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Clinical Applications of Capillary Electrophoresis-Based Immunoassays

Annette C. Moser¹, Corey W. Willicott¹, and David S. Hage^{2,*}

¹Chemistry Department, University of Nebraska, Kearney, NE

²Chemistry Department, University of Nebraska, Lincoln, NE

Abstract

Immunoassays have long been an important set of tools in clinical laboratories for the detection, diagnosis and treatment of disease. Over the last two decades there has been growing interest in utilizing capillary electrophoresis (CE) as a means for conducting immunoassays with clinical samples. The resulting method is known as a CE immunoassay. This approach makes use of the selective and strong binding of antibodies for their targets, as is employed in a traditional immunoassay, and combines this with the speed, efficiency, and small sample requirements of CE. This review discusses the variety of ways in which CE immunoassays have been employed with clinical samples. An overview of the formats and detection modes that have been employed in these applications is first presented. A more detailed discussion is then given on the type of clinical targets and samples that have been measured or studied by using CE immunoassays. Particular attention is given to the use of this method in the fields of endocrinology, pharmaceutical measurements, protein and peptide analysis, immunology, infectious disease detection, and oncology. Representative applications in each of these areas are described, with these examples involving work with both traditional and microanalytical CE systems.

Keywords

clinical chemistry; immunoassay; capillary electrophoresis; CE immunoassay; affinity capillary electrophoresis

1.INTRODUCTION

Immunoassays have been important tools in clinical chemistry for many decades [1–10]. In general, an immunoassay can be defined as a technique in which antibodies or antibody-related substances are used as selective binding agents for chemical detection [4–11]. Antibodies are a group of glycoproteins that are produced by the immune system in response to a foreign agent, or antigen. The binding of an antibody with an antigen is a reversible process that occurs through non-covalent interactions. However, the fit of the antigen within an antibody's binding sites and the large variety of interactions that can occur allow this process to create a highly selective and strong complex that often has an association equilibrium constant in the range of $10^5 - 10^{12} \text{ M}^{-1}$ [3,9–11].

There are several features of immunoassays that make them appealing for use in clinical testing. For instance, the highly selective and strong nature of antibody-antigen interactions makes antibodies or related agents valuable as reagents for the measurement and detection

* Author for correspondence: 704 Hamilton Hall, Chemistry Department, University of Nebraska, Lincoln, NE 68588-0304; Phone, 402-472-2744; FAX, 402-472-9402; dhage1@unl.edu.

of analytes in complex samples that include blood, plasma, serum, urine, cerebrospinal fluid and tissue samples [3–11]. There are also many different formats in which antibodies can be used as binding agents. These formats include those that involve competitive binding assays, sandwich immunoassays and direct binding assays, among others. Antibodies do require the use of a biological system for their production, such as a rabbit for the creation of polyclonal antibodies or a hybridoma cell line for the generation of monoclonal antibodies [9–11]. In addition, the use of large carrier agents and appropriate conjugates are needed to generate antibodies against small targets [1–3,9–11]. However, through these techniques antibodies can be obtained against a large variety of substances that range from small drugs and low mass hormones up to proteins, bacteria and viral particles. There are also many types of labels and detection schemes that can be utilized in immunoassays. These features mean that immunoassays can be developed against a wide range of clinical targets and can be employed for the measurement of both major and ultratrace sample components [1–11].

Many types of immunoassays involve some form of a separation step for examining either the various species that may contain a label or for separating the bound and non-bound forms of a labeled species from each other. Since the early 1990s, there has been growing interest in using capillary electrophoresis (CE) as a separation component in immunoassays [1–8,12,13]. The resulting technique has been referred to as a “CE immunoassay”. This method is a subset of a broader group of methods known as affinity capillary electrophoresis (ACE), in which a biological-related ligand is used in CE to selectively bind and recognize a given analyte or group of analytes [14–19].

There are a number of potential advantages to using CE as part of an immunoassay. For example, the high efficiency of CE and its ability to be easily automated have made this method appealing for the separation of labeled antibodies, targets and/or antibody-target complexes [14,20–27]. In some cases, the simultaneous separation and analysis of multiple targets has even been possible [16,22]. Another valuable feature of CE is the small amounts of sample and reagents that are typically required. This property has been exploited in CE immunoassays when looking at systems that may have only small amounts of sample available, such as in single cell analysis. This same feature has made it possible to modify many types of CE immunoassays for use with microanalytical devices [24–28].

This review will discuss the ways in which CE immunoassays have been used for clinical applications. An overview of the formats and detection modes that have been employed in these applications will first be considered. A more detailed examination will then be given on the various clinical applications that have involved CE immunoassays. Areas to be discussed will include the use of this method in the fields of endocrinology, pharmaceutical measurements, protein and peptide analysis, immunology, infectious disease detection, and oncology. Along with immunoassays, some closely-related methods that have made use of aptamers or other selective binding agents such as enzymes will also be briefly considered [29–34]. Representative applications in each of these areas are described, with examples involving work with both traditional and microanalytical CE systems.

2. GENERAL FORMATS FOR CE IMMUNOASSAYS

There are several ways in which CE immunoassays can be classified [14–16,21,22]. The two main divisions are homogeneous immunoassays, in which all of the assay components are present in solution, and heterogeneous immunoassays, in which one or more of the assay components is used in an immobilized form (e.g., on a solid support). CE immunoassays can be further divided into subcategories based on whether they involve a competition between an analyte and a labeled binding agent (i.e., a competitive binding immunoassay) or whether binding by only the analyte is required for detection (e.g., as occurs in non-competitive

immunoassays or immunometric assays). The following section will discuss each of these formats and their properties. Further divisions based on the type of label and detection method that are used will then also be considered in Section 3.

2.1. Homogeneous CE immunoassays

Homogenous immunoassays are the most common format currently used with CE [16,21,22]. This can be done by using either a non-competitive or competitive binding format. This section will examine the principles for these two formats and discuss their relative advantages and disadvantages.

2.1.1. Non-competitive methods—A homogeneous non-competitive binding immunoassay, as represented in Figure 1(a), involves the incubation of the sample with an excess of labeled antibodies (or labeled antibody fragments) prior to CE separation of the analyte-antibody complexes and the free labeled antibodies. Measurement of the analyte is most often performed in this method by monitoring the analyte-antibody complex, although the response for the free labeled antibodies can also be used. Because it is necessary for the analyte-antibody complex and the free antibodies to be well resolved during the separation step, non-competitive CE immunoassays are often limited to analytes that consist of relatively large targets, such as proteins, to ensure there is a large enough difference in the electrophoretic mobilities between the analyte-antibody complex and the labeled antibodies [22].

One important item to consider in a homogeneous non-competitive CE immunoassay is the degree of uniformity for the labeled antibodies or antibody-like binding agent. In this case it is desirable for the labeled antibodies or binding agents to have consistent electrophoretic mobilities. This property helps to provide a narrow CE peak for these species, which makes it easier to detect and measure the labeled binding agent or to separate it from the complex it forms with the analyte. For this reason, polyclonal antibodies or F_{ab} fragments from polyclonal antibodies are not generally used in this type of assay due to their variable electrophoretic mobilities. Instead, monoclonal antibodies or F_{ab} fragments that are produced from such antibodies are preferred [22]. Another option is to use labeled binding agents that are created using single chain variable (scFv) fragments, which are recombinant fusion proteins that consist of the variable regions of the heavy and light chains of an immunoglobulin connected by a linker peptide [35].

The response and limits in this method are affected by such factors as the binding strength of the labeled antibodies or binding agent, non-specific binding, and the detector's response for the label [22,31,36]. Because two of these factors are directly related to the binding affinity, care must be taken to ensure the activity of the antibodies is not destroyed or significantly altered during the labeling process. Labels can be attached to these binding agents through various approaches, including amine-, carbohydrate-, and sulfhydryl-based coupling methods [11,22,37–41]. The use of amine-based methods can sometimes create problems in this type of assay because many such groups are present throughout an antibody's structure, leading to a heterogeneous labeled agent, and these groups may be at or near an antibody's interaction site for the target, creating a possible decrease in binding activity. Carbohydrate-based coupling methods help to minimize such problems because these types of groups are typically located only in the F_c region of an antibody and away from the antibody's binding sites for a target analyte [11,37]. The production of F_{ab} fragments from antibodies and labeling the free sulfhydryl groups that are generated during this process can also be utilized for site-selective modification or immobilization and avoid altering the binding sites of such agents [11,37,40,41]. Yet another option to prevent blocking the binding sites with the label is to use affinity protection chromatography as part of the modification [42].

