Review

# The application of immunoassay in bioanalysis

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**Abstract:** Immunoassay technology is an analytical method with high sensitivity and specificity; it provides a technique to assay materials which cannot be measured by other methods, or are difficult to detect. It plays a very important role in biological sample pre-treatment, therapeutic drug monitoring and drug determination, and is one of the important means for in vivo drug analyses. This paper reviews immunoassays commonly used in bioanalysis, including immunoextraction and immunodepletion for pretreatment of biological samples, conventional immunoassay methods and new immunoassay technologies for determination of target drugs.

**Keywords:** Immunoassay, Sample pretreatment, Biological samples, Bioanalysis

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**Contents**



## 1. Introduction

Biopharmaceutical analysis is a subdiscipline of pharmaceutical analysis. It is performed by analyzing drugs and their metabolites in human or animal body fluids, tissues and organs to understand the quantitative changes in vivo, or to obtain the various parameters and changes of pharmacokinetics, metabolism, pathways and other information. The analysis objects of biopharmaceutical analysis are biological samples. Usually biological matrices are quite complex, and sometimes target analytes are not very stable and their concentration levels are generally very low. So analysis approaches with excellent selectivity, high sensitivity and time-saving are highly desirable.

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Chromatographic techniques are considered as the most powerful tools for qualifying and quantifying target molecules in biological samples. Chromatographic methods show high separation efficiency, fast analysis speed and high sensitivity. But, for some biological samples, these methods require high cost, skilled analysts, and complicated sample preparation steps. In 1960, Solomon Berson and Rosalyn Yalow described a method using radioimmunoassay for measuring endogenous insulin in plasma. From then on, immunoassay has been increasingly applied to biology and medical investigation and practice. With the emergence of new labeling methods, enzyme immunoassay, fluorescence immunoassay and chemiluminescence immunoassay are developed. These methods are simple, reliable, sensitive, specific and easy to automate, so they have been widely used in various fields of biopharmaceutical analysis. Immunoassay technologies can also be used for pretreatment of biological samples to remove non-targeted substances to enable better detection. Creaser[1] and Qiu[2] described the application of immunoextraction technology, and confirmed that the technology could really improve the sensitivity of analytical methods. Cellar et al[3] used immunoaffinity techniques to remove high abundant proteins (HAPs) from samples selectively prior to analysis, and proved that immunodepletion of HAPs could enable better detection of low abundance proteins. Immunoassay technologies have also been used for drug determination in biological matrices. For example, Wang et al[4] used a homogeneous enzyme immunoassay to detect fentanyl in human urine, and Wang et al[5] developed a highly sensitive and selective microplate chemiluminescence enzyme immunoassay for the determination of free thyroxine in human serum. New immunoassay technologies, such as biosensor immunoassay, capillary electrophoresis immunoassay and lateral-flow immunoassay, have also been widely used in biopharmaceutical analysis.

In this review, we summarize immunoassay technologies commonly used in biopharmaceutical analysis. Immunoextraction and immunodepletion for pretreatment of biological samples, conventional immunoassay methods and new immunoassay technologies for determination of target drugs are discussed as well. And we also take a further outlook for the development of immunoassay technology, and hope this review may provide useful information for further investigations and researches.

## 2. Pretreatment of biological samples

Sample pretreatment is an essential step in many analysis processes. The complexity of biological samples (such as urine, serum, plasma, tissues, etc.) makes them difficult to be directly measured by conventional analytical methods. Conventional procedures, including protein precipitation, liquid-liquid extraction and solid-phase extraction, are often used in combination with evaporation to dryness to concentrate samples, which may affect the activity or stability of biological samples. Immune pretreatment technology is based on the specific binding reactions of antigen and antibody. It employs a biologically related binding agent for selective purification of a target compound. At present, the widely used immune pretreatment methods mainly include immunoextraction and immunodepletion.

### 2.1. Immunoextraction

Immunoextraction is the most effective purification method for biologically active substances. Immunoaffinity chromatography is used to remove a specific analyte or group of analytes from a sample prior to analysis by a second analytical method; and selective adsorption and separation of biological molecules make it more specific and effective[6]. In addition, the samples in the concentrating process may bind to affinity ligands, which often make the samples more stable. Immunoextraction can be carried out either offline or online with the second analytical method. In the offline mode, antibodies are typically immobilized onto a low-performance support and packed into a small disposable syringe or SPE cartridge. Samples are then applied through the affinity support, which binds the analytes of interest while other sample components are washed away. Compared with common extraction methods such as liquid-liquid extraction, solid-phase extraction and supercritical fluid extraction, immunoextraction methods have exquisite specificity as a result of specific antibody-antigen interaction, and thus will lead to the highly selective adsorption of target analytes.

