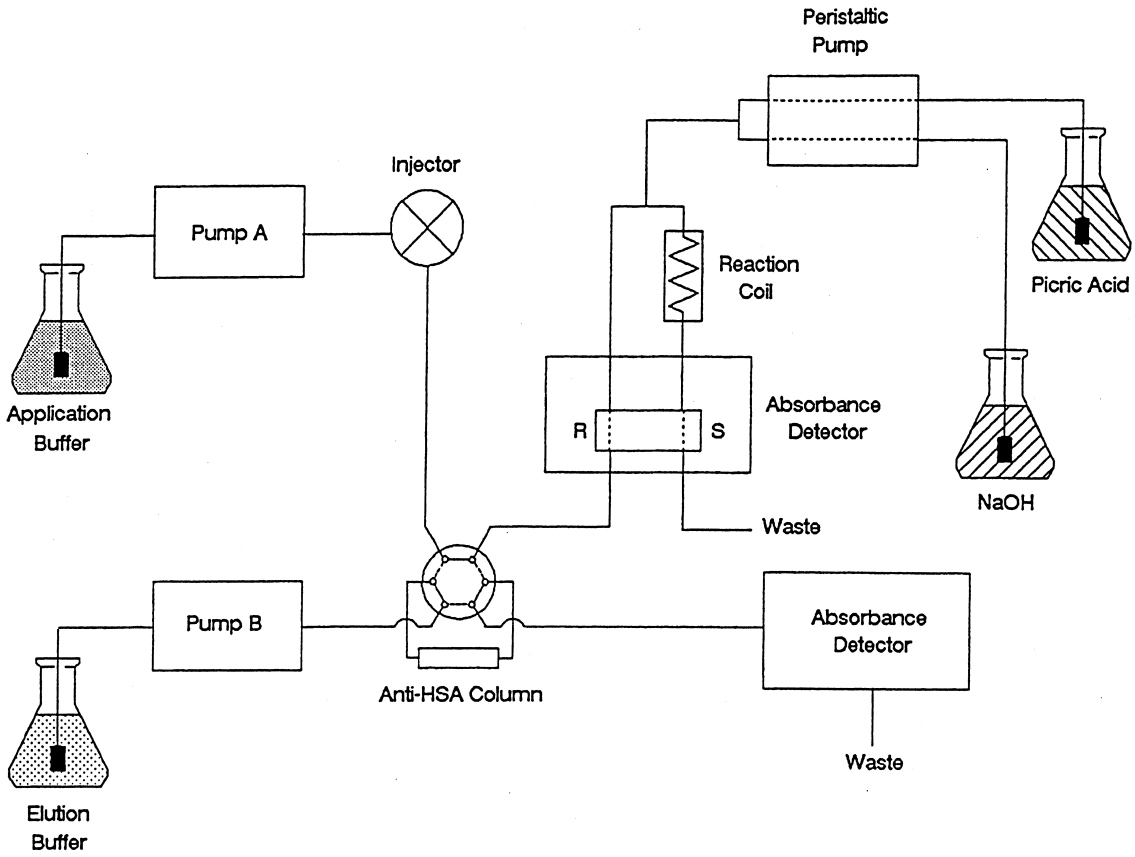

Fig. 3. Schematic of an HPIAC/FIA system for the simultaneous determination of human serum albumin and creatinine in urine samples. (Reproduced with permission from Ref. [30]).

that involve the direct coupling of immunoextraction with HPLC, GC or capillary electrophoresis.

3.1. Off-line immunoextraction

Off-line methods are generally the easiest way for combining immunoaffinity columns with other analytical techniques. This approach typically involves the use of antibodies that are immobilized onto a low-performance support (e.g., activated Sepharose or Trisacryl) that is packed into a small disposable syringe or solid-phase extraction cartridge [45–47]. After conditioning the immunoaffinity column with the necessary application buffer or conditioning solvents, the sample is applied and undesired sample components are washed away, as shown earlier in Fig. 1. An elution buffer is then applied and the test analyte is collected as it is removed from the column. In some cases this eluted fraction is analyzed directly by a second technique, but in most situations the collected fraction is first dried down and reconstituted in a solvent that is compatible with the method to be used for later quantitation. If needed, the collected solute fraction may also be derivatized before it is examined by other techniques in order to obtain improved detectability or more appropriate physical properties (e.g., an increase in solute volatility prior to separation and analysis by GC).

The use of off-line immunoextraction for sample preparation has been the subject of several recent reviews (e.g., see Refs. [45–48]) and research articles. Some specific applications reported for HPLC [49–68] and GC [69–81] are given in Table 2. The

Table 2
Examples of off-line immunoextraction in HPLC or GC[a]

Analyte	Analysis method	Samples [Refs.]
Immunoextraction/high-performance liquid chromatography		
Aflatoxin	RPLC–fluorescence[b]	Figs, animal feed [49], nuts [49,50], urine [51], dust [52]
	RPLC–fluorescence	Milk [53], cheese [54]
Albuterol	RPLC–fluorescence	Plasma [55]
Benzodiazepines	RPLC–ESI-MS	Synthetic libraries [56]
Cytokinins	RPLC–absorbance	Plant extracts [57]
Fumonisin	RPLC–fluorescence[b]	Corn [58]
Human chorionic gonadotropin	RPLC–ESI-MS	Urine [59]
Ivermectin & avermectin	RPLC–absorbance	Serum [60], plasma, meat, fruit [61]
Nortestosterone	RPLC–absorbance	Urine, bile [62]
Ochratoxin A	RPLC–fluoresence[b]	Serum, plasma, milk [63]
Oxytocin	RPLC–coulometry	Culture media [64]
Phenylurea herbicides	RPLC–absorbance	Food extracts [65]
Sendai virus protein	RPLC–absorbance	Viral and tissue extracts [66]
Trenbolone	RPLC–absorbance	Urine [67]
Triazine herbicides	RPLC–absorbance	Plant extracts [68]
Immunoextraction/gas chromatography		
Alkylated DNA adducts	GC–MS	DNA extracts [69], urine [70,71]
Chloramphenicol	GC–ECD	Urine, tissue samples [72]
Dexamethasone	GC–MS	Urine [73]
Estrogens	GC–MS	Plasma [74], urine [74,75]
Flumethasone	GC–MS	Urine [76]
Nortestosterone	GC–MS	Meat samples [77]
Prostaglandins and thromboxanes	GC–MS	Urine [78–81]

[a] Abbreviations: ECD, electron capture detector; ESI-MS, electrospray ionization mass spectrometry; GC–MS, gas chromatography/mass spectrometry; RPLC, reversed-phase liquid chromatography.
[b] Following precolumn derivatization with *o*-phthaldialdehyde [58] or postcolumn derivatization with iodine [49,50], bromine [51,52] or ammonia [63].

list in Table 2 demonstrates the wide applicability of off-line immunoextraction by the variety of samples that are represented. For example, this table includes samples ranging from body fluids (serum, plasma and urine) to foods (nuts, fruits, meat, milk, cheese and corn) to various biological extracts (extracts of cell cultures, tissue or plant samples) and even libraries of synthetic compounds.

