UDC 57.083.3
ISSN 1330–4862

review

Simultaneous Determination of Several Analytes Using Immunochemical Techniques – An Overview

*Ursula Bilitewski**

Gesellschaft für Biotechnologische Forschung mbH,
Mascheroder Weg 1, 38124 Braunschweig, Germany

Received: October 27, 1997
Accepted: April 7, 1998

Summary

Immunoanalysis is based on the recognition of analytes by antibodies with the determination being done either through suitable labelled compounds or directly using affinity sensor systems. Different analytes can only be distinguished by different antibodies requiring the development of multi-channel assays or sensors, which allow monitoring of the interactions of each single antibody. An obvious approach was the incubation of the sample with a mixture of all relevant antibodies together with the corresponding tracers. For each analyte another label had to be used to allow the independent analysis of the different affinity reactions. This approach was, however, limited by the number of tracers which could be distinguished within one assay under identical conditions. Alternatively, the different affinity reactions were spatially separated during the analytical procedure, which led to the applicability of only one label for all analytes. The determination of the different analytes was achieved by a corresponding spatially resolved signal transduction. As a consequence, the number of analytes, which could be determined simultaneously, was limited by the degree of spatial resolution which could be achieved by the coupling procedure, the tolerable complexity of the resulting system and the degree of spatial resolution achievable by the signal transduction device.

Keywords: combination of several labels, spatial separation of affinity reactions, enzyme immunoassays, fluorescent immunoassays, immunosensors

Introduction

Immunoanalytical techniques are usually applied to the specific determination of single analytes in even complex matrices, such as serum, urine or food. The specificity of these techniques originates from the specificity of the antigen-antibody-binding which is a non-covalent binding. Van der Waals forces, electrostatic and steric features lead to the ideal fit between the epitope on the antigen and the antigen-binding-site of the antibody *(1–3)*. In most of the routine well-established immunoassays one of the reaction partners, either the antigen or the antibody, is bound to a solid phase, for example the wells of a microtiter plate, the sample is added together with a labelled compound and incubated, unbound compounds are removed by washing and the signal is recorded either directly (radioactive or fluorescent labels) or after addition of suitable enzyme substrates (enzyme label). This basic concept led to various assay formats, depending on the analyte (*e.g.* whether it is the antigen or the antibody and whether only one or more epitopes are available), how separation of bound and unbound compounds is achieved (homogenous *vs.* heterogeneous assays) and whether a labelled antigen (competitive assay) or a labelled secondary antibody (sandwich assay) is used. To describe all these basic concepts in detail is beyond the scope of this article and the reader is referred to corresponding books and review articles *(1–5)*. Common to all these assays is the use of a single antibody and a single tracer, as one analyte is to be determined.

However, in some applications samples have to be investigated with respect to more than one analyte:

- In medical diagnosis of certain diseases or for screening purposes several analytes have to be determined in a single sample. For example, the levels of different hormones related to the human reproductive systems have to be determined to allow the

* E-mail: ubi@gbf-braunschweig.de

diagnosis of pathological abnormalities *(6,7)*, water samples are analysed with respect to several pollutants *(8,9)*, and urine or serum samples are screened for several drugs of abuse *(10)*. For each of these analytes another antibody is to be used, thus several immunoassays have to be performed on a single sample.

- Antibodies usually recognize only part of the analyte molecule, the so-called epitope. This epitope may be present on different compounds leading to cross-reactions of the antibody *(3,9,11)*. In the immunoassay all cross-reacting compounds present in the sample will bind to the antibody (Fig. 1, *(12)*), and consequently the quantification of a single compound is not possible anymore. The number of compounds and the corresponding degree of antibody-binding is described by the cross-reactivity pattern of the antibody. If it is possible to obtain several antibodies differing in cross-reactivities to a given class of analytes, their combination may allow a distinction, at least a classification, of analytes. However, data analysis has to be performed using statistical models *(9)* or mathematical models of pattern recognition, like principle component analysis (PCA) *(13)*, neural network analysis *(14)*, maximum likehood estimation (MLE) *(15)* or cluster analysis *(15)*.