An example of this approach is a homogeneous non-competitive CE immunoassay that was created to quantify carcinoembryonic antigen (CEA), a glycoprotein associated with certain types of cancer, as shown in Figure 1(b) [43]. This assay utilized horseradish peroxidase (HRP)-labeled antibodies that were conjugated with gold nanoparticles (i.e., with the gold nanoparticles being used for signal amplification). This method included a 40 min incubation step, followed by a 5 min separation. The results showed good agreement with those obtained by a reference method, and this approach was able to easily distinguish between serum CEA values from a small set of healthy individuals and cancer patients [43]. Other clinical analytes that have been examined by using homogeneous non-competitive CE immunoassays are follicle-stimulating hormone [44], luteinizing hormone [45], immunoglobulin G [46], and interferon gamma [47].

2.2.2. Competitive binding methods—The most common type of homogeneous CE immunoassay is the competitive binding format [16,21,22]. In this type of assay, all of the sample components and reagents are present in the solution phase. Competition is created between the analyte and a labeled analog of the analyte (or “label”) by using a limited amount of antibodies, as is illustrated in Figures 2(a–b) [48]. After these components have been incubated and allowed to bind, CE is used to separate the free and antibody-bound forms of the labeled analog. The amount of either the free or bound forms of the labeled analog is then measured and used as an indirect measure of the amount of analyte that was originally in the sample. This can be done because an increase in the amount of analyte will cause less of the labeled analog to bind to the antibodies, leading to an increase in the free form and a decrease in the bound form of the labeled analog.

Homogeneous competitive CE immunoassays have two distinct advantages over non-competitive immunoassays. First, it is often less challenging to create a labeled analyte that has a uniform electrophoretic mobility than it is to create a uniform group of labeled antibodies. This is especially true when the analyte to be labeled is a small molecule such as a drug that may have only one possible modification site. Second, competitive binding formats are not limited to the analysis of large targets like proteins because the electrophoretic mobilities of the free form of the labeled analyte analog and the complex for this labeled analog with antibodies are usually quite different, making it easy to separate these species by CE. However, non-competitive formats often have lower limits of detection than competitive binding methods, larger dynamic ranges, and can distinguish between cross reactive species such as protein variants [22,49].

Calibration curves for a homogeneous competitive binding CE immunoassay are often made by plotting the amount of the free labeled analyte analog versus the concentration of analyte in the sample. This approach is used because the free labeled analog is usually well-resolved from the other sample components. The response and limit of detection for this type of assay will depend on the concentrations of the antibodies and the labeled analog, the affinity of the antibody for the analyte and labeled analog, and the degree of non-specific binding in the system. In addition, the binding strength of the labeled analog with the antibody should be comparable to that of the analyte with the antibody to provide good competition between these species and an optimum change in signal [22,50].

A recent example of a homogeneous competitive binding CE immunoassay used a microchip system for carrying out both on-line sample clean-up and CE separations for the detection of human insulin-like growth factor-I [51]. Green fluorescent protein was used to label the insulin-like growth factor. In this method, a 30 min incubation step was used, followed by a 1 min separation. The detection limit was 0.68 ng/mL, or approximately 89 pM, which was about 200-fold lower than the limit of detection for a conventional enzyme-linked immunosorbent assay [51]. A large number of other homogeneous competitive

binding CE immunoassays, as related to clinical samples, have also been reported, as will be described in Sections 4–8 of this review.

2.2. Heterogeneous CE immunoassays

A heterogeneous format for a CE immunoassay has at least one assay component that is immobilized onto a solid support, while the other reagents or sample components are present at least initially in solution [22,36]. The most popular form of a heterogeneous CE immunoassay is one in which the antibodies, or an antibody-related binding agents, are immobilized and used to extract and concentrate the analyte. Another possible format is one in which the analyte, an analyte analog of the analyte, or a related target is immobilized to a solid support. Both of these formats will be discussed in this section.

2.2.1 Methods based on immobilized antibodies—The most common type of heterogeneous CE immunoassay is one in which antibodies or antibody-related agents are immobilized and used to extract analytes from a sample [22,27]. The use of antibodies for this purpose is also known as “immunoextraction” and is a subclass of immunoaffinity chromatography [11,52–55]. In a CE immunoassay, these immobilized antibodies can act to extract analytes from a sample prior to the separation and quantification of these chemicals by CE, as illustrated in Figure 3(a). This extraction step can be utilized to isolate a group of structurally related compounds or only a single compound, depending on the type of antibodies that are employed. If multiple compounds are to be extracted, a mixed bed containing more than one type of antibody can also be used [11,24,55]. The limit of detection of this type of heterogeneous CE immunoassay is generally determined by the total moles or mass of analyte applied to the system, rather than the concentration of the analyte. This situation occurs when using high affinity antibodies and relatively short extraction times because under these conditions the binding is essentially irreversible. One consequence of this behavior is that the concentration-based limit of detection can often be adjusted to lower levels by increasing the volume of the applied sample [54–56].

In order to get the antibodies to release the extracted analytes and to carry out CE on these targets, the conditions in the immunoextraction support must be altered to promote analyte dissociation. The pH of the running buffer is often lowered to disrupt antibody-analyte binding for this purpose [24,57,58]; changes to the ionic strength, polarity or composition of the running buffer can also be utilized (see Refs. [54,55]). However, if the immunoextraction support is to be used repeatedly, care must be taken to ensure that the immobilized antibodies are not permanently damaged during the release step, creating a loss in activity and/or an increase in nonspecific binding. To increase the degree of analyte extraction during the application step, the rate of sample application can be adjusted or the amount of immobilized antibodies can be increased. For instance, in one study at least a 15-fold mole excess of immobilized antibodies versus analytes was found to be optimal in a heterogeneous CE immunoassay for measuring various cytokines [58].

A heterogeneous CE immunoassay was utilized to simultaneously measure six chemokines in clinical samples, as shown in Figure 3(b) [59]. In this assay, six different F_{ab} fragments were immobilized onto glass fiber filter disks and placed into a CE microchip system. After the immobilized F_{ab} support had been used to extract the target chemokines from a sample, a reactive form of a fluorescent dye (AlexaFluor 633) was added and used to label the captured targets. The labeled analytes were then released from the immunoextraction support, separated by CE and measured by an on-line laser-induced fluorescence detector. The immobilized Fab support could then be regenerated for application of the next sample. The total analysis time was 35–40 min, which included all sample preparation and analysis steps [59]. Other studies have employed immunoextraction and CE for the examination of

clinical samples in assays for brain-derived neurotrophic factor [60], mixtures of various drugs [61], opioid peptides [62,63], isoforms of α_1 -acid glycoprotein [64,65], and multi-analyte methods for neuropeptides and various immunomodulating agents [24,58,66].

2.2.2 Methods based on immobilized analytes, analyte analogs or related targets—

Heterogeneous CE immunoassays can also be created using an immobilized form of the analyte or of a target for antibodies [22]. For example, such a format could be utilized by combining labeled antibodies with the analyte in a sample and then using an immobilized form of the target analyte to extract the remaining non-bound antibodies from the sample/reagent mixture. This approach is a form of a one-site immunometric assay [11,54] and has been used with CE for measuring human serum albumin (HSA) [67].

A variation of this format has been reported in which proteins or peptides in a sample, representing the target analytes, were first separated using capillary isoelectric focusing [68,69]. At the end of this separation step, the targets were immobilized using photochemically-activated groups that line the capillary. Once immobilized, the targets were tagged by combining them with both primary antibodies against the target and then labeled secondary antibodies that could bind to the primary antibodies. This method has been utilized with HRP as the label and chemiluminescent detection to distinguish between protein isoforms and to determine the extent of phosphorylation that occurs at particular sites on proteins [68]. This method has also been used to analyze amino-terminal variants of amyloid- β peptides, allowing the detection of multiple low abundant peptide isoforms in cerebrospinal fluid [69].