Like any IAC method, off-line immunoextraction requires the availability of an antibody preparation that is selective for the desired analyte or group of analytes. If such antibodies are available, then immunoextraction offers the potential of much greater specificity than traditional liquid–liquid or solid-phase extraction methods [45]. It should always be kept in mind when using immunoextraction that most antibodies will show some binding, or cross-reactivity, with solutes that are close to the desired analyte in structure. Ideally, this cross-reactivity should be evaluated for each immunoextraction support by performing binding and interference studies with any solutes or metabolites that are related to the analyte and that may be present in the samples of interest. However, even if several solutes do bind to the same IAC column, this will not present a problem as long as the analyte can be resolved or discriminated from these other compounds by the method that is to be used for quantitation.

The ability of an IAC column to bind to several types of solutes has actually been used as an advantage in a number of procedures involving immunoextraction. For instance, the ability of antibodies to cross-react with a parent compound and related agents or metabolites has been used for the

development of immunoextraction methods for 17α- and 17β-trenbolone [67]; 17α- and 17β-nortestosterone [62]; and diethylstilbestrol, dienestrol and hexestrol [74]. This idea can be taken one step further by placing multiple types of antibodies into the same IAC column and using this for multiresidue analysis. Such an approach is sometimes referred to as multi-immunoaffinity chromatography (MIAC) and has been used in situations where several classes of compounds are to be analyzed simultaneously. One example is a method developed by Ginkel and coworkers in which an IAC column containing seven different antibodies was used along with HPLC for the analysis of testosterone, nortestosterone, methyltestosterone, trenbolone, zeranol, estradiol, diethylstilbestrol and related compounds in urine [45] (see Fig. 4); another example is one in which MIAC and GC–MS were combined for the detection of nortestosterone and methyltestosterone in meat samples [77].

One advantage of off-line immunoextraction is that the samples collected from the IAC column can be readily derivatized or placed into a different solvent between the sample purification and quantitation steps. This advantage is particularly important when combining IAC with GC, where (1) it is desirable to remove any water from the collected sample before injection onto the GC system and (2) solute derivatization is often required to improve solute volatility or detection. Another advantage of off-line immunoextraction is that it is relatively easy to set up once an appropriate antibody preparation has been obtained. The cost of an immunoextraction cartridge is typically much higher than it is for conventional solid-phase extraction; however, this difference can be minimized by carefully selecting the application and elution conditions so that the same IAC cartridge can be used for multiple samples [45].

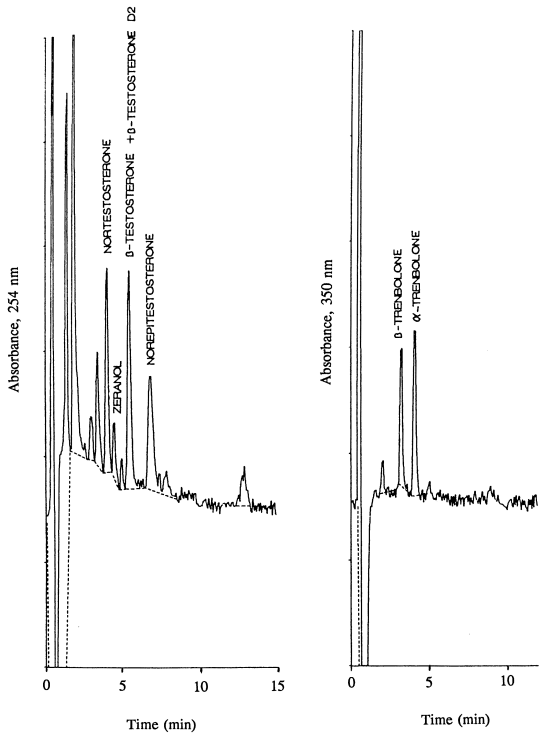

Fig. 4. Example of multiimmunoaffinity chromatography (MIAC) used for off-line extraction with reversed-phase HPLC and UV absorbance detection for the quantitation of norepitestosterone, nortestosterone, β-testosterone, 17α- or 17β-trenolone and zeranol in a spiked urine sample. The β-testosterone D2 is deuterated testosterone that was added to the sample as an internal standard. (Reproduced with permission from Ref. [45]).

3.2. Immunoextraction/high-performance liquid chromatography

The fact that off-line immunoextraction is generally a manual sample preparation method does limit the potential speed and precision of this approach. To overcome this problem, a large number of recent reports have examined the use of various on-line immunoextraction methods. The direct coupling of immunoextraction with HPLC has been of particular interest. The relative ease with which IAC can be incorporated into a HPLC system makes this appealing as a means for automating immunoextraction methods and for reducing the time required for sample pretreatment. Also, the relatively high precision of HPLC pumps and injection systems provides on-line immunoextraction with better precision than off-line methods, since the on-line approach has more tightly controlled sample application and elution conditions.

The topic of on-line immunoextraction/HPLC has previously been discussed in a review by de Frutos and Regnier [82]. Specific examples of such techniques are provided in Table 3 [40–42,83–108] and

Table 3
Examples of on-line immunoextraction in HPLC, GC and CE[a]

Analyte	Detection method	Samples [Refs.]
Immunoextraction/reversed-phase liquid chromatography		
Aflatoxin M1	Fluorescence	Milk [83]
β-Agonists	API-MS	Urine [84]
α1-Antitrypsin	UV absorbance	Plasma [85]
Atrazine	UV absorbance	Water [40,41]
Atrazine metabolites	UV absorbance	Water [41,42]
Benzylpenicilloyl-peptides	UV absorbance	Tryptic digests [86]
Bovine serum albumin	UV absorbance	Aqueous standards [87]
Carbendazim	Diode array, API-MS	Water [88]
Carbofuran	API-MS	Water, potato extract [89]
Chloramphenicol	UV absorbance	Milk, muscle tissue [90]
Clenbuterol	UV absorbance	Urine [91]
	API-MS	Urine [84]
Cortisol	UV absorbance	Plasma, urine, milk, saliva [92]
Dexamethasone	UV absorbance, API-MS	Urine [93]
Diethylstilbestrol	UV, flow-FAB-MS	Urine [94]
Digoxin	Postcolumn fluorescence	Serum [95]
Estrogens	UV absorbance	Plasma [96], urine [96,97]
Hemoglobin	UV absorbance	Aqueous standards [87]
Human epidermal growth factor	Fluorescence	Bacterial extracts, urine [98]
Interferon α-2	UV absorbance	Cell extracts [99]
LSD	UV absorbance, API-MS	Urine [100]
LSD analogs and metabolites	UV absorbance, API-MS–MS	Urine [101]
Lysozyme variants	UV absorbance	Aqueous standards [87,102]
17β- and 17α-19-Nortestosterone	UV absorbance	Urine, bile, tissue samples [103,104]
Phenytoin	UV absorbance	Plasma [105]
Propranolol	UV absorbance, API-MS	Urine [100]
Δ^9-Tetrahydrocannabinol	UV absorbance	Saliva [106]
Tolubuterol	API-MS	Urine [84]
Transferrin	UV absorbance	Serum [107], plasma [85]
17β- and 17α-Trenbolone	UV absorbance	Aqueous standards [103]
Immunoextraction/size exclusion chromatography		
Human growth hormone variants	UV absorbance	Aqueous standards [108]
Immunoextraction/ion-exchange chromatography		
Lysozyme variants	UV absorbance	Aqueous standards [87,102]
Immunoextraction/gas chromatography		
β-19-Nortestosterone and related steroids	FID	Urine [109]
Immunoextraction/capillary electrophoresis		
Cyclosporin	UV absorbance	Tears [110]
Immunoglobulin E	UV absorbance	Serum [111]
Insulin	UV absorbance	Serum [112]