These practical requirements led already almost 20 years ago to the development of immunoassay formats in which several affinity reactions were performed simultaneously on a single sample. In this paper an overview will be given of the basic principles of these multi-analyte-immunoanalytical formats together with short discussion of the respective perspectives and limitations. The different approaches will be illustrated by representative examples, but not all experimental details will be mentioned so as not to extend this contribution too much. For more details the reader is referred to the literature.

Classical immunoanalytical techniques rely on the use of labelled compounds. Well-established markers are radioisotopes, fluorophores and enzymes *(2)*. If several immunological reactions have to occur within one sample, they must finally be distinguished from each other in order to lead to the desired results. This distinction can be achieved by two basically different concepts, which are illustrated in the following chapters:

The classical approach for multi-analyte immunoassays is the use of a specific label for each analyte followed by its specific determination. All antibodies and tracers are incubated simultaneously in the sample and the different antibody-tracer-complexes are distinguished by characteristic features of the respective labels. This was shown to be applicable to the simultaneous determination of up to three analytes with enzymes, radioisotopes, fluorophores or metal ions as labels. The extension of this approach is limited by the number of labels which can be distinguished in a common solution.

As an alternative, common labels are used for all analytes. As a prerequisite spatial separation of the immunoreactions is required together with an appropriate spatial resolution of the detector. Corresponding assays were performed as enzyme-linked immunoassays in microtiter plates or in automated or manual immunosensor systems. Improvement of technical possibilities leads to investigations with respect to the applicability of immunosensor systems, in which either fluorescent markers in combination with optical fibres or no markers are used. The latter systems are based on direct monitoring of the antigen-antibody-binding by suitable physical, mainly optical, transducers. This approach of spatial separation

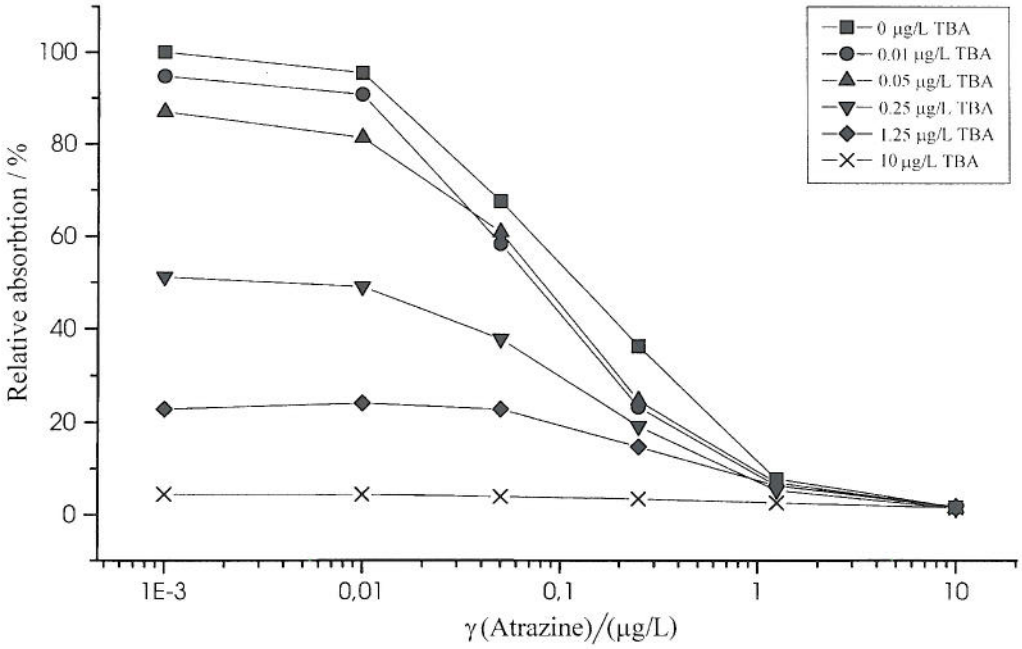

Fig. 1: B/B_0 data obtained with a microtiter plate ELISA based on the monoclonal antibody K4E7 for mixtures of the s-triazines, atrazine and terbuthylazine (TBA), which both bind to the antibody.

of the immunoreactions is not limited to any type of marker or assay format but by the complexity of the resulting systems and the achievable degree of resolution of the coupling procedure and the detector.