Another application for this format is to use an immobilized target to extract and detect antibodies in a sample against the target. An example of this approach was recently used with serum samples for the detection of antibodies against the bacterium *Helicobacter pylori* (see Figure 4) [70]. In this method, antigens from *H. pylori* were immobilized onto magnetic nanobeads, which were used to extract *H. pylori* antibodies from human serum. Labeled secondary antibodies against human IgG-class antibodies were then applied to bind antibodies that had been captured by the support. All of these antibodies were later released and separated by CE, with the labeled antibodies being detected by LIF and providing a measure of the *H. pylori* antibodies that were in the original sample [70].

3. DETECTION IN CE IMMUNOASSAYS

A variety of detection methods and types of labels can be used in CE immunoassays. As was indicated in Section 2, these labels may be attached to antibodies, the analyte or analogs of the analyte. This section will examine labels and detection schemes that have been used in clinical applications of CE immunoassays, including schemes based on fluorescence, chemiluminescence, enzyme labels, electrochemical detection, and mass spectrometry.

3.1. Fluorescence Detection

Most CE immunoassays have detection based on laser-induced fluorescence (LIF) and labels that can be used in this detection mode [16,21,22]. LIF detection can provide extremely low limits of detection and can be used with small sample volumes, which make this approach useful in both traditional and microanalytical CE systems [16,21,22,27]. There are also a number of labels that can be used for fluorescence detection and that can be readily coupled to antibodies, analytes or analyte analogs for use in CE immunoassays. These labels have included various activated forms of fluorescein [22], Cyanine 5 (Cy5) [48,66,71–73], tetramethylrhodamine [49,74], AlexaFluor 633 [24,58], green fluorescent protein [51], and B-phycoerythrin [75], among others [22,76,77]. Examples of these labels are given in Figure 5.

Fluorescein is the most popular label for CE immunoassays. The optimum excitation wavelength range of this dye is 488–495 nm (with emission at 520 nm), which is a good fit with the light emission that is produced by an argon laser at 488 nm, making it easy to monitor this label in CE through LIF detection. This label has a good quantum yield that can be as high as 0.75, although the fluorescence does decrease at a pH below 7 or when fluorescein is exposed to light and stored for extended periods of time. Another advantage of this dye is that it can easily be added to amine-containing analytes or binding agents by using fluorescein isothiocyanate (FITC) as a reagent. This feature has made fluorescein tags, as obtained by using FITC or other activated forms of fluorescein, a common way of labeling proteins, peptides, antibodies, and antibody-related agents for use in CE immunoassays [16,22].

Tetramethylrhodamine, Cy5 and AlexaFluor 633 are three other dyes that have been employed as labels in CE immunoassays. Tetramethylrhodamine has an excitation maximum at 540 nm and emission maximum at 567 nm. One advantage of this dye for LIF detection in CE is it can be excited by using either an argon ion laser or a He-Ne laser [49]. Cy5 (i.e., Cyanine 5) is a near-infrared fluorescent dye that has also been employed as a label in CE immunoassays. It has an excitation wavelength at 650 nm and an emission maximum at 670 nm. This type of label is attractive because many biological samples have little or no background absorption or emission in the near-infrared range. A limitation of Cy5 is that it does not have as many commercially-available activated forms as fluorescein, and it has a lower fluorescence quantum yield [22]. AlexaFluor 633 is another near-infrared fluorescent dye. This label has an excitation maximum at 632 nm and an emission maximum at 647 nm, allowing it to be used with a He-Ne laser. AlexaFluor 633 also has high photostability, good water solubility for its succinimidyl ester form, and little change in its absorption of light when it is conjugated with proteins, oligonucleotides, or nucleic acids [22].

B-Phycoerythrin and green fluorescent protein (GFP) are two other labels that have been used for fluorescent detection in immunoassays. B-Phycoerythrin is a fluorescent protein with an absorbance maximum at 545 nm and maximum emission at 575 nm. In addition, B-phycoerythrin has an isoelectric point of 5.5, which makes it possible to separate from antibodies by CE when using a running buffer such as borate [78,79]. GFP has an absorbance maximum at 488 nm and an emission maximum at 508 nm [80].

3.2. Chemiluminescence

Another detection approach that has been used in several clinically-oriented CE immunoassays is chemiluminescence. This process is based on the production of light as a result of a chemical reaction. To use this detection scheme in a CE immunoassay, either an antibody or analyte analog is labeled with an agent that can produce light when later combined with a given set of reagents. One advantage of this detection approach is that chemiluminescent reactions are often quite rapid and can provide good limits of detection with low background signals [11,81]. The main disadvantages include the need to use post-capillary reagents to initiate chemiluminescence and the need to carefully adjust these reaction conditions to optimize both the detection limit and system reproducibility [22].

Luminol (i.e., 5-amino-2,3-dihydro-1,4-phthalazinedione) is a common tag that is used for chemiluminescence-based detection in CE. The reaction that is involved in this process involves the formation of an excited state molecule of 3-aminophthalate through the reaction of luminol with hydrogen peroxide under basic conditions. This excited state species then releases some visible light as it falls to the ground state. *p*-Iodophenol can be used to improve the production sensitivity of light by this reaction [82]. A scheme based on the use of gold nanoparticles as labels to catalyze luminol chemiluminescence has also been

employed for detection in a CE immunoassay [46]. In addition, luminol-based chemiluminescence has been combined in several studies with the use of HRP as an enzyme label [44,45,81,83–86], as will be discussed in the next section.

3.3. Enzyme Labels

Enzymes are often used in traditional immunoassays as labels. Such labels are detected by later adding a substrate that can be converted by the enzyme into a product that is easily measured. The fact that this is a catalytic process and can lead to many product molecules per enzyme label is a great advantage to this approach. This same feature has made enzyme labels useful for detection in CE immunoassays. Another advantage of enzyme labels is that they can be used to generate products that can be examined through many techniques, including absorbance, fluorescence, chemiluminescence and electrochemical detection.

HRP, or horseradish peroxidase, is the enzyme that is most often used as a label in CE immunoassays. This enzyme has a molar mass of 40 kDa and catalyzes the oxidation of a variety of chemicals in the presence of hydrogen peroxide or related agents. HRP can be used as a label for either large agents such as antibodies or small compounds for CE immunoassays [9,10]. This label has been used with luminol for chemiluminescent-based detection [44,45,81,83–86]. In addition, HRP has been used along with the creation of products that can be monitored by electrochemical detection [87,88]. For instance, an HRP label has been used in a CE immunoassay for thyroxine, in which this enzyme catalyzed the oxidation of 3,3',5,5'-tetramethylbenzidine. The product was then reduced at a carbon fiber microdisk bundle electrode and detected [88].

3.4. Mass Spectrometry

An additional detection approach that has seen some use in CE immunoassays is mass spectrometry. Some care must be taken in this approach to use buffer and separation conditions in the CE method that are compatible with the ionization source for the mass spectrometer (e.g., an electrospray nozzle). However, the use of a CE immunoassay can be a powerful approach for the measurement and identification of analyte, particularly when multiple chemicals in a sample are to be examined simultaneously.

One example of this approach was a method created to detect opioid peptides in clinical samples. This approach used a support that contained immobilized antibodies or antibody F_{ab}' fragments to first extract the desired targets from a sample. The captured analytes were then released by an elution buffer for their on-line analysis by CE and mass spectrometric detection. This technique was employed for the simultaneous analysis of endomorphins 1 and 2 in human plasma at levels in the low-to-mid ng/mL range (see Figure 6) [62,63]. Other reports that have combined CE immunoassays with mass spectrometry for the analysis of urinary codeinoids [89] and the measurement of drugs or drug metabolites such as methadone, amphetamines, morphine, and benzoylecgonine [90,91].