[a] Abbreviations: API-MS, atmospheric pressure ionization mass spectrometry; FAB-MS, fast atom bombardment–mass spectrometry; FID, flame ionization detector; LSD, lysergic acid diethylamide.

in Fig. 5. The vast majority of these applications have involved the use of IAC along with RPLC [34–42,83–107], but there have also been techniques involving protein analytes that have used IAC coupled to size exclusion [108] or ion-exchange chromatography [87,102]. Most of the examples in Table 3 have employed antibodies that are coupled to high-performance supports; however, low-performance immunoaffinity media have also been used in about a fourth of these applications. These supports can be based either on covalently immobilized antibodies or antibodies that are adsorbed to protein A or protein G as secondary ligands. In the latter case, an additional step in the method must be added for the reapplication of antibodies to the protein A or G column before each sample injection [84,100,101,107]. Also, the use of protein A or G requires that the second, HPLC analytical column be capable of separating any dissociated antibodies from the analyte [107].

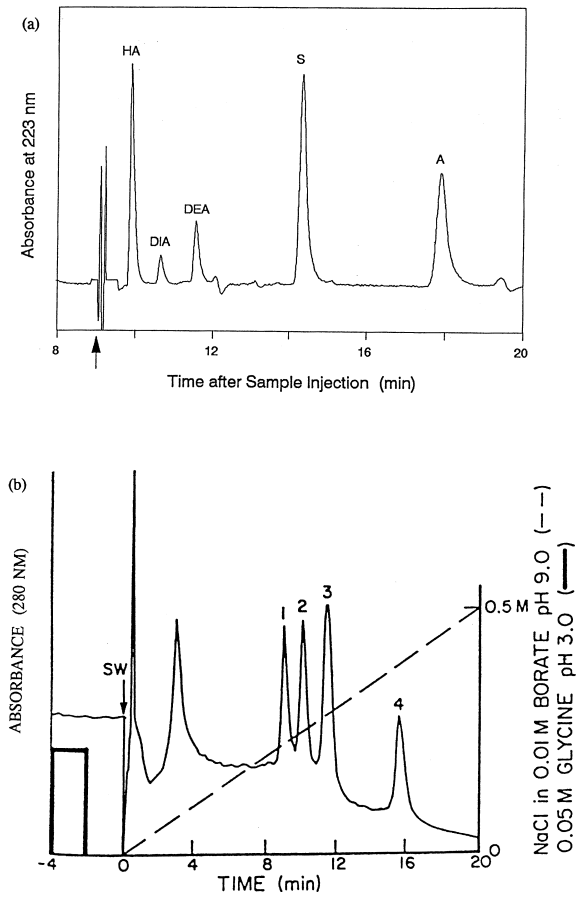

Fig. 5. Examples of separations performed with IAC coupled on-line with HPLC. The chromatogram in (a) was obtained by an antiatrazine immunoextraction column and a RPLC analytical column for a 250 μl river water sample spiked with hydroxyatrazine (HA), deisopropylatrazine (DIA), deethylatrazine (DEA), simazine (S) and atrazine (A). The chromatogram in (b) was obtained by an antilysozyme immunoextraction column and cation-exchange analytical column for a 20 μl aqueous sample containing (1) ringed-neck pheasant, (2) duck A, (3) duck B and (4) duck C lysozymes. (Reproduced with permission from Refs. [40] and [102]).

One reason for the large number of reports involving the combination of on-line immunoextraction with RPLC undoubtedly has to do with the popularity of RPLC in routine chemical separations; but there is also a more fundamental reason that involves the underlying nature of both IAC and RPLC. For instance, the fact that the elution buffer for an IAC column is an aqueous solvent that generally contains little or no organic modifier is convenient since this same elution buffer will act as a weak mobile phase for RPLC. In other words, as a solute elutes from an IAC column, it will tend to have strong retention on any on-line reversed-phase support, leading to analyte reconcentration (note: reconcentration can also occur when coupling IAC with an ion-exchange column if the elution buffer contains a low concentration of competing ions [87,102]). This reconcentration phenomenon is valuable in dealing with analytes that have slow desorption from their immobilized antibodies, a factor which makes these solutes impractical to analyze by the more traditional on/off mode of IAC (see Section 2).

A two-column scheme for performing on-line immunoextraction in RPLC is shown in Fig. 6; similar systems are used when coupling IAC with size-exclusion or ion-exchange supports [87,102,108]. More advanced IAC/RPLC systems that employ an additional RPLC precolumn or trapping column are also commonly used to provide improved detection limits by giving smaller background peaks due to column switching (e.g., see Refs. [40,83,84]). The general format for using a system like that in Fig. 6 involves injecting the sample onto the IAC extraction column, with the nonretained components being allowed to go to a waste container. The IAC column is then switched

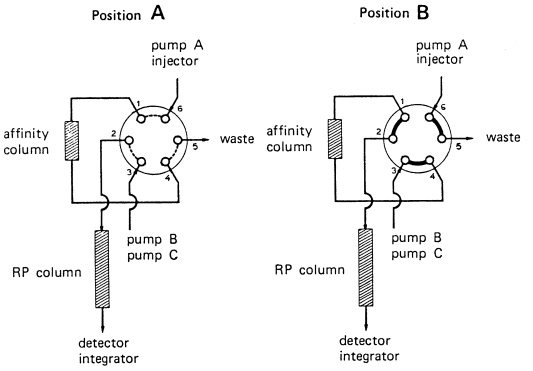

Fig. 6. Schematic of a two-column system for use in on-line immunoaffinity extraction in HPLC. Position A is the valve position used on the IAC column for loading sample, washing away nonretained components and regeneration prior to the next sample injection; the same position is used for the separation of sample components from a previous injection on the HPLC analytical column (a reversed-phase support in this case). Position B is used for eluting solutes from the IAC column onto the analytical column. (Reproduced with permission from Ref. [99]).

on-line with a RPLC column and an elution buffer is applied to the IAC support to dissociate any retained analyte. As these analytes elute, they are captured and reconcentrated at the head of the RPLC column. After all solutes have left the IAC column, this column is then switched back off-line and regenerated by passing through the initial application buffer. Meanwhile, the RPLC column is developed with either an isocratic or gradient elution scheme involving the application of a mobile phase with an increased organic modifier content. This latter step causes analytes at the head of the RPLC column to move through this support and to be separated based on their differences in polarity. As these solutes elute, they are then monitored by and quantitated at an on-line detector.