In this paper some of the above mentioned concepts are described in more detail, starting with the classical approach of analyte-specific-labels, focussing however on systems based on spatially separated reaction zones, as this approach is of major importance for the development of immunosensor-arrays.

Simultaneous immunoassays using analyte-specific labels

Several analytes can simultaneously be determined in a single sample by the addition of all immunoreagents to the sample using a specific label for each analyte, followed by the separation of bound and unbound tracers and finally the determination of each bound tracer. These combined assays are only possible, if the antibodies used in the different assays are not influenced by the presence of the others. Moreover, suitable labels and derived tracers have to be chosen, which means that the determination of each label must be possible in the presence of the others and without their interference, and each tracer should bind only to the corresponding antibody. Mainly the requirement, that different labels have to be determined specifically under almost identical conditions, limits the extension of this approach.

An assay for the simultaneous determination of lutropin and follitropin was described using the radioisotopes ^{57}Co and ^{125}I as labels, which have different scintillation energies and can be discriminated by gamma-counters *(7)*. Each of the hormones was labelled with another of these two isotopes, thus competing with the hormones present in the sample for the binding sites of the added antibodies. Free ligand was separated from the bound by use of a goat anti-rabbit antiserum and a precipitation accelerator. As the detector could distinguish between the two isotopes, each of the analytes could be determined.

Comparable principles were used, when proteins were labelled with metal ions through the use of bifunctional chelating agents. The proteins could be either the antigens *(16,17)* or the antibodies *(18)*, *i.e.* the assays were performed as competitive and non-competitive assays, respectively. Those assays were mainly developed for medical applications and used for serum analysis. They were based on general common principles and thus, the following descriptions should be taken as representative examples:

Myoglobin (Mb) and carbonic anhydrase III (CA III) were simultaneously determined in serum using fluorescent chelates of europium (Eu^{3+}) and samarium (Sm^{3+}) as analyte-specific markers *(16)*. Mb was labelled with Sm^{3+}, CA III with Eu^{3+} and both tracers were incubated together with Mb- and CA III-specific polyclonal rabbit antibodies with the serum sample or protein standard. Bound and non- bound ligands were easily separated by washing, as the immunoreactions took place in microtiter plates coated with anti-rabbit antibodies leading to a binding of the analyte-specific antibodies. An enhancement solution containing 2-naphthoyl-trifluoroacetone dissociated the lanthanides from the proteins into the solution where they formed highly fluorescent chelates with emission peaks at 613 nm for Eu^{3+} and at 643 nm for Sm^{3+}. Using a time-resolved fluorometer both compounds could easily be distinguished.

The same detection principle was described by Hemmilä *et al. (18)* for the simultaneous non-radioactive determination of lutropin and follitropin. However, they labelled the specific anti-β-chain antibodies with Tb^{3+} and Eu^{3+}, respectively, and used microtiterplates coated with anti-β-chain antibodies as common capture antibody. Thus, the assay was performed as non-competitive assay. The enhancer to be used for this combination was a β-diketone (pivaloyl trifluoroacetone) and the chelates could be distinguished through their emission peaks at 614 nm for Eu^{3+} and at 544 nm for Tb^{3+}.

Differences in the electrochemical properties of metal ions were utilized by Hayes *et al. (17)*, who developed a simultaneous immunoassay for human serum albumin (HSA) and immunoglobulin G (IgG) using In^{3+} and Bi^{3+} as nonisotopic metal ion labels. The assay was performed as competitive assay with a mixture of both analyte-specific antibodies coated on the surfaces of polystyrene tubes. After the immunoreaction the metal ions were released from the proteins by 1.0 M hydrochloric acid, and this acidic solution was transferred to an electrochemical cell, where the ions were detected by differential pulse anodic stripping voltammetry at a hanging mercury drop electrode.