Chen et al[7] prepared and evaluated an affinity monolithic capillary for immunoextraction with testosterone (T) as a model analyte. The preparation of the monolith was based on copolymerization of 2-vinyl-4,4-dimethylazlactone (VDA), 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EDMA). Anti-testosterone polyclonal antibody was immobilized onto the monolith via the reaction with VDA. Fluorescence-labeled testosterone at C3 was designed as a tracer to estimate the extraction ability of this immunoaffinity column, and to optimize the immunoextraction process, such as washing, eluting, incubation and injection. Assay was carried out by competitive immunoassay mode and on-line laser-induced fluorescence (LIF) detection. The immunological studies showed that T3F (Fluorescence-labeled testosterone at C3) had almost the same interaction as that of testosterone. T3F was used for the estimation and conditions optimization of the affinity column. On the contrary, the main binding sites of T17F (Fluorescence-labeled testosterone at C17) were covered by fluorescein isothiocyanate (FITC). Thus T17F was taken as the control labeled tracer to test the specificity of the affinity column. Results showed that the target tracer (T3F) had a recovery rate of 0.26, higher than that of 0.02 in the case of control tracer (T17F). These data indicated that this immunoaffinity monolith had the ability of extracting its target specifically. This rapid immunoextraction system within capillary can be applied to develop on-line IA-CE which can be used to analyze a group of related compound simultaneously. Kim et al[8] developed an on-line immuno-extraction and liquid chromatography/mass spectrometry (LC/MS) method for the estimation of R,R′-fenoterol, R,R′-methoxyfenoterol and R,S′-naphthylfenoterol in rat plasma. Sample preparation involved immunoextraction of analytes using an antibody raised against R,R′- and R,S′-aminofenoterol that was immobilized onto chromatographic support. The data demonstrated that affinity-purified rabbit anti-fenoterol antibodies were successfully immobilized onto chromatographic support and packed into HPLC column. The specificity of immuno-extraction column was investigated in cross-selectivity studies using structurally related compounds and the immunoextraction column, which showed high selectivity toward R,R′-fenoterol, R,R′-methoxyfenoterol and R,S′-naphthylfenoterol. Furthermore, all other structurally similar β2-AR agonists showed minimal recoveries, indicating that the antibody column is highly selective for R,R′-fenoterol, R,R′-methoxyfenoterol and R,S′-naphthylfenoterol. Optimization of immuno-extraction procedures was performed using the design of experiment (DoE) approach and showed that the flow rate and amount of organic modifiers in elution buffer significantly affect the overall analysis time. A pseudo-homogeneous immunoextraction method based on gold-coated magnetic nanoparticles (MNPs) for the specific extraction of epitestosterone (ET) from human urine samples was developed by Qiu et al[2]. Half-IgG of anti-ET monoclonal antibodies were covalently immobilized onto ($Fe_3O_4$) core-Au shell ($Fe_3O_4$@Au) MNPs. An external magnetic field was applied to collect the MNPs, which were then rinsed with distilled water and eluted with absolute methanol to obtain ET as the analyte. The obtained extraction solution was analyzed by HPLC with UV detection (244 nm) within 12 min. The antibody-conjugated $Fe_3O_4$@Au MNPs are novel materials for immunoaffinity extraction. Compared with the conventional technique using immunoaffinity column, this method for sample pretreatment is fast, highly specific and easy to operate.

### 2.2. Immunodepletion

Immunodepletion is a powerful pretreatment tool because it can selectively remove non-target proteins to enable better detection and identification of low abundant proteins. For immunodepletion, an antibody column is used to remove abundant analytes from a complex sample before using a second method to analyze the minor sample components. One common application is to split a complicated mixture (such as serum, cell lysate and homogenized tissue) into two batches, then extract a targeted molecule from one batch by immunodepletion. Depletion typically works by adding an antibody targeting the molecule of interest and then followed by adding either protein A or anti-IgG. After mixing, the samples are centrifuged and the supernatant is treated as the depleted (or control) product. In contrast to other methods that can be used to remove high- and mid-abundant proteins (e.g. precipitation, SPE, ultracentrifugation, molecular-weight separation and pI separation), immunodepletion can provide highly selective depletion of multiple high-abundant proteins simultaneously[6].