Like off-line immunoextraction, on-line techniques can be used to monitor either a single analyte or group of analytes. Some examples of multiresidue analysis for related classes of solutes are shown in Fig. 5. Such techniques are attractive since they allow both simple sample pretreatment, as made possible by the IAC extraction column, and good resolution between closely-related compounds, as accomplished by using an appropriate HPLC analytical column. Along with these types of applications, there have also been a number of alternative uses reported for immunoextraction/HPLC. For example, Flurer and Novotny used immunoextraction to selectively remove major proteins from human plasma prior to the analysis of minor proteins by RPLC [85]. Yvon and Wal examined the covalent binding sites of benzylpenicillin on human serum albumin (HSA) by using immunoextraction/RPLC to study tryptic digests of HSA [86]. An on-line dialysis system was combined by Farjam et al. with IAC and RPLC for the determination of aflatoxin in milk [83]. In addition, immunoextraction/HPLC methods have been used in several studies by Regnier and coworkers for the analysis of protein variants and aggregates [87,102,108].

3.3. Immunoextraction/gas chromatography

Although off-line immunoextraction combined with gas chromatography has been reported for many compounds (see Table 2), there has been little work done in directly coupling IAC with GC. An exception is work performed by Farjam et al. in a method developed for the quantitation of β-19-nortestosterone and related steroid compounds in urine samples [109]. The basic scheme that they used is shown in Fig. 7. The first part of this system includes an IAC column coupled to a RPLC column. After the sample is applied to the IAC column, the retained solutes are passed onto the RPLC column, as described earlier for immunoextraction/HPLC in Sec-

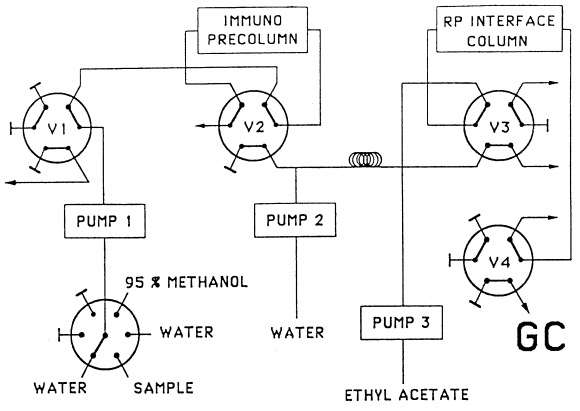

Fig. 7. Schematic of a system used for performing on-line immunoextraction in GC. Valves V1–V4 are shown in their initial position, as used during sample injection onto the IAC column. (Reproduced with permission from Ref. [109]).

tion 3.2, but with the main purpose for the RPLC column now being to capture these solutes and remove any water from them prior to injection onto the GC system. This is done by later applying a volatile organic solvent, like ethyl acetate, to the RPLC column and passing a portion of this eluted solute fraction into a GC injection gap. Once the solute/organic solvent plug has entered the GC system, a temperature program is then begun for solute separation. One advantage of this approach, like immunoextraction/HPLC, is that large volumes of sample can be applied to the IAC column, thus allowing for low detection limits. The main disadvantage is the greater complexity of this method versus off-line immunoextraction or even on-line immunoextraction/HPLC.

3.4. Immunoextraction/capillary electrophoresis

Several recent studies have considered the possibility of combining on-line immunoextraction with capillary electrophoresis (CE) [110–112], as shown in Table 3. One example is work by Phillips and Chmielinska, in which on-line immunoextraction and CE were used for the analysis of cyclosporin and its metabolites in tear samples from corneal transplant patients [110]. In this case, CE alone gave rise to many peaks and poor limits of detection for these samples (Fig. 8A). However, when antibody Fab fragments directed against cyclosporin were covalently immobilized to part of the CE capillary, cyclosporin and related solutes could be selectively extracted from the sample and concentrated prior to their separation, thus producing a faster separation with better resolution and improved detection limits (Fig. 8B). A number of other system designs have been examined for directly coupling IAC with CE. One example is work by Guzman in which antibodies were covalently immobilized in bundles of microcapillaries or in laser-drilled glass rods [111]. Both types of supports were then connected by sleeves to a traditional CE capillary for the on-line immunoextraction and detection of immunoglobulin E in serum. A second approach, as reported by Cole and Kennedy [112], involved the use of a capillary that was packed with a protein G chromatographic support. This packed capillary was then used to adsorb antibodies for the extraction and concentration of insulin. Off-line immunoextraction was performed by eluting the retained insulin (and adsorbed antiinsulin antibodies) into a collection vessel and injecting part of this fraction onto a CE system for separation. The same packed capillary was also combined directly with a CE capillary by means of a flow-gated interface for the on-line immunoextraction and quantitation of insulin in serum.

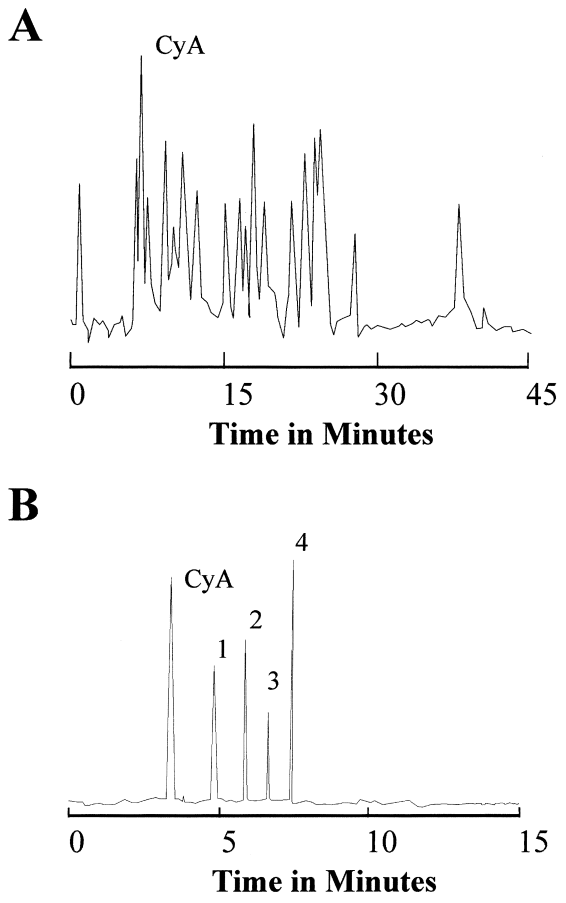

Fig. 8. Analysis of cyclosporin in tear samples by (A) normal CE and (B) immunoaffinity extraction coupled on-line with CE. CyA represents cyclosporin and peaks 1–4 in (B) represent various cyclosporin metabolites. (Reproduced with permission from Ref. [110]).