4. APPLICATIONS IN ENDOCRINOLOGY

Immunoassays are frequently utilized for the examination of samples in endocrinology, which is the field that involves the study of hormones and their effects on the body [1,2]. Hormones are chemicals which are secreted by endocrine glands and enter the circulation, where they are then carried to their target tissue or organ to produce an effect. Examples of hormones include insulin, steroids, and thyroxine, among many others [1,2,92]. Because of their importance in the creation and regulation of many biological functions, either the production of too much or too little hormone can create problems in the body. A common example of this is the production of too little insulin in type 1 diabetes, which will lead to an increase in glucose concentrations in blood [1,2].

Many hormones are present in the body at only small concentrations and may have a number of closely-related chemicals present in the same sample (e.g., as occurs for steroid hormones or thyroxine) [1,2]. This makes the routine, selective measurement of these trace chemicals a challenge and often requires the use of immunoassays for this purpose in clinical laboratories [3,4]. Thus, it is not surprising that there have also been a relatively large number of reports that have examined the use of CE immunoassay for this application. Examples of such reports are provided in Table 1. Many of these studies have used homogeneous competitive binding immunoassays with fluorescent detection, but other formats have also been employed (e.g., non-competitive immunoassays).

Insulin has been the subject of several studies that have examined the use of CE-based immunoassays for clinical applications [23,25,71,93–95]. This peptide hormone is produced by the islets of Langerhans in the pancreas and plays a central role in controlling blood glucose levels, as well as carbohydrate and fat metabolism [1,2,92]. Several papers have taken advantage of the small sample size requirements of CE to create immunoassays for measuring insulin secretion from the islets of Langerhans. For instance, a homogeneous competitive binding CE immunoassay with detection based on fluorescein as a label was developed and employed for the measurement of as little as 3 nM insulin in such an application [25,93,94]. This method was further used to estimate equilibrium constants for the binding of insulin with anti-insulin antibodies [96]. A similar approach has been modified for the detection of both insulin and glucagon [23] or insulin, glucagon and islet amyloid polypeptide [95]. This strategy has also been employed with a combination of fluorescein and Cy5 as labels for the separate measurement of insulin and glucagon [71].

Glucagon is another peptide hormone that is produced by the islets of Langerhans in the pancreas. This hormone acts to raise the concentration of glucose in blood when this level is too low [1,92]. Glucagon has been used as a model analyte with aqueous samples and fluorescence detection to examine the various factors that affect homogeneous competitive binding immunoassays [97]. In this study, the effects on assay response and peak resolution were examined for factors such as injection time, voltage ramp time and separation time. A microchip-based system and a fluorescein tag have been recently employed with this approach for the dynamic detection of glucagon secretion of living cell groups of islets of Langerhans, as shown in Figure 7, which made it possible to obtain results from this system in 6 s intervals [98]. A technique that combined reversed-phase capillary liquid chromatography with a homogeneous competitive binding immunoassay was used to differentiate between several cross-reacting forms of glucagon. When this approach was used with a fluorescein label it provided a limit of detection of 20 pM for glucagon and could be used to measure the production of this hormone by islets of Langerhans [99]. A related technique was reported for the simultaneous detection of both glucagon and neuropeptide Y [100]. In addition, multi-analyte homogeneous competitive binding immunoassays have been developed for detecting the secretion of both glucagon and insulin from islets of Langerhans. This method made use of fluorescein as a label and provided limits of detection of 4 nM for glucagon and 5.5 nM for insulin [23]. Related methods have been reported in which islet amyloid polypeptide was detected by this approach [95] or in which fluorescein and Cy5 were both utilized as labels for the simultaneous analysis of glucagon and insulin [71].

The hormone thyroxine has also been examined in a few reports that have used CE-based immunoassays. Thyroxine is the main hormone produced by the thyroid gland and is important in the regulation of metabolism in the body. Several studies have used homogeneous competitive binding immunoassays to detect this hormone, with HRP being employed as the label [88,101]. In one of these reports, an HRP label was used to catalyze the oxidation of 3,3',5,5'-tetramethylbenzidine in a reaction capillary that was part of the CE

system, giving a product that was then reduced for electrochemical detection [88]. Based on this approach, a detection limit of 3.8 nM was obtained for thyroxine. Another report used a similar scheme with *o*-aminophenol as the substrate for HRP, giving a limit of detection of 1.3 nM for thyroxine [102]. This same assay format was carried out on a microchip-based electrophoresis system but with detection now being based on the HRP-catalyzed production of chemiluminescence from luminol [101].

Several types of steroid hormones have also been measured using CE immunoassays. For instance, homogeneous competitive binding immunoassays based on fluorescein as a label have been created for both estradiol [50] and estriol [103]. Estradiol is important in the control of the female reproductive cycle, while estriol is produced by the placenta during pregnancy [1,92]. In work with estradiol, aqueous standards were used to optimize a CE immunoassay conducted on a microchip system, which was found to provide a final detection limit of 310 pM [50]. A study that used a CE immunoassay to measure estriol in serum from normal pregnant women gave a detection limit of 109 pM for this hormone [103]. In another report, testosterone was part of a group of four hormones that were measured in blood, saliva and urine samples through the use of immunoextraction and the addition of a fluorescent tag (AlexFluor 633) to each analyte, followed by a CE separation of the captured and labeled analytes [104].

Cortisol is another steroid that has been measured by a CE immunoassay. Cortisol is the primary glucocorticoid hormone that is produced by the adrenal cortex. This hormone balances the effects of insulin and helps to maintain a homeostasis during the metabolism of carbohydrates, fats and proteins and has a normal serum concentration in the mid- to upper-nanomolar range [1,92]. A homogeneous competitive binding immunoassay with detection based on a fluorescein label was developed to measure cortisol in serum, with a reported limit of detection of 30 nM [105].

Vasopressin is another endocrine-related substance that has been examined using a CE-based immunoassay. Also known as antidiuretic hormone, this chemical is a peptide hormone that is made in the hypothalamus and regulates the body's retention of water [1,92]. A method for detecting vasopressin in samples such as cerebrospinal fluid was described in which a homogeneous competitive binding immunoassay was utilized along with a fluorescein label. In the final approach, it was possible to measure this hormone at levels down to at least 400–500 nM [106].

A number of other glycoprotein-based reproductive hormones have been examined by CE immunoassays. Follicle-stimulating hormone was measured in serum by using a noncompetitive immunoassay format. A limit of detection of 0.08 IU/L was obtained when employing HRP as a label to catalyze the production of chemiluminescence by luminol [44]. The same type of method was employed to measure luteinizing hormone in serum [45]. Both of these hormones, along with testosterone and thyroid stimulating hormone, were determined in blood, saliva and urine samples using a heteromogeneous CE immunoassay based on immunoextraction and fluorescent tagging of the captured analytes [104].

5. APPLICATIONS IN PHARMACEUTICAL ANALYSIS

Another area that has seen a variety of applications of CE-based immunoassays is in the determination of drugs in clinical samples. This work has included the development of these assays for toxicology, therapeutic drug monitoring, forensic analysis, and screening assays for drugs of abuse [26,34,61,75,107–116]. As is shown in Table 2, most of these methods have again involved the use of homogeneous competitive binding immunoassay and fluorescence detection. However, some of these methods further involved the detection of

multiple analytes [26,61] or used a direct assay with a binding agent other than an antibody [34]. Related work with CE-based immunoassays for drug analysis has also employed other labels and systems for detection besides fluorescein and fluorescent detection [75,84,115].

Several of reports listed in Table 2 have investigated the use of CE-based immunoassays in the detection of drugs of abuse [26,107,109]. This has included the use of these methods for the detection of methamphetamine [107,109] and amphetamine, as well as related compounds [113] in urine. The same general approach has been employed to determine morphine in serum [110]. Another study described a multi-analyte method in which amphetamines, the cocaine metabolite benzoylecgonine, methadone and opiates were simultaneously examined in urine samples [26]. This technique involved the combination of a urine sample or standard with a mixture of antibodies for each of the target analytes, along with fluorescein-labeled analogs for each group of targets. It was then possible to separate and examine the remaining non-bound labeled targets by using CE (see Figure 8). This method gave good correlation with reference methods and provided limits of detection in the range of 50–200 nM for these analytes [26]. In another study, a homogeneous competitive binding immunoassay with fluorescence detection was described for the analysis of urine samples for testosterone as a possible doping agent by athletes [116]. A homogeneous competitive binding immunoassay using HRP as a label and with chemiluminescent detection has also been described for the detection of erythropoietin, as a doping agent, in serum [115].