In all cases, simultaneous determinations of the metal ions were only possible because conditions could be found, which allowed the simultaneous efficient release and complexation of both ions, combined with differences in the fluorescence spectra or the electrochemical behaviour, respectively.

The situation is even more complex, if enzyme immunoassays for several analytes are to be combined. The selection of the enzymes involved in a common assay is an important step, as they have to fulfil certain criteria *(19)*:

- they should be readily available and have high turnover numbers;
- they must be stable under simultaneous assay conditions and not susceptible to interference from another enzyme reaction;
- they must have a similar optimum pH;
- the products of the enzyme reactions are to be distinguished clearly, *e.g.* by not-overlapping absorption spectra.

The enzyme pairs β-D-galactosidase / alkaline phosphatase *(19)* and glucose-6-phosphate dehydrogenase / β-galactosidase *(20)* fulfil most of these criteria and corresponding simultaneous enzyme immunoassays were described. For simultaneous triiodothyronine- and thyroxin-determination *(19)* and biotin- and vitamin B_{12}-determination *(20)*, respectively, competitive assays were developed which were performed either with a homogeneous immunoreaction and precipitation of the immunocomplexes by second antibodies *(19)* or with a heterogeneous immunoreaction with the specific binding proteins immobilized to Sepharose 4B *(20)*. The enzyme conju-

gates were added together with the sample to the antibodies and after removal of unbound tracers the substrates of both enzymes were added. The enzyme reactions of alkaline phosphatase and β-D-galactosidase were distinguished with phenolphthalein monophosphate (540 nm) and *o*-nitrophenyl-β-galactoside (420 nm) as enzyme substrates *(19)*. The distinction of β-galactosidase and glucose-6-phosphate dehydrogenase was possible with *o*-nitrophenyl galactopyranoside (415 nm) and glucose-6-phosphate / β-nicotinamide adenine dinucleotide (347 nm), respectively, as enzyme substrates *(20)*. In both examples there was no interference of one enzyme reaction by the presence of the substrates of the other. Thus the simultaneous assays could be performed with similar accuracy and sensitivity as the single assays.

Though all these principles can be widely varied with respect to assay format and detection principle and though the performance does not require additional steps compared with the single-analyte assay besides mixing of reagents, the further improvement of this approach is stagnant at present, as it is limited in the number of analytes / antibodies used within one assay. The number of labels which can be determined reliably within a common solution is limited, independent on the type of label, as in any case compromises between different optimum conditions have to be found.

That is why at present more effort is put on immunoassay-arrays based on spatially separated immunoreactions, even if first reports on this approach originate already in the early 1980s (*e.g. 21*).

Systems based on spatially separated immunoreactions

As mentioned above, the number of labels which can be used simultaneously has been limited up till now to two or three. Moreover, change of a label together with a possibly required change of assay conditions may lead to a deterioration of the assay performance. Therefore, possibilities to use common labels for several analytes were evaluated. This, however, requires the spatial separation of the immunoreactions together with a separated analysis of the reaction zones. In an early report *(21)* on a multi-analyte assay system two different antibodies were immobilized on different halves of a reaction tube, the sample was added together with the two analyte-specific tracers labelled with the same radioisotope ^{125}I. The two halves were separated again and counted separately *(21)*.

As RIAs are avoided nowadays as much as possible, alternatives are under development with this early basic concept being modified and adapted to technical possibilities and requirements.