4. Chromatographic immunoassays

Another area that has received increasing attention in recent years has been the use of immobilized

antibody (or immobilized antigen) columns to perform various types of immunoassays. Such an approach is known as a chromatographic (or flow-injection) immunoassay. Some recent reviews on this topic are included in Refs. [82,113–115]. Examples of specific applications are provided in Table 4 [116–151]; related studies concerning the optimization of detection schemes for such assays are described in Refs. [152–155]. The use of IAC to perform immunoassays is particularly valuable in determining trace analytes that, by themselves, may not produce a readily detectable signal. This problem is overcome in chromatographic immunoassays by using a labeled antibody or labeled analyte analog that can be used for indirect analyte detection. There are several types of formats, as well as labels, that can be employed in such methods. Particular formats to be discussed in this section will include competi-

Table 4
Examples of chromatographic immunoassays[a]

Analyte	Label/detection method[b]	Sample [Refs.]
Competitive binding immunoassays		
Adrenocorticotropic hormone	Lucifer yellow/fluorescence	Aqueous standards [116]
α-Amylase	HRP/absorbance	Aqueous standards [117]
Atrazine/triazines	HRP/fluorescence	Water [118–120]
2,4-Dinitrophenyl lysine	I^{125}/radioactivity	Aqueous standards [121]
	Fluorescein/fluorescence	
Human chorionic gonadotropin	HRP/absorbance	Serum [122]
Human serum albumin	HSA/absorbance	Aqueous standards [123], serum [124]
	HRP/absorbance	aqueous standards [125]
Immunoglobulin G (human)	GOD/electrochemical detection	Serum [126]
	Fluorescein/fluorescence	Serum [127]
Immunoglobulin G (mouse)	Fluorescein/fluorescence	Aqueous standards [128,129]
Insulin	ALP/thermometric detection	Aqueous standards [130]
Isoproturon	HRP/absorbance	Water [131]
Testosterone	Texas red/fluorescence	Serum [132]
Theophylline	Liposomes/fluorescence	Serum [133–135]
	Fluorescein/fluorescence	Serum [136]
	ALP/electrochemical detection	Serum [137]
Thyroid stimulating hormone	HRP/absorbance	Serum [122]
Thyroxine	HRP/chemiluminescence	Aqueous standards [138]
Transferrin	Lucifer yellow/fluorescence	Serum [139]
Transferrin	Transferrin/absorbance	Serum [124]
Trinitrotoluene	Fluorescein/fluorescence	Water [140]
Sandwich immunoassays		
Anti-bovine IgG antibodies	GOD/electrochemical detection	Serum [141]
Human serum albumin	Fluorescein/fluorescence	Ferment. broth [142]
Immunoglobulin G (bovine)	HRP/chemiluminescence	Serum [143]
Immunoglobulin G (human)	Acridinium ester/chemiluminescence	Serum [144]
Immunoglobulin G (mouse)	Acridinium ester/chemiluminescence	Aqueous standards [145]
	HRP/fluorescence	Aqueous standards [146]
Interferon	GOD/electrochemical detection	Cell culture [147]
Parathyroid hormone	Acridinium ester/chemiluminescence	Plasma [148,149]
One-site immunometric assays		
α-(Difluoromethyl)ornithine	HRP/fluorescence	Plasma [150]
17-β-Estradiol	Liposomes/fluorescence	Aqueous standards [151]

[a] Abbreviations: ALP, alkaline phosphatase; GOD, glucose oxidase; HRP, horse radish peroxidase; IgG, immunoglobulin G.
[b] For ALP, GOD and HRP, the means of detection is based on the substrate/product systems that are used along with these enzyme labels. For liposome labels, detection is based on the agent incorporated within the liposomes (e.g., carboxyfluorescein in Refs. [133–135]).

tive binding immunoassays, sandwich immunoassays and one-site immunometric assays.

4.1. Competitive binding immunoassays

As indicated by Table 4, this is the most common format used in performing immunoassays by IAC. The basic principle behind a competitive binding immunoassay involves the incubation of analyte in the sample with a fixed amount of a labeled analyte analog in the presence of a limited amount of antibodies that bind to both the native analyte and labeled species. Because there is only a limited amount of antibodies present, the sample and labeled analyte molecules must compete for binding sites on these antibodies. After this competition has been allowed to take place, the analyte and labeled analog that are bound to the antibodies are separated from the analyte and labeled analog molecules that remain free in solution. The amount of the labeled analog that is present in either the bound or free fraction is then measured. In the absence of any sample analyte, the largest amount of labeled analyte in the bound fraction should be observed. But as the amount of sample analyte increases, the level of bound labeled analyte will decrease, giving rise to an indirect measure of the amount of native analyte that is present in the sample.

There are several different ways in which competitive binding immunoassays can be performed by IAC. The simplest approach is to mix the sample and labeled analyte analog (i.e. the ''label'') and simultaneously apply these to the IAC column. This is known as a simultaneous injection competitive binding immunoassay and is the format used in most of the competitive binding techniques listed in Table 4. A number of different labels have been used in such assays, including enzymes [122,126,130,131,137, 138], fluorescent tags [116,127–129,136,139] and liposomes [133–135]. Detection can be performed by either examining the amount of label that elutes nonretained from the column or by measuring the labeled species that dissociate from the IAC column during the elution step. Either covalently immobilized antibodies or antibodies adsorbed to protein A/protein G can be used in these assays. When working with protein A or G, the separation of dissociated antibodies from the analyte and label is not necessary as long as the antibodies do not interfere with detection of the label. However, the protein A or G column must still be regenerated by applying additional antibodies to the column between each sample/label injection cycle.

An alternative format involves the application of only sample to the IAC column, followed later by a separate injection of the label. This method is known as a sequential injection competitive binding immunoassay [117,118,123–125]. An example of this type of assay is shown in Fig. 9. The advantage of this approach is that even an unlabeled preparation of analyte can potentially be used as the ''label'', provided that this species produces a sufficient signal for detection; this makes this method particularly useful for complex samples that contain the analyte at intermediate to high concentrations [123,124]. For lower concentration analytes, labeled analyte analogs can also be used [117,118,125], as discussed in the previous paragraph. Like the simultaneous injection format, an indirect measure of the sample analyte is obtained by examining the amount of label that elutes in either the nonretained or retained IAC fractions (see Fig. 9b). One advantage of this technique over the simultaneous injection format is that there are no matrix interferences present during detection of the label in the nonretained fraction; however, the sequential injection method also has the slight disadvantage of requiring an additional step for the separate application of sample and label to the IAC column.