High-abundant protein species have a huge impact on the dynamic range in mass spectrometric analysis by masking low-abundant proteins and thereby reduce the identification potential. Therefore, the depletion of high-concentrated proteins is vital. Steinsträßer et al[9] applied the ProteoPrep® 20 plasma Immunodepletion kits (Sigma-Aldrich, Stein-heim, Germany) to an assay of wound fluids and the results were comparable qualitatively to 1D-SDS-PAGE and quantitatively to MudPIT experiments, indicating that the immunodepletion is successful. They also tested the Proteo-Prep® 20 plasma immunodepletion kits for the protein depletion in chronic and acute wound fluids. SDS-PAGE separations and BCA assay measurements indicated a substantial decrease in the amount of proteins in the depleted samples. MudPIT experiments confirmed that the reduced protein concentration was resulted from the depletion of the most abundant proteins. The bound fraction contained 17 of the 20 most abundant proteins, while the remaining three were not detectable. In the flow-through fractions of three acute and three diabetic samples, six high-abundant proteins could not be identified (e.g. complement system proteins and immunoglobulins). The majority of the detected abundant proteins in the depleted samples showed a spectrum count percentage of less than 1% each, compared to the overall count. The concentrations of the 20 most abundant proteins in the depleted fraction were helpful for following tolerability investigations. Brand et al[10] carried out a study to examine the "multi-affinity removal system", an immunoaffinity depletion column targeting against six plasma proteins. The first-generation custom-made 10 mm×6100 mm MARS column (Agilent Technologies, Palo Alto, USA) attached to an Äkta Purifier HPLC System (Amersham Biosciences, Freiburg, Germany) was used through all the experiments. As determined by sandwich ELISA, the depletion rate for each target protein was >99% over 200 cycles of regeneration. To estimate a potential loss of samples after the immunodepletion, they performed spiking/recovery experiments with a selection of tumor markers at concentrations of lower to medium ng/mL range. The average recovery of 9 out of 11 markers was 78%. They concluded that the selective depletion of plasma proteins by immunoaffinity chromatography is a valid strategy for the enrichment of potential biomarkers sought by proteomics methodologies. Tu et al[11] evaluated the effects of top 7 or top 14 immunodepletion on the shotgun proteomic analysis of human plasma. Analysis of unfractionated and immunodepleted plasma made by peptide isoelectric focusing (IEF) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) demonstrated enrichment of non-targeted plasma proteins by an average of 4-folds, as assessed by MS/MS spectral counting. Either top 7 or top 14 immunodepletion resulted in a 25% increase in identified proteins compared to unfractionated plasma. Although 23 low abundant (<10 ng/mL) plasma proteins were detected, they accounted for only 5%–6% of total protein identified in the immunodepleted plasma. In both unfractionated and immunodepleted plasma, the 50 most abundant plasma proteins accounted for 90% of cumulative spectral counts and precursor ion intensities, leaving little capacity to detect lower abundant proteins. Therefore, untargeted proteomic analyses using current LC-MS/MS platforms, even with immunodepletion, can not be expected to efficient enough to discover low abundant, disease-specific biomarkers in plasma.

## 3. Conventional immunoassay methods

### 3.1. Radioimmunoassay

Radioimmunoassay (RIA) is based upon the competition between labeled and unlabeled antigen for specific antibody sites to form antigen-antibody complexes. It was developed by Berson and Yalow in the late 1950s, which represented a milestone in the history of the application of radionuclide methodology to biology and to medical investigation and practice. RIA is simple, reliable, sensitive, specificity and easy to automate, and it offers a technique to assay materials otherwise unmeasurable or detectable. But as isotopes have short half-life, the kit is not suitable for long-term storage. In addition, although the use of radioactive material is small, there still are certain radiation and pollution problems, demanding extra attention during the operation.

Ai et al[12] developed a RIA for the measurement of exendin-4 concentration in rhesus monkey serum. This assay produced antiserum with a Ka value of $3.2\times10^{10}$ L/mol, indicating sufficient binding to the radio-ligand. The resulting antibody dilution for the assay was set at

1:30 000. Antibody specificity was determined using glucagon-like peptide (GLP), erythropoietin (EPO), parathyroid hormone (1–34) PTH (1–34) and brain natriuretic peptide (BNP). Cross-reactivities of <1% were observed, showing good selectivity and specificity of the antibody. The use of magnetic beads in this assay realized easy separation and in some cases improved sensitivity. Obviously, the exendin-4 radioimmunoassay showed satisfactory accuracy and precision along with good selectivity, demonstrating that it is a useful quantitation method for this drug in monkey serum. Welp et al[13] developed a high-throughput direct IRA to detect the concentrations of melatonin (*N*-acetyl-5-methoxytryptamine) in mice serum and plasma. This method allowed the analysis of melatonin in different biological fluid samples with small sample volumes (e.g. mice and rats) and wide working range. The flexible standards covered a working range from 12 to 4000 pg/mL with a sample volume of 50 μL. The limits of detection in mouse serum and mouse plasma were 9 pg/mL and 7 pg/mL, respectively. The recovery of melatonin was 108% in mouse serum and 99% in mouse plasma. The variation coefficients of the assay, within and between runs, ranged between 7% and 13% in mouse serum and between 5% and 8% in mouse plasma. The wide working range made it possible to analyze low and high melatonin concentrations. Sara el al[14] evaluated the validity of measuring fentanyl concentrations in equine plasma using RIA by comparing it to the established method of liquid chromatography-mass spectrometry (LC-MS). The cross-reactivity of the primary equine fentanyl metabolite, *N*-[1-(2-phenethyl-4-piperidinyl)] maloanilinic acid (PMA), in the RIA was determined. Fentanyl concentrations determined by RIA and LC-MS were correlated, but the RIA overestimated low fentanyl concentrations and underestimated high fentanyl concentrations. The overestimation of low fentanyl concentrations is most likely due to the 29% cross-reactivity of PMA in the RIA. Consequently, when compared with LC-MS, fentanyl concentrations determined by RIA in equine plasma could be misleading, especially for the calculation of fentanyl pharmacokinetics.