Therapeutic drug monitoring is a second potential use for CE-based immunoassays. The goal in this type of analysis is to measure and follow concentrations of a drug in a patient during treatment to determine if the dosage is within an acceptable range. One study described a microchip-based CE and immunoassay system for the measurement of theophylline in serum [111]. Other reports examined the development and use of CE immunoassays for measuring the antibiotic vancomycin in plasma samples [112] and the immunosuppressant cyclosporine A in blood [108]. A method based on this approach but using B-phycoerythrin as a fluorescent label has further been reported for the determination of digoxigenin in serum [75]. A multi-analyte method employing a heterogeneous competitive binding immunoassay was also described, which allowed the simultaneous analysis of carbamazepine, phenobarbital, phenytoin and theophylline [61].

In the field of toxicology, drug measurements may be required to identify the cause of toxic effects in an individual or to determine the source or extent of exposure to a possible toxic agent. A CE immunoassay has been described for this purpose in the analysis of clenbuterol in urine or other biofluids. Possible applications given for this method included the determination of clenbuterol exposure from contaminated foods and in the identifying cases of accidental exposure to this drug [114].

Several related studies examined the use of homogeneous competitive binding immunoassays for drugs in aqueous solutions or in non-clinical samples. As an example, initial work was described in the use of a homogeneous competitive binding immunoassay with fluorescein as a label for the determination of methotrexate, both alone and in the presence of vancomycin at therapeutically-relevant levels in aqueous standards [117]. The synthetic hormone medroxyprogesterone was measured in porcine tissue samples by the same general format, but with HRP being utilized as a label coupled with chemiluminescent detection [84]. In addition, a direct assay based on affinity probe CE was developed for the drug dorzolamide, in which this drug was allowed to bind with fluorescently labeled human carbonic anhydrase II. The resulting method could be carried out in 10 min and provided a limit of detection of 62.5 pM in urine and plasma samples [34].

6. APPLICATIONS IN THE ANALYSIS OF PROTEINS & PEPTIDES

A variety of proteins and peptides have been examined by CE immunoassays (see Table 3). These have been used as model analytes in the development of these methods and as potential biomarkers for the detection of disease [24,48,51,60,62, 63, 95, 100, 118, 119]. For instance, HSA has been used as a model analyte with aqueous samples to examine the use of homogeneous competitive binding immunoassays based on a Cy5 fluorescent label plus on-column mixing of the sample and label with antibodies (see Figure 9) [73] and in the use of isoluminol isothiocyanate as a label with chemiluminescence detection [120]. A similar method using a Cy5 label and semiconductor laser-induced fluorescence detection has been employed in measuring HSA in serum [48].

Two examples of peptides that have been examined by CE immunoassays are [methionine]enkephalin and neuropeptide Y [100,119]. [Methionine]enkephalin, also known as opioid growth factor, is a peptide that has neurotransmitter properties. This peptide has been measured by a homogeneous competitive binding immunoassay with a fluorescein label. The resulting method was used to measure and compare the concentrations of this peptide in plasma samples from normal individuals and cancer patients, providing detection in the low nM range [119]. Neuropeptide Y also acts as a neurotransmitter. This peptide was measured using a multi-analyte competitive binding immunoassay to examine the production of both this peptide and of glucagon by the islets of Langerhans, as described earlier in Section 4 [100]. A multi-analyte method based on immunoextraction coupled with CE and fluorescein labeling was also employed for the detection of neuropeptide Y along with other neuropeptides and cytokines in skin biopsy samples (see Section 7) [24].

Several other proteins or peptides have been examined by CE immunoassays. Insulin-like growth factor-I was determined by a microchip system that combined on-line electrophoretic sample clean up (i.e., to aid in the detection of low abundance proteins) with a homogeneous competitive binding immunoassay. This method was utilized with serum samples and provided a detection limit of 89 pM when using green fluorescent protein as the label [51]. Brain-derived neurotropic factor was measured in skin biopsy samples using a heterogeneous CE immunoassay based on immunoextraction and a fluorescent tag for labeling and detecting this neuropeptide [60]. The secretion of islet amyloid polypeptide was examined in islets of Langerhans from mice by using a multi-analyte homogeneous competitive binding immunoassay [95]. Opioid peptides such as endomorphins 1 and 2 have been measured in plasma through the combination of immunoextraction, CE and mass spectrometric detection (see previous example given in Figure 6) [62,63]. Immunoextraction has been combined with CE for analyzing the isoforms of alpha 1-acid glycoprotein [64,65]. Furthermore, aptamers have been used as alternatives to antibodies for protein assays using a homogeneous competitive binding format, as demonstrated in assays that used a fluorescein tag to measure thrombin in aqueous samples [31,33].

Some studies have employed CE immunoassays to examine recombinant proteins and modified forms of these proteins. As an example, methionyl recombinant growth hormone and its deamidated variants have been measured in a non-competitive immunoassay by using antibody F_{ab}' fragments as the binding agent and tetramethylrhodamine as a fluorescent label in combination with capillary isoelectric focusing [49]. Human growth hormone was used to examine the separation of antigen-antibody complexes by CE [12]. In addition, recombinant hirudin has been analyzed in human plasma through the use of a homogeneous competitive binding immunoassay and detection based on fluorescein as a label [118].

7. APPLICATIONS IN IMMUNOLOGY & INFECTIOUS DISEASE DETECTION

There have been a number of applications reported for CE immunoassays in the field of immunology, as illustrated in Figure 4 [24,31,32,46,47, 58, 66, 72, 121]. Some of these methods have looked directly at components of the immune system. A noncompetitive immunoassay was reported for the measurement of immunoglobulin G-class antibodies in human serum using gold nanoparticles as labels for catalyzing the chemiluminescence of luminol [46]. In another report, a homogeneous competitive binding format was utilized with aptamers to develop and optimize a method for the determination of immunoglobulin E-class antibodies that was applied to both aqueous standards [33] and serum samples [31]. Other studies used this approach with Cy5 or fluorescein as fluorescent labels for the analysis of secretory immunoglobulin A in saliva [72,122] or with fluorescein as a label for measurement of immunoglobulin A in serum [121].

Another group of CE immunoassays has been developed to detect and measure agents that can lead to infectious disease. A homogeneous competitive binding immunoassay using a fluorescein label was described for the determination of enterotoxin A from *Staphylococcus aureus*. This method gave a detection limit in the low nanomolar range in work with standard samples of the toxin [123]. Aptamers were used as binding agents in a competitive binding CE assay to determine reverse transcriptase from human immunodeficiency virus type 1 in cell cultures [32]. A heterogeneous CE immunoassay based on immunoextraction and fluorescence detection was utilized to measure IgG-class antibodies against *H. pylori* in serum [70]. In addition, several reports have appeared on the use of homogeneous competitive binding immunoassays and fluorescent labels in CE to detect prion proteins in blood and brain samples from normal sheep and elk or scrapie-infected animals [124–126].

Work with immobilized antibodies and CE has been used in heterogeneous immunoassays for the measurement of immunomodulating agents such as interferons and interleukins [24,47,58,66,127]. For instance, one report used a microchip-based system and a mixed bed of immobilized antibodies to extract various inflammatory biomarkers, which were then labeled with AlexaFluor 633 as a fluorescent tag, separated and detected by CE (see Figure 10). These biomarkers included the cytokines interleukin-1 beta, interleukin-6, interleukin-8, interferon gamma, transforming growth factor-beta, macrophage inflammatory protein 1 alpha, macrophage chemoattractant protein 1, and tumor necrosis factor-alpha. Neuropeptides such as calcitonin gene-related peptide, neuropeptide Y, vasoactive intestinal peptide, and substance P were also measured. Skin biopsies were examined by this system, which could process approximately six samples per hour [24]. A similar approach has been used to examine CC and CX cytokines in skin biopsy samples [59]. In addition, immunoextraction has been used in a CE immunoassay to detect various cytokines in cerebrospinal fluid from patients with head trauma, providing detection limits in the low pg/mL range [58]. A non-competitive CE immunoassay was employed to separate and measure forms I-III of interferon gamma in single natural killer cells, providing mass detection limits of 0.4–0.5 zmol [47].