A third type of format is the displacement competitive binding immunoassay [121,140]. In this technique, the IAC column is first saturated with the labeled analog, followed by the application of sample to the column. As the sample passes through the column, the unlabeled analyte is able to bind to any antibody binding regions that are momentarily unoccupied by the label as this label undergoes local dissociation/reassociation. The net result is displacement of label from the column by mass action, with the degree of displacement increasing with the amount of applied sample analyte. One advantage of this approach is that a single application of label onto an IAC column can be used for multiple sample injections [121]. However, this approach is highly dependent on the rate of labeled analyte dissociation

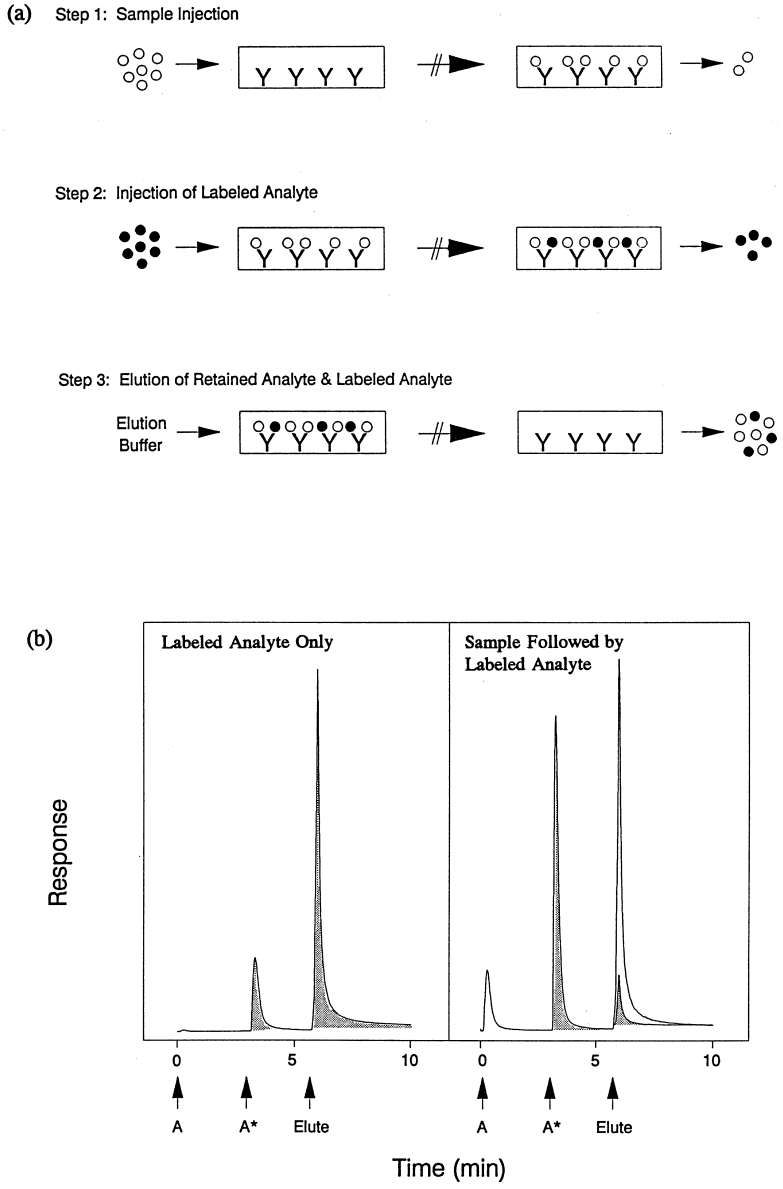

Fig. 9. (a) Format and (b) typical chromatograms obtained for a sequential injection competitive binding assay performed by HPIAC. The chromatograms in (b) represent the application of only the labeled analyte analog, or sample followed by the labeled analyte analog. (Reproduced with permission from Ref. [123]).

from the immobilized antibodies and can potentially produce very broad peaks for high affinity systems. Also, it is known that slow flow-rates work best in these assays for maximizing label displacement and increasing signal production; this occurs because such conditions increase the amount of time allowed for label dissociation and sample analyte binding in the column [121,140].

4.2. Sandwich immunoassays

A sandwich immunoassay, or two-site immunometric assay, involves the use of two different types of antibodies that each bind to the analyte of interest. The first of these two antibodies is attached to a solid-phase support and is used for extraction of the analyte from samples. The second antibody contains an easily measured tag (e.g., an enzyme or fluorescent label) and is added in solution to the analyte either before or after this extraction; this second antibody serves to place a label onto the analyte, thus allowing the amount of analyte on the immunoaffinity support to be quantitated. An important advantage of sandwich immunoassays is that they produce a signal for the bound label that is directly proportional to the amount of sample analyte; this is in contrast to most competitive binding immunoassays (Section 4.1), which give a decrease in the amount of bound label with an increase in sample analyte levels. The fact that two types of antibodies are used in sandwich immunoassays tends to give this technique much higher selectivity than competitive binding immunoassays. The main disadvantage of sandwich immunoassays is that they can only be used for analytes that are large enough to bind simultaneously to two separate antibodies.

One scheme for performing a sandwich immunoassay by IAC is shown in Fig. 10. In this case, the sample and labeled antibody are allowed to incubate and bind before injection onto an IAC column that contains immobilized antibodies for sample extraction. Alternatively, the sample and labeled antibody can be injected sequentially onto the IAC column; this has the advantage of eliminating the preincubation step but produces worse limits of detection and/or requires more labeled antibodies than the simultaneous injection mode [148]. Specific examples of IAC-based sandwich immunoassays are provided in Table 4. These can potentially be performed with any of the labels used in competitive binding immunoassays. For example, specific applications that have already been reported have included chromatographic sandwich immunoassays based on enzyme labels [141,143,146,147], fluorescent agents [142] and chemiluminescent tags [144,145,148,149].

4.3. One-site immunometric assays

A third format for an IAC-based immunoassay is the one-site immunometric assay. In this technique, the sample is first incubated with a known excess of labeled antibodies or Fab fragments that are specific for the analyte of interest. After binding between the sample analyte and antibodies has occurred, this mixture is then applied to a column that contains an immobilized analog of the analyte. This column serves to extract any antibodies or Fab fragments that are not bound to sample analyte. Meanwhile, those antibodies or Fab fragments that are bound to sample analyte will pass through the column in the nonretained peak. Detection is then performed either by looking at the nonretained labeled antibodies/Fab fragments, which will give a signal directly proportional to the sample analyte concentration, or by monitoring the amount of excess antibody/Fab fragments that later dissociate from the column during the elution step.

Although this approach has been used in only a few studies [150,151], it does have a number of potential advantages over other chromatographic immunoassays. For instance, like a competitive binding immunoassay, this method is able to detect both small and large solutes. Also, like a sandwich immunoassay, it gives a signal for the analyte-bound label that is directly proportional to the amount of analyte in the original sample. In addition, the fact that an immobilized analyte analog, rather than an immobilized antibody, is used creates the possibility of employing a fairly wide range of elution conditions for column regeneration. One disadvantage of this approach is that a different immobilized analog column must be produced for each analyte of interest. In addition, pure and highly-active labeled antibodies or Fab fragments are needed for this method when monitoring the nonretained fraction in order to provide a low background signal for label detection.