### 3.2. Enzyme immunoassay

Enzyme immunoassay (EIA) is the most current clinical application of immunoassay technology, and it's well known and adopted in medical laboratories, manufacturers of in vitro diagnostic products, regulatory bodies, and external quality assessment and proficiency-testing organizations. It is based on the principle of immunoassay with an enzyme rather than radioactivity as the reporter label. The main advantages of enzyme immunoassay using enzyme labels in devising competitive binding assays is that analytes can be detected at extremely low levels in relatively short periods of time via kinetic methods[15,16]. EIA demonstrates good selectivity and simple operation while no needs of expensive equipments, and it doesn't pollute the environment. Additionally, the enzyme markers are fairly stable, thus long shelf-life and wide range of applications, making them favored by the majority of researchers. Enzyme immunoassay can be divided into two categories: homogeneous enzyme immunoassay and heterogeneous enzyme immunoassay.

#### 3.2.1. Homogeneous enzyme immunoassay

Homogeneous enzyme immunoassay is a competitive binding immunoassay. It is mainly used in the determination of small molecule hormones and haptens. Although less sensitive, homogeneous methods do not need time-consuming washing steps to separate bound and unbound labels, therefore are more rapid and amenable to automation[17]. The biggest drawback of the homogeneous enzyme immunoassay is its vulnerability to non-specific endogenous enzyme inhibitors in samples and the cross-interference of the reactants. Due to the competitive binding assay principle, the sensitivity is not as good as heterogeneous enzyme immunoassay though its sensitivity can reach $10^{-9}$ mol/L.

Snyder et al[18] evaluated a new fentanyl homogeneous enzyme immunoassay (HEIA) for its ability to detect fentanyl accurately in 307 urine samples from patients receiving chronic opioid therapy. Samples were screened by HEIA and confirmed by LC-MS/MS and ELISA for diagnostic comparison. HEIA diagnostic sensitivity, specificity, precision and accuracy studies were performed to comparison with LC-MS/MS and ELISA. HEIA detected 37 of the 38 LC-MS/MS positive samples identified in their initial evaluation, including some samples at very low fentanyl concentration levels (<1 ng/mL (<3 fmol/L)). HEIA showed minimal cross-reactivity with other opioid analgesics and drugs commonly encountered. While interferences by common urine

contaminants were negligible, they observed considerable signal suppressions in acidic samples (pH<4.0). In a way, the immunoanalysis of urine fentanyl HEIA is a simple, quick (<8 min) and reliable screening method with high sensitivity and specificity in patients suffering from chronic pain. Rebollo et al[19] carried out a study to evaluate a modified automated enzyme multiple immunoassay technique (EMIT) for the routine therapeutic drug monitoring (TDM) of free mycophenolic acids (fMPAs). The method was linear over the concentration range between 0.01 and 1.25 μg/mL. The within-run coefficients of variation (CV) varied from 3.02% to 10.68% and the between-day CV ranged from 7.80% to 12.72%, and the error ranged between 3% and 14%. Ansermot et al[20] compared a validated LC-MS method with the commercial EMIT for cyclosporine and tacrolimus quantification in whole blood. Whole blood samples from liver, kidney, lung and bone marrow transplant patients were analyzed successively using EMIT and LC-MS. Overestimations of the concentrations were found while measured with EMIT comparing to LC-MS, which were observed with means of 23% (range: 6% to 46%) for cyclosporine and 30% (range: −3% to 73%) for tacrolimus. The EMIT demonstrated significant positive biases due to cross-reactions with metabolites. This indicates that, in some clinical situations, a selective method such as LC-MS might be a better choice than EMIT for therapeutic drug monitoring in transplant patients.

**3.2.2. Heterogeneous enzyme immunoassay**

Heterogeneous enzyme immunoassay belongs to the most widely used class of immunoassay technologies. It employs a solid phase component which allows the separation of bound from unbound components. Separation method is based on a fixed reactant in the solid phase carrier which can be bound with other reactants, and the separation is made through washing and centrifugation to remove other substances in the liquid phase, such reactions are known as solid-phase enzyme immunoassay. The enzyme-linked immunosorbent assay (ELISA) is accepted as the general term for heterogeneous enzyme immunoassays. ELISA is a sensitive, easy to operate method and the development of standardization and automation of operational procedures may bring the further improvements. So, this method is widely used to analyze protein components, antigens or antibodies, blood and other body fluids, cells and other samples. The main drawback is that there are so many influencing factors in the experiment, which will cause distortion of the results. Besides, the operating requirements and difficulty to realize automation make batch tests hard to achieve.