8. APPLICATIONS IN ONCOLOGY & DETECTION OF CANCER BIOMARKERS

The detection of cancer and the assessment of damage that has been caused by cancer are other areas in which CE immunoassays have been utilized. The assessment of DNA damage due the polyaromatic hydrocarbon benzo[*a*]pyrene was determined by measuring the diol epoxide-DNA adduct of this agent in cells. This assay was carried out by means of a homogeneous competitive binding immunoassay with tetramethylrhodamine being used as a fluorescent label [74]. The same general format but instead using AlexaFluor 546 as a

fluorescent tag was utilized to measure benzo[a]pyrene diol epoxide adducts in mononuclear white blood cells [128]. Furthermore, CE immunoassays have been employed to detect DNA methylation [129] and to screen environmental agents in terms of their ability to lead to DNA methylation [130].

Some studies have explored the use of CE immunoassays for cancer biomarkers. One such report used a noncompetitive immunoassay for measuring alpha-fetoprotein (AFP), a biomarker for hepatocellular cancer. This method was used to determine AFP in serum and gave a detection limit of 0.48 ng/mL (or roughly 6.9 pM) when using HRP as the label, *o*-aminophenol as the substrate and electrochemical detection of the product [102]. This method has further been modified for the simultaneous detection of AGP and carcinoembryonic antigen (CEA) in serum [131]. A microchip-based CE immunoassay that employed a sandwich-type immune complex and fluorescent tag allowed for the detection of AFP down to a concentration of 5 pM [40]. This technique has also been combined with the use of lectin affinity chromatography to more selectively monitor the AFP-L3 form of this biomarker, as illustrated in Figure 11 [41,132]. A noncompetitive immunoassay with HRP as the label, but with luminol-based chemiluminescent detection, has been described for the measurement of tumor marker carbohydrate antigen 15-3 (or CA15-3) in serum, resulting in a detection limit of 0.035 U/mL [133]. This approach has further been employed in an assay for CEA, in which gold nanoparticles were utilized to amplify the chemiluminescent signal. This latter method was used with serum and provided a detection limit for CEA of 0.034 ng/mL, or approximately 0.18 pM [43]. A microchip system using immunoextraction, fluorescent labeling and CE has also been described for the measurement of AFP, CEA, heat shock protein 90 and cytochrome C in serum [134].

Another application of a CE immunoassay has been its use to measure multidrug resistance-associated protein (MRP) in cancer cells. This involved the use of a noncompetitive format and fluorescein as the label. The resulting method provided a limit of detection of 0.2 nM for MRP and was utilized to examine the expression of this protein by various cancer cell lines [135].

9. SUMMARY

It has been shown in this review how CE immunoassays have been applied in various ways for the analysis of clinical samples. The most popular format for this work has been the homogeneous competitive binding method, although use of the non-competitive CE immunoassay has also been employed for a number of targets. Research in the use of various types of heterogeneous CE immunoassays has also progressed in the field of clinical analysis. Many of these methods have employed fluorescent labels and laser-induced fluorescence detection. However, additional research has been conducted in detection methods based on chemiluminescence, mass spectrometry, and enzyme labels and associated methods based on electrochemical measurements. These methods have been carried out using both traditional and microanalytical CE systems, with the creation of such schemes and devices representing an active area of research.

Many applications for CE immunoassays have now been reported for targets and samples of clinical interest. In the field of endocrinology, these methods have been created to measure such analytes as insulin, glucagon, thyroxine, steroid hormones, vasopressin, follicle-stimulating hormone and luteinizing hormone. Pharmaceutical applications have included the use of CE immunoassays for detecting drugs of abuse, therapeutic drug monitoring, and the measurement or study of potential toxic agents. Many proteins and peptides have also been examined by CE immunoassays, ranging from serum proteins and recombinant proteins to endomorphins and peptide-based neurotransmitters. Another use of CE

immunoassays has been in immunology to monitor and profile agents such as interferons and interleukins, or in the detection of infectious agents such as bacteria and prion proteins. Finally, various applications of CE immunoassays have been reported in the field of cancer, with examples including the detection of cancer biomarkers, the assessment of DNA damage by potential carcinogens, and the determination of multidrug resistance in cancer cells.

Based on this past work, it is expected that CE immunoassays will continue to be explored for use in other clinical applications and to eventually mature into a method that is suitable for use in routine clinical laboratories. Advantages of CE in these methods are its speed, efficiency, and ability to work with small samples (e.g., such as may appear in work with single cells or in microanalytical systems). As was shown in this review, these benefits are already being exploited in the use of CE immunoassays for biomedical research. Although CE is still often seen as a complementary tool to HPLC and traditional electrophoresis in routine clinical and pharmaceutical laboratories [136,137], the various formats and possible applications of CE immunoassays should lead to an increase in the use of this method in such laboratories. The adoption of this method should be further enhanced as advances continue to be made in the stability, precision and cost of CE systems for routine chemical analysis [136,138,139].

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(a) **Step 1. Incubate sample with excess of labeled antibodies (Ab)**

Step 2. Separate labeled Ab and labeled analyte-Ab complexes by CE

Figure 1.

(a) Scheme for a homogeneous non-competitive CE immunoassay and (b) some typical results for this method, as obtained by a technique for measuring carcinoembryonic antigen (CEA) in a serum sample from a cancer patient. In this type of assay, the sample is first mixed and incubated with an excess of labeled antibodies against the desired target analyte. CE is then used to separate the complex of the labeled antibodies with the target from the free, labeled antibodies that remain. The size of the peaks for either the labeled antibody complex with the target or the free labeled antibodies can then be used to determine how much of the target was in the sample. The electropherogram shown in (b) is reproduced with permission from Ref. [43].

(a) **Step 1. Incubate sample and fixed amount of labeled analyte analog with a limiting amount of antibodies**

Step 2. Separate and measure bound and non-bound labeled analogs by CE

Figure 2.

(a) Scheme for a homogeneous competitive binding CE immunoassay and (b) some typical results for this approach, using a method reported for human serum albumin (HSA) as an example. In this technique, the sample and a fixed amount of a labeled analog of the analyte are combined with a limited amount of antibodies against the desired target. After being allowed to bind, the components of this mixture are separated by CE and the labeled species are detected. The amount of analyte in the original sample can be determined by measuring the relative amount of the labeled analog that is bound to the antibodies or that remains free in solution, with this response being compared to what is obtained when standard samples

are measured under the same conditions. Electropherograms shown in (b) are reproduced with permission from Ref. [48], in which Cyanine 5 (Cy5) was used as a fluorescent label.

Figure 3.

(a) Scheme for a heterogeneous CE immunoassay based on the use of immobilized antibodies for immunoextraction and (b) some results for this approach, using a method reported for the analysis of several chemokines as an example. In this technique, the immobilized antibodies are first used to extract the analytes from a sample. After the non-retained sample components have been washed away, the retained analytes are then released for their separation and detection by CE. The electropherograms shown in (b) is reproduced with permission from Ref. [59], in peaks 1–6 represent fluorescent labeled analytes and “*” is the free dye label.

Figure 4. Immobilization of antigens from the bacterium *Helicobacter pylori* onto magnetic nanobeads (MNB) and the use of these immobilized antigens with CE to extract and analyze *H. pylori* antibodies in human serum. Once the antibodies against *H. pylori* had been captured, they were bound tagged with labeled secondary antibodies. After the antibodies had been released and separated by CE, they were detected by laser-induced fluorescence (LIF). Reproduced with permission from Ref. [70].

Figure 5.
Structures of several common fluorescent dyes, in their activated forms, that have been used in CE immunoassays.