Microtiter plate enzyme immunoassays

A routine format of immunoassays, mainly in centralized analytical laboratories, is the heterogeneous enzyme immunoassay performed in 96-well microtiter plates. Each well is separated from the other, which allows the simultaneous investigation of a number of samples even in duplicate or triplicate together with corresponding standards. Data for each well are obtained at the end of the assay using multi-channel photometers or, nowadays, also fluorimeters. Utilising this built-in separation of the different wells leads to multi-analyte assays by coating different wells in a microtiter plate with different antibodies, reducing, of course, the number of samples which can be investigated on one plate. If large numbers of samples are to be analysed, separate assays for each analyte are, hence, preferred, and only some descriptions of combinations of assays performed in microtiter plates are found in literature. They are mainly those where same conditions for the different immunoreactions were more important than a high sample throughput. The application of different immunoreactions to one sample in one assay results in a pattern indicative for a characteristic of this sample. For example, the ELISA-diagnosis of Lyme disease is based on the detection of antibodies which are formed after an infection by *Borrelia burgdorferi (Bb)* against *Bb* antigens *(22)*. To avoid the use of whole proteins which may result in false positive signals due to cross-reactivities of antibodies, chemically synthesized peptides were used representing the epitopes on the proteins recognized by the antibodies. These peptide-epitopes were found from screening with ELISA-formats peptide libraries representing the amino acid sequences of four different proteins, which were found through Western blotting of Lyme disease sera. Typical patterns were obtained showing the epitops of each protein. Eight epitope peptides were chosen for further use. In the normal ELISA format the antigen is adsorbed to the plate, which is not possible with small peptides. Therefore, the epitope peptides were prolonged with additional residues of β-alanine and cysteine as spacer and to allow coupling to bovine serum albumine (BSA). The BSA-conjugates of each peptide were applied to separate wells of the ELISA plate. Unknown serum samples were investigated with respect to the presence of antibodies to each of these peptide epitopes. It was found that none of the peptides alone allowed diagnosis of the disease with 100% correlation to the clinical signs. Using, however, positive indication by at least two peptides improved the reliability, but could not exclude false positive diagnosis completely *(22)*.

Another example of pattern analysis is the application of ELISAs to pesticide determination. Antibodies used for the determination of pesticides in environmental smaples usually show cross-reactivities to other compounds of a similar chemical structure, because pesticides cannot induce an immune response by themselves, but only when they are coupled to carrier proteins *(23–25)*. A wide range of antibodies is formed in response to immunization with the immunogen and only a few recognize the part of the immunogen representing the analyte. All compounds sharing the structural elements which are accessible for the antibodies will cross-react. Thus, the structure of the immunogen strongly influences the specificity of the resulting antibodies *(23,26)*. This is of practical importance, for example, for the determination of s-triazine herbicides *(15,24,25,27)*, where a number of chemically related compounds are in practical use as herbicides, and also the metabolites still possess some of the major residues on the triazine ring. In these situations the application of a single antibody only al-

lows quantification in terms of equivalents of the main analyte of the antibody, but does not allow quantification of a completely unknown compound or even of a mixture of compounds. Additional information can be obtained by assaying a sample with a number of antibodies against the chemical class of compounds, *e.g.* the s-triazines, but of different cross-reactivities. Interpretation of the results was done using response paths for two antibodies *(9)* (see description below, Fig. 2) or, if more antibodies are used, by an extended four-parameter logistic model *(9,27)* or mathematical methods of pattern recognition, such as principle component analysis (PCA) *(13)*, maximum likelihood estimation (MLE) *(15)*, cluster analysis *(15)* or neural networks *(14)*. This allowed within a limited concentration range at least classification of the compound present in an unknown single analyte solution and also analysis of binary mixtures of analytes. The basic idea is illustrated in Fig. 2, where signals obtained from competitive ELISAs based on only two different monoclonal antibodies for solutions of different concentrations of different triazines were combined (see also *(9)*). Due to the assay format (competitive ELISA) low analyte concentrations lead to high signals, and high affinity antibodies are characterized by low analyte concentrations leading to a decrease of the signal. For each triazine a typical curve (response path *(9)*) was obtained influenced by the ratio of the affinities of these two antibodies for the given compound. The affinities of the antibody K4E7 (obtained from Prof. B. Hock, Dr. T. Giersch, TU Munich, Germany, *(24)*) for the s-triazines, atrazine and propazine, were higher than of the antibody K1F4 *(28)*. Therefore the corresponding typical curves were placed in the upper part of the diagram. Both antibodies showed comparable affinities for terbuthylazine, as a consequence the resulting curve divided the diagram approximately in halves. As K1F4 had a higher affinity for terbutryne than K4E7, the typical curve for this compound was placed in the lower part of the diagram (Fig. 2). Each unknown solution could be analysed again with these two antibodies giving a point in this diagram. The compound present in the sample could be identified through the curve on which it was placed. Thus, using only two antibodies allowed a classification of compounds at least in three groups according to the three different possible ratios of affinities (group I: high affinity to antibody 1 and low affinity to antibody 2, *e.g.* atrazine and propazine in Fig. 2; group II: low affinity to antibody 1 and high affinity to antibody 2, *e.g.* terbutryne in Fig. 2; group III: similar affinities to both antibodies, *e.g.* terbuthylazine in Fig. 2). It is obvious that this model can only work within the linear ranges of the assays, because at very low and at high saturating concentrations all compounds give the same results with all antibodies. The more distinct the typical curves for the different compounds are, the easier is the identification of the compounds. However, the more compounds have to be considered, the more difficult the distinction is. For example, in the above mentioned system propazine and atrazine could not be distinguished, because both compounds differ only in one residue of the triazine ring, which was removed for the preparation of the immunogen *(24,28)* and thus both antibodies showed similar affinities to both compounds. Moreover, due to the combination of data of independent assays the experimental errors multiply, leading to a reduced accuracy and the need for more standards