Lei et al[21] developed a novel immunomagnetic bead ELISA based on IgY (egg yolk immunoglobulin) to detect circulating schistosomal antigen (CSA) in sera of hosts infected with *S. japonicum*. The results showed that the CSA levels in urine of heavily and lightly infected mice reached the peaks 8 and 10 weeks after infection, respectively. The CSA level in urine of heavily infected mice was much higher than that of lightly infected mice from 8 to 14 weeks after infection. Their findings suggested that the IgY-IMB-ELISA was valuable to detect CSA in urine of infected mice and to monitor the efficacy of schistosome chemotherapy, which provided scientific basis for its further application for detecting circulating antigens in urine of human schistosomiasis. A new double-antigen sandwich ELISA for detecting antibody against the human hepatitis B core antigen (anti-HBc) was developed by Deng et al[22], with recombinant HBc (rHBcAg) immobilized on the solid phase of the plate and a HRP-rHBcAg conjugation for detection. The rHBcAg was expressed in *Escherichia coli* and purified by a monoclonal antibody (against HBcAg)-specific affinity chromatography. The CV within- and between-runs ranged from 2.99% to 4.08% and from 5.53% to 9.00%, respectively, indicating that the reproducibility of the sandwich ELISA kit is good. Totally, 942 sera from both normal individuals and patients with HBV infection were tested in parallel with the double-antigen sandwich assay and the competitive assay. The relative specificity value for the sandwich assay was 95.7% in comparison to the competitive assay, with 98.4% (927 of 942 cases) agreement between the two assays. And the discrepancies were shown in 15 specimens. These 10 positive specimens seen in the sandwich assay were because of the improved sensitivity over the commercial assay. In summary, this sandwich ELISA has been proven to possess characteristics of good sensitivity, specificity, precision and stability, and it will be a reliable method for screening anti-HBc in the blood in general population.

### 3.3. Fluorescence immunoassay

Fluorescence immunoassay (FIA) is an immune analysis technology with fluorescein-labeled antibody or antigen as a tracer, and the principle is similar to ELISA. The method can be used in the measurement of many compounds, including drugs, hormones and proteins, in the identification of antibodies, and in the quantification of antigens such as viral particles and, potentially, bacteria. Time-resolved fluoro-immunoassay, fluorescence polarization immunoassay, fluorescence quenching immunoassay and enhanced fluorescence immunoassay all belong to fluorescence immunoassay. Fluorescence immunoassay is the mainstream of non-RIA analysis. With the application of a variety of long-lasting fluorescent markers and fluorescence enhancers, the application of fluorescence immunoassay is widespread. For example, fluorescence polarization immunoassay can be used to detect the concentrations of gentamicin[23], teicoplanin[24], methotrexate[25], cyclosporine[26] and vancomycin[27] in biological samples. Time-resolved fluorescence immunoassay can detect chlormadinone acetate[28], medroxyprogesterone acetate[29], isoflavones[30] and Varicella-Zoster virus immunoglobulin G[31] in serum.

Wu et al[32] established a rapid and simple fluorescence polarization immunoassay method for determination of vancomycin serum concentration. The drug concentrations were measured by the established HPLC method and FPIA with vancomycin kit. A FPIA algorithm for the determination of vancomycin concentration was established according to the correlation between the FPIA and HPLC results. HPLC determination showed a good linear correlation within the range of 0.5–100 mg/L. Correlation analysis between the measurements of HPLC and FPIA in 300 serum samples gave the linear regression equation: (concentration by HPLC) = 0.760 × (concentration by FPIA)–0.577 ($P$<0.001, $R^2$ = 0.982). An algorithm was derived from this correlation for measuring the serum vancomycin concentrations with FPIA. When it was validated in additional 70 serum samples from patients, 'FPIA algorithm' showed good accuracy versus HPLC: 'FPIA algorithm' = 0.93 (HPLC) + 0.63, $R^2$ = 0.962, and 94.3% of the results from FPIA algorithm fell within the range of ±20% of HPLC. This algorithm developed in this study can be easily used for determination of vancomycin using TDx analyzer with vancomycin kit indirectly. It may also be useful for vancomycin therapeutic drug monitoring. Fiet et al[33] developed a serum chlormadinone acetate (CMA) time-resolved fluoroimmunoassay (TR-FIA). The detection limit was 51 pg/mL. Interassay reproducibility CVs were between 2.6% and 4.5%. This TR-FIA thus appeares to be a sensitive, specific, precise, and consequently well-suited method for measurement of serum CMA during a pharmacokinetic studies.

### 3.4. Chemiluminescent immunoassay

Chemiluminescent immunoassay (CLIA) is the combination of high sensitivity of chemiluminescence measurement techniques and high specificity of the immune response. It's an immunoassay technology that is developed following the RIA, enzyme immunoassay and fluorescence immunoassay. CLIA is currently the fastest development and application of the immune analysis methods, and the most advanced immunoassay technology, which has better sensitivity and accuracy than the enzyme immunoassay and fluorescence method in several orders of magnitude. Because of its high sensitivity and specificity, low reagent cost, reagent stability, and stability (6–18 mon.), the method is stable and fast with wide detection range, simple in operation with high degree of automation. It is replacing the traditional biopharmaceutical analysis detection technologies gradually. Especially in recent years, as a variety of CLIA automated analyzers become available, the CLIA provides broad prospects for development in biopharmaceutical analysis.