Figure 6.

Electropherograms obtained by using on-line immunoextraction coupled with CE and mass spectrometry for the analysis of a plasma sample spiked with 100 ng/mL of endomorphin 1 (End1) and endomorphin 2 (End2). Reproduced with permission from Ref. [62].

Figure 7.

(a) A microfluidic system for continuous monitoring of glucagon release from live islets of Langerhans in contact with a perfused solution containing a controlled concentration of glucose, and (b) the average release of glucagon over time from the cells in this system when going from an initial solution that contained 15 mM glucose to one that contained 1 mM glucose. The error bars in (b) represent ± 1 standard error of the mean ($n = 4$) and are shown for every fifth data point for the sake of clarity. Reproduced with permission from Ref. [98].

Figure 8.

Use of a homogeneous competitive binding CE immunoassay to simultaneously examine several drugs of abuse in urine samples for patients (PM, PC, HB and SD) or a urine blank. The peaks shown in these electropherograms represent the free labeled tracers for methadone (M), D-amphetamine (A), morphine (O), benzoylecgonine (C), and the internal standard (IS). Terms: RFU, relative fluorescence unit. Adapted with permission from Ref. [26].

(A) On-column binding assay

(B) Stepwise reaction immunoassay

anti-HSA (○) Cy5-HSA (●) native HSA (○)

Figure 9.

Scheme for a homogeneous competitive binding CE immunoassay with on-line mixing of reagents for human serum albumin (HSA) and using Cyanine 5 (Cy5) as a fluorescent label. This method is based on the introduction of the sample and reagents in different zones, which then react as the analyte and reagent bands cross each other due to their different electrophoretic mobilities. The on-line binding assay in (a) shows the expected result in this case for the addition of antibodies and a labeled analog of HSA, while the stepwise reaction immunoassay in (b) also includes a zone in which sample is applied between these two reagent layers. Reproduced with permission from Ref. [73].

Figure 10.

Use of immunoextraction, fluorescent labeling and CE for the simultaneous analysis of 12 inflammatory biomarkers. (a) Typical electropherogram for a standard mixture of the biomarkers and (b) content of the biomarkers that were measured in patient lesions (open bars) versus a control group (filled bars). The error bars represent a range of ± 1 standard error of the mean. Abbreviations: IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; TNFα, tumor necrosis factor-alpha; IFNγ, interferon gamma; TGFβ, transforming growth factor-beta; MIP-1α, macrophage inflammatory protein 1 alpha; MCP-1, macrophage chemoattractant protein 1; SP, substance P; CGRP, calcitonin gene-

related peptide; NY, neuropeptide Y; VIP, vasoactive intestinal peptide. Adapted with permission from Ref. [24].

Figure 11.

Analysis of L3 isoform of α -fetoprotein (AFP-L3) by a CE immunoassay combined with lectin affinity chromatography; AFP-L1 is the L1 isoform of the same protein. The results in (a) show a typical electropherogram for this method, in which the two markers represent fluorescent dyes that were used as mobility references for identification of AFP-L3 versus AFP-L1. The plot in (b) is a correlation chart that was obtained with the results of this method, in terms of %AFP-L3, were compared to those of a reference technique for a series of 98 patient serum samples. Adapted with permission from Ref. [41].

Table 1**Clinical Applications of CE Immunoassays in Endocrinology**

Analyte	Analysis Method	Sample & Limit of Detection ^a
Cortisol	Homogeneous competitive binding immunoassay, fluorescein label	Serum, <28 nM (1 µg/dL) [105]
Estradiol	Homogeneous competitive binding immunoassay, fluorescein label	Standards, 310 pM [50]
Estriol	Homogeneous competitive binding immunoassay, fluorescein label	Serum, 110 pM (31.6 pg/mL) [103]
Follicle-stimulating hormone	Noncompetitive immunoassay, HRP label with luminol-based chemiluminescent detection	Serum, 0.08 IU/L [44] ^c
Glucagon	Homogeneous competitive binding immunoassay, fluorescein label Multi-analyte homogeneous competitive binding immunoassay (with insulin), fluorescein label Multi-analyte homogeneous competitive binding immunoassay (with insulin and islet amyloid polypeptide), fluorescein label Reversed-phase capillary liquid chromatography + homogeneous competitive binding immunoassay, fluorescein label Multi-analyte homogeneous competitive binding immunoassay, Cy5 label Multi-analyte reversed-phase capillary liquid chromatography + homogeneous competitive binding immunoassay, fluorescein label	Islets of Langerhans, 1 nM [98] Islets of Langerhans, 760 pM (glucagon alone) or 4 nM (with insulin) [23] Islets of Langerhans, 3 nM [95] Islets of Langerhans, 20 pM [99] Islets of Langerhans, 3 nM [71] Islets of Langerhans, <500 pM [100] ^b
Insulin	Homogeneous competitive binding immunoassay, fluorescein label Multi-analyte homogeneous competitive binding immunoassay (with glucagon), fluorescein label Multi-analyte homogeneous competitive binding immunoassay (with glucagon and islet amyloid polypeptide), fluorescein label	Islets of Langerhans, <0.3 nM [25], 0.3 nM [94], 3 nM [93] or 7 nM [71] Islets of Langerhans, 5.5 nM [23] Islets of Langerhans, 2 nM [95]
Luteinizing hormone	Noncompetitive immunoassay, HRP label with luminol-based chemiluminescent detection	Serum, 0.08 IU/L [45] ^c
Thyroxine	Homogeneous competitive binding immunoassay, HRP label with electrochemical detection Homogeneous competitive binding immunoassay, HRP label with chemiluminescence detection Homogeneous competitive binding immunoassay, HRP label with electrochemical detection	Serum, 3.8 nM (1.0 ng/mL) [88] Serum, 2.2 nM [101] Serum, 1.3 nM [102]
Vasopressin	Homogeneous competitive binding immunoassay, fluorescein label	Cerebrospinal fluid, <460 nM (500 ng/mL) [106] ^{b,d}

^aThis table summarizes methods that actually used clinical samples during part of their application or development. Additional examples are given in the main text of reports that have used analytes in the given category but that worked only aqueous standards. All limits of detection have been converted to units based on molarity, where possible; if a conversion was made, the original concentration units that were reported are also provided in parentheses. When more than one analyte was being examined by a given method, the concentration limit of detection that is listed is for a representative member of the analyte class.

^bInsufficient information was given in this reference to determine the true limit of detection; the range provided here is based on the lowest concentration standard that was examined or typical results that were presented.

^cThe limit of detection in this study was given in terms of activity by using international units per liter (IU/L).

^dThis work was stated as being suitable for cerebrospinal fluid samples but may have been evaluated using only aqueous standards.