5. Postcolumn immunodetection

The various formats described in Sections 2 and 4 for IAC can be used either as stand-alone assay

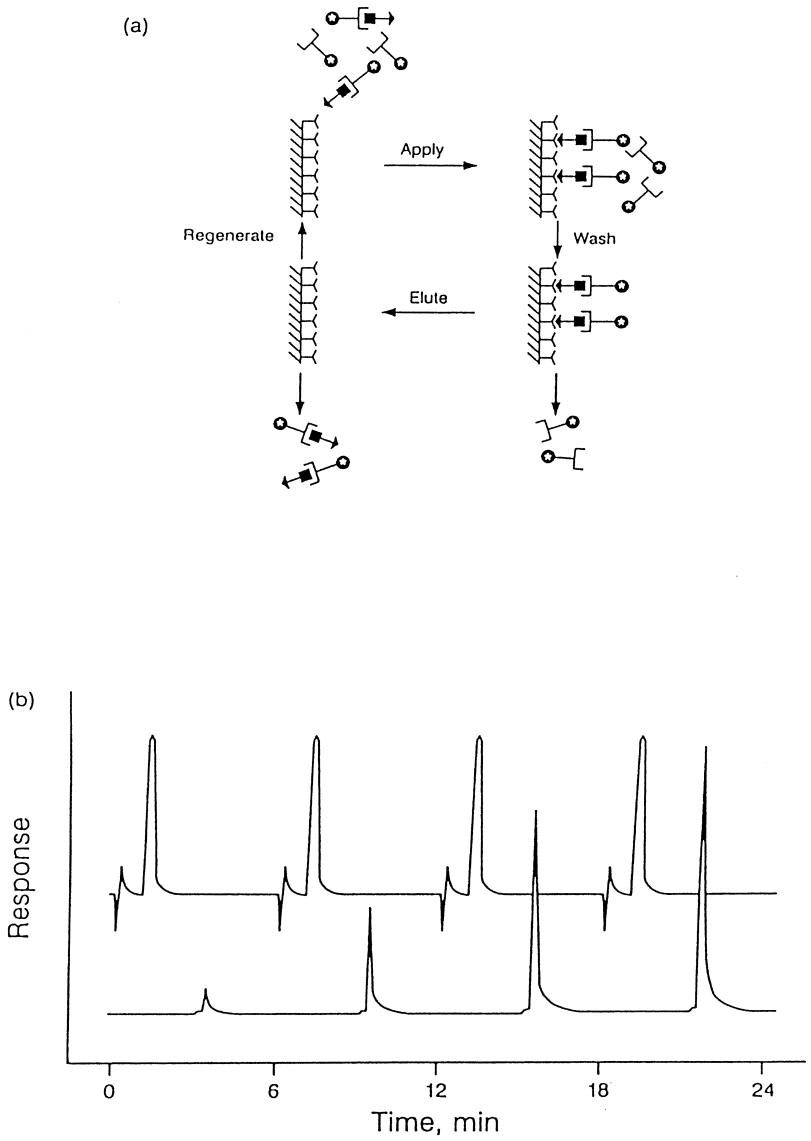

Fig. 10. (a) Format and (b) typical chromatograms obtained in a sandwich immunoassay for parathyroid hormone, as performed by HPIAC. The chromatograms in (b) represent the nonretained sample components (top) and the retained analyte plus associated labeled antibodies (bottom) for a sample containing increasing analyte concentrations. (Reproduced with permission from Ref. [148]).

systems or as a means for monitoring the presence of a specific solute eluting from other chromatographic columns. This latter approach is often referred to as immunodetection. This technique typically involves the use of a postcolumn reactor and an immobilized antibody or antigen column attached to the exit of an analytical HPLC column. The use of immunodetection was the subject of a recent article by Irth et al. [156]; examples of specific applications are provided in Refs. [157–162]. Several potential strategies have been presented in these articles for performing postcolumn immunodetection, including use of the on/off mode of IAC and the use of competitive binding immunoassays, sandwich immunoassays

or one-site immunometric assays based on IAC. Each of these formats will be examined in some detail in the following section.

5.1. On/off immunoaffinity reactors

As discussed in Section 2, the on/off mode of IAC represents the simplest approach for quantitating an analyte if this solute is capable of directly generating a sufficiently strong signal for detection. One example of where the on/off mode has been used for postcolumn immunodetection is in work described by Vanderlaan et al. for the analysis of acetylcholinesterase (AChE) in amniotic fluid by size exclusion chromatography [162]. The method developed in this report used an IAC column containing anti-AChE antibodies to capture AChE as it eluted from the analytical column. After the AChE was adsorbed to the IAC column, a substrate solution for AChE was passed through the column and the resulting colored product was detected by an on-line absorbance detector. A second example was provided by Cho et al. in their work with bovine growth hormone releasing factor (GHRF). In this case, an IAC column containing anti-GHRF antibodies was used to capture GHRF from the eluent of a RPLC column; the GHRF was then directly monitored by its UV absorbance as it was later dissociated from the immunodetection column [160].

As discussed earlier in Section 2, the main advantage of the on/off mode of IAC is the relative simplicity of this technique. The main disadvantages include the need for an analyte with a sufficiently high concentration for direct detection and the need for fast analyte dissociation from the IAC column for the production of sharp, well-defined peaks. When used for postcolumn detection, an additional item that needs to be considered for the on/off mode is the need to adjust the eluent of the HPLC analytical column to a pH, organic modifier content and ionic strength that is appropriate for use as an IAC application buffer [160]. This item is especially important when using immunodetection for RPLC, where an appreciable amount of organic modifier may be present in the mobile phase leaving the HPLC analytical column. One solution to this problem is to combine the analytical column eluent with a dilution buffer prior to sample application onto the IAC detection column. Immunodetection by the on/off mode also requires that the eluting analyte be present in, or be able to rapidly convert into, a conformation that is recognized by antibodies in the IAC column. This latter item is of particular concern when detecting the elution of proteins from RPLC columns, since many proteins can undergo denaturation in the presence of the organic solvent levels that are used in RPLC mobile phases.

5.2. Competitive binding immunoassay reactors

Although IAC-based competitive binding immunoassays (see Section 4.1) have not yet received direct use in immunodetection, a method has been proposed by Oosterkamp and coworkers for performing this type of assay in postcolumn reactors [156,161]. In this approach (see Fig. 11b), solutes eluting from an analytical HPLC column are first combined with unlabeled antibodies that will bind to the analyte of interest. This mixture is passed through a reaction coil and combined with a second reagent stream containing a labeled analyte analog for binding to any remaining, unoccupied binding sites on the antibodies. After passing this new mixture through another reaction coil, it is then taken through a restricted-access RPLC support for separation of the free and bound labeled analog. As these bound and free fractions are separated, they are monitored by an on-line detector. The amount of bound versus free label is then used as an indirect measure of the amount of sample analyte that was present in the analytical column eluent. This approach has been successfully used with the biotin-avidin system and fluorescein labels [157,161] and is potentially amenable for use with any size analyte. However, the same precautions as described in Section 5.1 must again be used when coupling this method with RPLC columns in order to minimize the effects of organic modifiers on the antibody–analyte binding process.