Theophylline is one of the antiasthmatic drugs most commonly used in clinic. As it is apt to result in the serious side-effect when the blood drug level exceeds 20 mg/L, monitoring the blood drug level of theophylline is necessary. Zhou et al[34] established a CLIA for quantitative determination of theophylline levels in human serum. The linear range of the CLIA method was 0.51–40 mg/L (Y = 1.02X + 0.44, $r$ = 0.995). The intra- and inter-CVs of CLIA were 3.20% and 3.57%, respectively. This method was free of interference from brilirubin (<200 μmol/L), hemoglobin (<10 g/L), and triglycerides (<15 mmol/L). They also compared this CLIA method with FPIA method, and the result showed there was no statistical significance between them ($P$>0.05). In summary, this method is simple, convenient

and precise for clinical pharmacokinetics study of theophylline. To facilitate pharmacokinetic comparisons of carboxyl-terminal B-chain analogues of human insulin, Cao et al[35] established a noncompetitive sandwich chemiluminescence ELISA. This method was validated for the quantification of carboxyl-terminal B-chain insulin analogues in human serum over a concentration range from 5 to 3125 pM. The mean bias (RE%) within the validated range varied from –10.3% to 4.3%, with an intermediate precision (inter-assay CV%) from 4.2% to 11.5%. The two-sided 90% expectation tolerance interval for total measurement error was within 25% of the nominal concentration for all levels of validation samples. Insulin lispro, human insulin, proinsulin, despentapeptide insulin (DPI) and porcine insulin displayed comparable cross-reactivity in ELISA. Potential utility of the new assay for insulin bioanalysis in non-human species was investigated by assessing the pharmacokinetic profile of DPI in rats following the administration of a single subcutaneous dose. The results indicated that the sensitive chemiluminescence detection method was simple to perform and should be readily adaptable for ELISAs of other therapeutic proteins. A quantitative, one-step, competitive enhanced chemiluminescence (ECL)-based immunoassay for the determination of a fully human anti-$TNF_{\alpha}$ monoclonal antibody in human serum was developed by Horninger et al[36]. In this method, the antibody in the test sample competed with Ru-labeled antibody for binding to an immobilized anti-variable region monoclonal antibody. A single incubation step of 2 h followed by ECL detection was used. The assay was capable of measuring the analyte in clean serum over approximately a 1600-fold range with higher concentrations measured following a single dilution. Assay accuracy, precision, and reproducibility were suitable to support pharmacokinetic studies of the analyte. This competitive assay format offers an alternative approach to the development of immunoassays for the measurement of macromolecules in complex matrices to support preclinical and clinical studies.

## 4. New immunoassay technology

### 4.1. Biosensor immunoassay

Biosensor immunoassay (BIA) is a biosensor based on the principle of immunoassay. Immune biosensing technology is a highly sensitive sensor technology with the antigen, antibody-specific reaction combined in a detection method. Compared with the traditional immunoassay methods, immune biosensors use the principle of electrophysical and electrochemical reactions. Signals from immune binding reaction are captured as electrical signals and converted to digital and amplified to detect the concentration of antigen or antibody. It's not high-throughput, but highly selective, sensitive, fast, low cost, and capable of on-line continuous monitoring in complex environments. It has broad application prospects in biopharmaceutical analysis.

Ivermectin is used at substantially lower doses than other veterinary medicines (as low as 300 μg/kg). This makes the residual detection in animal tissues very challenging. New biosensor techniques which combine very specific antibody-antigen interaction with very sensitive signal transduction offer the possibility to develop faster, more sensitive and reliable techniques that can be applied to routine monitoring programs. Samsonova et al[37] developed a rapid and sensitive immunobiosensor method for ivermectin residue determination in bovine liver. The assay development was fully validated, and the detection limit of the assay (19.1 ng/g) was calculated as the concentration corresponding to the average in 20 negative bovine liver samples minus three times the standard deviation. Within-run repeatability (CV%) was determined to be between 18.3%–21.3% and 13.5%–16.4% for concentrations of 50 and 100 ng/g, respectively. Mytych et al[38] developed a SPR-based BIA, in which epoetin alfa and darbepoetin alfa were covalently immobilized onto consecutive flow cells in a carboxymethyl dextran-coated sensor chip. Positive samples were further characterized to determine the relative concentration of the antibodies using an affinity-purified rabbit anti-epoetin alfa antibody as a reference control. The assay can detect 80 ng/mL and 100 ng/mL of antibody for epoetin alfa and darbepoetin alfa, respectively. The detection range of the assay was from 0.078 μg/mL to 10 μg/mL using a rabbit antibody. For the detection of Flumequine (Flu) residue in blood serum of broilers, Haasnoot et al[39] developed a BIA which was fast (7.5 min per sample) and specific (no cross-reactivity with other fluoro-quinolones). This assay was based on a rabbit polyclonal anti-Flu serum and a CM5 biosensor chip coated with Flu which could be detected in the range of 15–800 ng/mL. Muscle

samples were analyzed by the BIA and LC-MS/MS, and a strong correlation was found ($R^2$ = 0.998). In summary, the polyclonal antiserum-based BIA for Flu in broiler serum and muscle could be a quantitative screening assay, and it has been proven to be robust (thousands of cycles per chip), specific (no cross-reactivity with other quinolones), fast (7.5 min per sample) and, due to the simplicity of the sample preparation procedures, it is easy and suitable to use in large range of measurements (15–800 ng/mL for serum and 24–4000 ng/g for muscle), which can simply be adapted by changing the sample (extract) volumes.