Table 2**Clinical Applications of CE Immunoassays in Pharmaceutical Analysis**

Analyte	Analysis Method	Sample & Limit of Detection ^a
Amphetamine & related analogs	Homogeneous competitive binding immunoassay, fluorescein label	Urine, 590 nM (80 ng/mL) [113]
D-Amphetamine	Multi-analyte homogeneous competitive binding immunoassay, fluorescein label	Urine, 200 nM (27 ng/mL) [26]
Benzoylcegonine	Multi-analyte homogeneous competitive binding immunoassay, fluorescein label	Urine, 93 nM (27 ng/mL) [26]
Carbamazepine	Multi-analyte heterogeneous competitive binding immunoassay, fluorescein label	Serum, 2.0 nM [61]
Clenbuterol	Homogeneous competitive binding immunoassay, fluorescein label	Urine, 2.2 nM (0.7 ng/mL) [114]
Cyclosporine A	Homogeneous competitive binding immunoassay, fluorescein label	Blood, 0.9 nM [108]
Digoxigenin	Homogeneous competitive binding immunoassay, B-phycoerythrin label	Serum, 1.1 nM [75]
Dorzolamide	Direct assay, human carbonic anhydrase II as binding agent, fluorescein label	Urine and plasma, 62.5 pM [34]
Erythropoietin	Homogeneous competitive binding immunoassay, HRP label with luminol-based chemiluminescent detection	Serum, 26 pM (0.9 ng/mL) [115]
Methadone	Multi-analyte homogeneous competitive binding immunoassay, fluorescein label	Urine, 52 nM (16 ng/mL) [26]
Methamphetamine	Homogeneous competitive binding immunoassay, fluorescein label	Urine, <130 nM (20 ng/mL) [109], 270 nM (40 ng/mL) [107]
Morphine	Homogeneous competitive binding immunoassay, fluorescein label Multi-analyte homogeneous competitive binding immunoassay, fluorescein label	Serum, 140 nM (40 ng/mL) [110] Urine, 49 nM (14 ng/mL) [26]
Phenobarbital	Multi-analyte heterogeneous competitive binding immunoassay, fluorescein label	Serum, 1.8 nM [61]
Phenytoin	Multi-analyte heterogeneous competitive binding immunoassay, fluorescein label	Serum, 2.5 nM [61]
Testosterone	Homogeneous competitive binding immunoassay, fluorescein label	Urine, 3.8 nM (1.1 ng/mL) [116]
Theophylline	Homogeneous competitive binding immunoassay, fluorescein label Multi-analyte heterogeneous competitive binding immunoassay, fluorescein label	Serum, 1.4 μM (0.26 mg/L) [111] Serum, 2.2 nM [61]
Vancomycin	Homogeneous competitive binding immunoassay, commercial fluorescent label	Plasma, 0.68 nM (0.98 ng/mL) [112]

^aThis table summarizes methods that actually used clinical samples during part of their application or development. Additional examples are given in the main text of reports that have used analytes in the given category but that worked only aqueous standards. All limits of detection have been converted to units based on molarity; if a conversion was made, the original concentration units that were reported are also provided in parentheses.

Table 3**Clinical Applications of CE Immunoassays in Protein or Peptide Analysis**

Analyte	Analysis Method	Sample & Limit of Detection ^a
Brain-derived neurotropic factor	Heterogeneous assay with immunoextraction followed by CE, fluorescent label	Skin biopsies, 0.02 pg [60]
Calcitonin gene-related product	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 0.83 pg [24]
Endomorphin 1	Heterogeneous CE assay with immunoextraction, detection by mass spectrometry	Plasma, 1.7 nM (1 ng/mL) [62]; plasma, 0.8 nM (0.5 ng/mL) [63]
Endomorphin 2	Heterogeneous CE assay with immunoextraction, detection by mass spectrometry	Plasma, 1.7 nM (1 ng/mL) [62]; plasma, 8.7 nM (5 ng/mL) [63]
Hirudin	Homogeneous competitive binding immunoassay, fluorescein label	Plasma, 14.2 nM [118]
Human serum albumin	Homogeneous competitive binding immunoassay, Cy5 label	Serum, 300 nM (0.02 mg/mL) [48]
Insulin-like growth factor	Homogeneous competitive binding immunoassay with on-line electrophoretic sample clean up, green fluorescent protein label	Serum, 89 pM (0.68 ng/mL) [51]
Islet amyloid polypeptide	Multi-analyte homogeneous competitive binding immunoassay (with glucagon and insulin), fluorescein label	Islets of Langerhans, 3 nM [95]
[Methionine]-enkephalin	Homogeneous competitive binding immunoassay, fluorescein label	Plasma, <1.7 nM (1.0 ng/mL) [119] ^b
Neuropeptide Y	Multi-analyte capillary liquid chromatography plus homogeneous competitive binding immunoassay, fluorescein label Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Islets of Langerhans, 40 pM [100] Skin biopsies, 1.1 pg [24]
Substance P	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 0.42 pg [24]
Vasoactive intestinal peptide	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 1.1 pg [24]

^a This table summarizes methods that actually used clinical samples during part of their application or development. Additional examples are given in the main text of reports that have used analytes in the given category but that worked only aqueous standards. All limits of detection have been converted to units based on molarity, where possible; if a conversion was made, the original concentration units that were reported are also provided in parentheses.

^b Insufficient information was given in the cited reference to determine the true concentration limit of detection for this analyte. The range provided here is based on data that was obtained for human plasma samples.

Table 4**Applications of CE Immunoassays in Immunology and Infectious Disease**

Analyte	Analysis Method	Sample & Limit of Detection ^a
HIV-1 reverse transcriptase	Homogeneous competitive binding assay using aptamers, fluorescein label	Cell culture, <7 nM [32] ^b
Immunoglobulin A	Homogeneous competitive binding immunoassay, Cy5 label Homogeneous competitive binding immunoassay, fluorescein label	Saliva (secretory), 2.6 nM (1.0 ng/mL) [72] Serum, 0.66 nM (0.1 µg/mL) [121]
Immunoglobulin E	Homogeneous competitive binding immunoassay, fluorescein label	Serum, 46 pM [31]
Immunoglobulin G	Noncompetitive immunoassay, gold nanoparticles as labels to catalyze luminol chemiluminescence	Serum, 7.1 pM [46]
Interferon alpha	Multi-analyte heterogeneous assay with immunoextraction followed by CE, Cy5 label	Plasma/serum, 0.3–0.5 nM (5–10 pg/µL); urine, 0.3–1.1 nM (5–20 pg/µL); saliva, 0.5–1.1 nM (10–20 pg/µL); cerebrospinal fluid, 0.3–0.8 nM (5–15 pg/µL) [66] ^c
Interferon gamma	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label Non-competitive immunoassay, fluorescein label Multi-analyte heterogeneous assay with immunoextraction followed by CE, Cy5 label	Skin biopsies, 0.55 pg [24] Natural killer cells, 0.4–0.5 pM [47] Plasma/serum, 0.3–0.9 nM (5–15 pg/µL); urine, 0.6–1.2 nM (10–20 pg/µL); saliva, 0.6–1.2 nM (10–20 pg/µL); cerebrospinal fluid, 0.3–1.2 nM (5–20 pg/µL) [66] ^c
Interleukin-1 beta	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 0.51 pg [24]; cerebrospinal fluid, 0.05 pM (0.9 pg/mL) [58]
Interleukin-2	Multi-analyte heterogeneous assay with immunoextraction followed by CE, Cy5 label	Plasma/serum, 0.3–0.6 nM (5–10 pg/µL); urine, 0.6–1.6 nM (10–25 pg/µL); saliva, 0.6–1.6 nM (10–25 pg/µL); cerebrospinal fluid, 0.3–1.0 nM (5–15 pg/µL) [66] ^c
Interleukin-6	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 0.63 pg [24]; cerebrospinal fluid, 0.03 pM (0.6 pg/mL) [58]
Interleukin-8	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 1.0 pg [24]; cerebrospinal fluid, 0.26 pM (2.1 pg/mL) [58]
Interleukin-10	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Cerebrospinal fluid, 0.04 pM (1.1 pg/mL) [58]
Macrophage chemoattractant protein-1	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 1.1 pg [24]
Macrophage inflammatory protein 1 alpha	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 1.3 pg [24]
Prion protein	Homogeneous competitive binding immunoassay, fluorescein label	Blood (sheep), 2.3 nM (80 ng/mL) [126]; blood (sheep or elk), 15 amol [125]; brain samples (sheep), 2 fmol [124]
Tumor necrosis factor-alpha	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 0.37 pg [24]; cerebrospinal fluid, 0.06 pM (0.8 pg/mL) [58]

Analyte	Analysis Method	Sample & Limit of Detection ^a
Transforming growth factor-beta (1)	Multi-analyte heterogeneous assay with immunoextraction followed by CE, AlexaFluor 633 label	Skin biopsies, 0.82 pg [24]; cerebrospinal fluid, 0.12 pM (1.5 pg/mL) [58]

^aThis table summarizes methods that actually used clinical samples during part of their application or development. Additional examples are given in the main text of reports that have used analytes in the given category but that worked only aqueous standards. All limits of detection have been converted to units based on molarity; if a conversion was made, the original concentration units that were reported are also provided in parentheses.

^bInsufficient information was given in the cited reference to determine the true concentration limit of detection for this analyte. The range provided here is based on results that were reported for a low concentration standard.

^cThese values are all for samples spiked with recombinant forms of the given analyte.