5.3. Sandwich immunoassay reactors

Chromatographic-based sandwich immunoassays (see Section 4.2) have also been used as a means for postcolumn immunodetection. For example, this was one of the modes investigated by Cho et al. in the

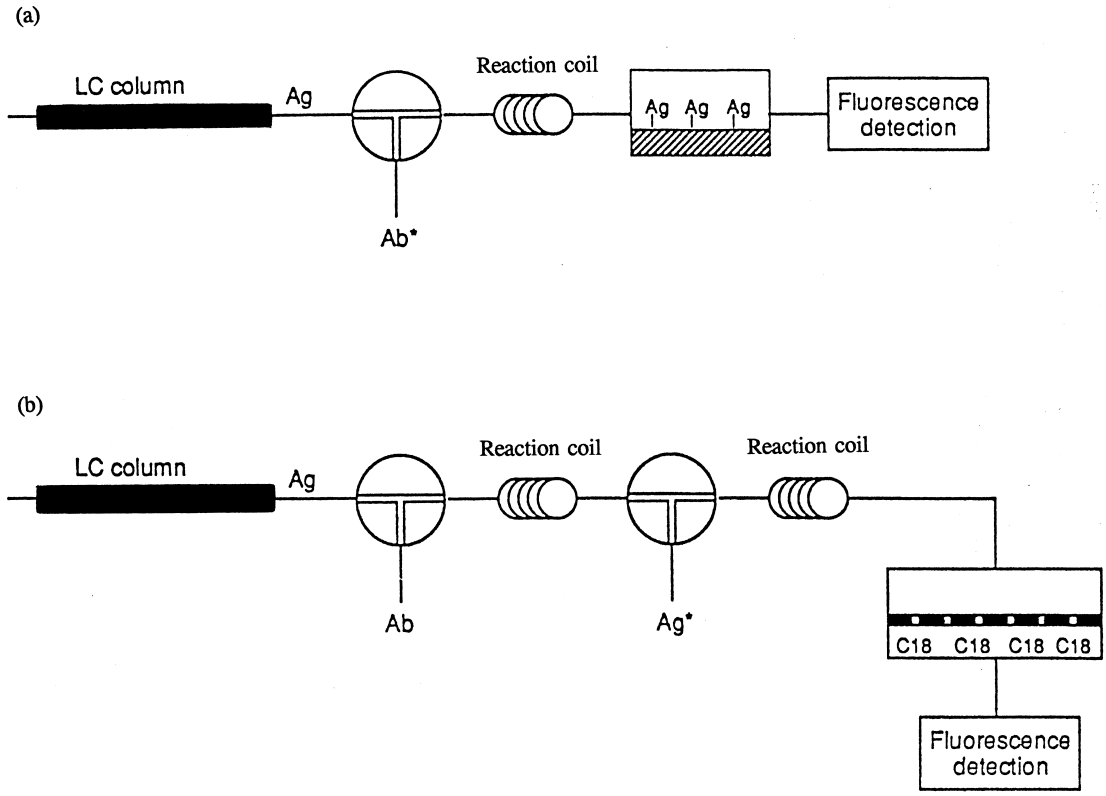

Fig. 11. Schemes for postcolumn immunodetection by using (a) a one-site immunometric assay and (b) a competitive binding immunoassay format. Ab and Ag (or Ag*) represent the antibody and antigen (or labeled antigen), respectively. (Reproduced with permission from Ref. [156]).

combined use of RPLC and immunodetection for the analysis of bovine GHRF [160]. In this case, an IAC column containing antibodies against GHRF was placed after the RPLC analytical column and used to capture any eluting GHRF. This captured GHRF was then quantitated by sending through a solution of excess fluorescein-labeled antibodies which were also capable of binding GHRF. After washing the excess labeled antibodies from the IAC column, the GHRF and associated labeled antibodies were eluted and monitored by an on-line fluorescence detector. Although this approach was able to quantitate GHRF at reasonably low levels, it was also noted that several special measures had to be taken to minimize the background signal caused by nonspecific adsorption of the labeled antibodies. In addition, the same precautions for the RPLC/IAC interface, as listed in Section 5.1, must be observed in order to promote the initial binding of the analyte to the immunodetection column.

5.4. One-site immunometric reactors

The one-site immunometric assay (see Section 4.3) currently represents the most common approach for performing postcolumn immunodetection. Fig. 11a contains a diagram which shows how this can be used for the detection of analytes eluting from an HPLC column. The basic operation involves taking the analytical HPLC column eluent and combining this with a solution of labeled antibodies or Fab fragments that will bind the analyte of interest. The column eluent and antibody or Fab mixture is then allowed to react in a mixing coil and passed through an immunodetection column that contains an immobilized analog of the analyte. The antibodies or

Fab fragments that are bound to the analyte will pass through this column and onto the detector, where they will provide a signal that is proportional to the amount of bound analyte. If desired, the immunodetection column can later be washed with an eluting solvent to dissociate the retained antibodies or Fab fragments; but a sufficiently high binding capacity is generally used so that a reasonably large amount of analytical column eluent can be analyzed before the immunodetection column must be regenerated.

The one-site immunometric approach was originally used by Irth and coworkers to quantitate digoxin and digoxigenin as they eluted from a standard RPLC column [157]. This was performed using fluorescein-labeled Fab fragments (raised against digoxigenin) and an immobilized digoxin support in the postcolumn detection system. This method was then used to successfully monitor both digoxin and its metabolites in plasma and urine samples [157]. The same general system was later used along with a restricted-access RPLC column to monitor digoxin, digoxigenin and related metabolites in serum samples [158]. A similar approach based on an RPLC analytical column was used by Miller and Herman for the immunodetection in serum of human methionyl granulocyte colony stimulating factor (GCSF) and GCSF modified with poly(ethylene glycol) [159]. In this particular case, a column containing immobilized GCSF and fluorescein-labeled Fab fragments against GCSF were used in the postcolumn reactor. To further help in providing assay specificity, an IAC column containing anti-GCSF antibodies was used for on-line immunoextraction before RPLC analysis.

6. Conclusions

In summary, this review has shown that the use of IAC and HPIAC in quantitative chemical analysis is a rapidly growing area of research and development. Applications such as direct analyte detection, immunoextraction, chromatographic immunoassays and postcolumn immunodetection have already been reported for a large variety of biological and non-biological agents in fields ranging from clinical chemistry and biochemical research to food science and environmental analysis. The selectivity of immunoaffinity methods makes them particularly appealing for the development of fast and simple methods for complex samples. The ability to combine IAC with other methods, such as HPLC, GC or CE, is also attractive as a means for obtaining even greater selectivity or more information on a group of structurally-related solutes. Because of these advantages, it is expected that IAC will continue to see even greater use in the future for the quantitation of chemicals in real-world samples.

7. List of abbreviations

CE	capillary electrophoresis
FIA	flow injection analysis
GC	gas chromatography
HPIAC	high-performance immunoaffinity chromatography
HPLC	high-performance liquid chromatography
HSA	human serum albumin
IAC	immunoaffinity chromatography
IgG	immunoglobulin G
MIAC	multi-immunoaffinity chromatography
RPLC	reversed-phase liquid chromatography

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