### 4.2. Capillary electrophoresis immunoassay

Capillary electrophoresis immunoassay (CEIA) is a new type of immunoassay technique and it combines the effective separation power of capillary electrophoresis and the ligand specificity of immunoassay, and appears as an effective technique for complex biological compound assays. Particularly, the assay time is shorter because the immunoreaction occurring in solution is much faster as a result of solution phase kinetics compared to tradition immunoassay[40]. It still has some limitations. For example, CEIA cannot be automated completely, and a lot of work has to be completed manually. The current CEIA detection limit also mainly stays at nmol level and needs to be further improved. So far, CEIA has been employed to detect a wide range of compounds including proteins[41], pesticides and veterinary drugs[42,43] and hormones[44].

A competitive immunoassay for clenbuterol (CLB) based on capillary electrophoresis with chemiluminescence (CL) detection was reported[45]. The method was based on the competitive reaction of horseradish peroxidase (HRP)-labeled CLB (CLB-HRP) and free CLB with anti-CLB antiserum. Under the optimal conditions, the tracer CLB-HRP and the immunoassay complex were separated, and the linear range and the detection limit (S/N = 3) for CLB were 5.0–40 and 1.2 nmol/L, respectively. Thus this method could be applied to the determination of free CLB in urine samples. Multidrug resistance-associated proteins (MRPs) are of particular interest because of their ability to efflux a broad range of substrates. Since MRP1 is the most prominent member of the MRP family, a simple technique is needed for its quantification. Mbuna et al[46] developed a simple, fast (total analysis time of 3 h) CEIA for the quantification of MRP1 in cancer cells. MRP1 antibody was labeled with fluorescein isothiocyanate. The labeled antibody was incubated with the cell lysate for a fixed interval (1 h), after which the cell lysate mixture was directly injected into the capillary to separate the complex of MRP1 and its antibody from free antibody. The non-competitive CEIA method had a limit of detection of 0.2 nM and a good linear range (1.7–14.9×$10^4$ cells), and was fairly reproducible (RSD <10%). The results showed that two cell lines, A549 and RDES, expressed MRP1 in the absence of doxorubicin (DOX) and A549 was a higher expression line. The results obtained in this work indicate that the CEIA method is useful for relative quantification of MRPs in cancer cells.

### 4.3. Lateral-flow immunoassay

Diagnostic immunoassays rely on specific antigen-antibody binding for accurate results. Although there are different formats, lateral-flow immunoassay (LFIA) generally uses a hydrophobic reaction membrane strip containing bound antibodies. Specimen, treated with reagents to produce target antigen conjugates, is allowed to flow along the membrane, producing a colored line if the antigen-conjugate is captured by the bound antibodies. Result interpretation is subjective and dependent on the strength of the color signal[47]. LFIA is stable at room temperature, has a rapid turnaround time, requires very little technical skill, and can be performed with minimal laboratory infrastructure[48]. Advantages demonstrated by LFIA have made them an important tool in biopharmaceutical analysis.

Because of the potential risk of β-adrenergic agonist residues for human health and for monitoring the illegal use of them, there is an urgent need for a sensitive method for β-adrenergic agonist analyses. Zhang et al[49] have developed a colloidal gold-based LFIA for the rapid and simultaneous detection of clenbuterol (CLE) and ractopamine (RAC) in swine urine. The assay could be accomplished within 5 min without any sample preparation steps. When applied to the swine urines, the detection limit and the half maximal inhibitory concentration ($IC_{50}$) of the test strip under an optical density scanner were calculated. Results from visual evaluation of the lateral-flow tests of spiked swine urine samples showed that the cut-off values of CLE and

RAC were 1.0 and 1.0 ng/mL, respectively. Rundstrom et al[50] developed a fast lateral immunochromatographic (ICR) assay for eosinophil protein X (EPX) and human neutrophil lipocalin (HNL) as measures of the concentration of eosinophils and neutrophils in blood using europium (III) chelated-microparticles and time-resolved fluorescence. The optimized assays showed analytical detection limits below the clinical ranges of 3.36 μg/L and 2.05 μg/L for EPX and HNL, respectively. The imprecision was 3%–17% CV for EPX over the whole range and 5%–16% CV for HNL. Zhu et al[51] evaluated the use of LFIA modified with nanoparticles for simultaneous and high-sensitive detection of cardiac troponin I (hs-cTnI) and myoglobin with the aim of excluding acute myocardial infarction (AMI). Specimens from 173 patients with symptoms suggestive of AMI were collected to measure hs-cTnI and myoglobin using an electrochemiluminecence immunoassay (ECLI) and the LFIA with modified nanoparticles. The results were compared between the modified method and a commercial LFIA kit for detection of the two proteins. Consistency was observed in the quantitative comparison of 173 clinical samples using the modified LFIA and ECLI, and the modified method was more sensitive than the commercial LFIA kit. The accuracy of the modified LFIA was <12% for both hs-cTnI and myoglobin. Thus, the new approach has great potential to improve LFIAs test, demonstrating its usefulness for simple screening applications and for sensitive and quantitative immunoassays for diagnosis of AMI.

## 5. Future prospect

The most common immune analytical methods in biopharmaceutical analysis are conventional immunoassay methods. They present several drawbacks because of the nature of the antibodies. First, the sensitivity and selectivity of the immunoassay methods are less than chromatographic techniques because cross-reactions may generate deviation of determination. Secondly, an immune kit is generally only suitable for the detection of single molecules, but for several drugs in a sample simultaneously. Thirdly, as a kind of biological detection technology, immune analysis inevitably involves a variety of biological factors. Finally, there is no fully automated immunoassay technology, and most of them still require manual operation. The experimental efficiency needs to be improved. These drawbacks limit the further development of immunoassay technology. Currently, with the emergence of the element-tagged antibodies, microfluidic chip immunoassay technology, automatic immunoassay analyzer and other advanced technologies, immunoassay techniques will have a broader application prospect. Once these methods become mature, their high sensitivity and high specificity will create step change in biopharmaceutical analytical technology.

Razumienko et al[52] investigated the possibility of using element-tagged antibodies for protein detection and quantification in microplate format using inductively coupled plasma mass spectrometry (ICP-MS), and compared the results to conventional immunoassays. The advantages of ICP-MS detection for routine performance of immunoassays include increased sensitivity, wide dynamic range, minimal interference from complex matrices, and high throughput. The approach advances the ICP-MS technology and demonstrates its applicability to proteomic studies through the use of antibodies directly labeled with polymer tags bearing multiple atoms of lanthanides. Development of this novel methodology could enable fast and quantitative identification of multiple analytes in a single well. Suligoi et al[53] compared a third-generation immunoassay (AxSYM HIV 1/2 gO, Abbott Diagnostics) to a fourth-generation immunoassay (Architect HIV Ag/Ab Combo, Abbott Diagnostics, which detects anti-HIV antibody and p24 antigen) in terms of AI performance in distinguishing between recent and established HIV infections. The two assays showed the same accuracy in identifying a recent infection (91.5%), using an AI cutoff of 0.80, although Architect HIV Ag/Ab Combo was slightly more sensitive (89.4% versus 84.8%, $P>0.05$) and yet less specific (93.4% versus 97.4%, $P>0.05$). The correlation between assays was high ($r^2 = 0.87$). When 20 specimens falling in the gray zone around the cutoff point ($0.75 \leq AI \leq 0.84$) were excluded, the accuracy of AI with Architect HIV Ag/Ab Combo was 94.7%, and the concordance between the two assays was 99.2%. He et al[54] designed a polymeric microfluidic biochip to reduce assay time and lower consumption of reagents in cytokine ELISA analysis. The proper flow sequencing was achieved using the superhydrophobic capillary valves. The burst frequency of each valve was experimentally

determined and compared with two capillary force equations and the fluent finite element simulation. This fully automated microfluidic biochip with an analyzer is able to provide high fluorescence signal of ELISA with a wider linear detection range and a much shorter assay time than 96-well microtiter plates. It is applicable to a variety of non-clinic researches and clinically relevant disease conditions.

## 6. Conclusions

Immunoassay technology is a kind of analytical methods. With high sensitivity and specificity, it offers a technique to assay materials otherwise unmeasurable or hard to detect. It plays a very important role in the biological sample pretreatment, therapeutic drug monitoring and drug content determination, and it is one of the important means for in vivo drug analysis. We have reviewed immunoassay technologies commonly used in biopharmaceutical analysis including immunoextraction and immunodepletion for pretreatment of biological samples, conventional immunoassay methods and new immunoassay technologies for determination of the target drugs. With the emergence of novel advanced technologies, immunoassay techniques will have broader application prospects. Once these methods become established, their high sensitivity and specificity will bring step change in biopharmaceutical analytical technology.

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# 免疫分析方法在体内药物分析中的应用

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**摘要:** 免疫分析方法十分灵敏, 而又具有很好的特异性。当常规的药物分析方法在样本前处理或是检测环节遇到困难时, 免疫分析方法常可提供解决办法, 尤其是在涉及到生物样本的前处理或生物药物浓度检测时更为重要。本文对免疫分析方法在体内药物分析中的应用进行了综述, 包括生物样本前处理时的免疫萃取与免疫耗竭, 以及目标药物测试过程中的各种常用的及新的免疫分析方法。

**关键词:** 免疫分析; 样本前处理; 生物样本; 体内药物分析