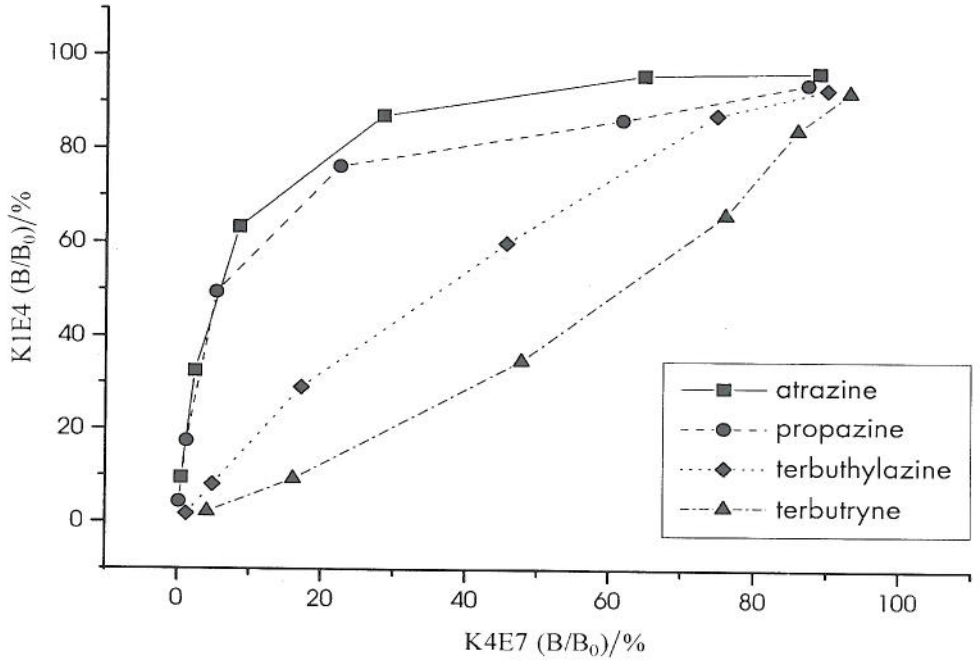

Fig. 2: B/B_0 data obtained in microtiter plate ELISAs based on the monoclonal antibodies K4E7 and K1F4 using different concentrations of some s-triazines. The concentration range was from 0.01 μg/L to 10 μg/L. Low concentrations lead to high B/B_0 data due to the competitive assay format. The antibodies were obtained from Prof. B. Hock, TU Munich, FRG.

to be run on the same microtiter plate. This reduces again the number of samples which can be analysed. Therefore, efforts are required to improve the reliability of microtiter plate assays to allow at least the application of single standard curves to several microtiter plates *(29)*.

Automated immunoassay formats

Immunoassays performed in microtiter plates are designed for the analysis of large numbers of samples using enzyme tracers. High sample throughput is achieved as 96-well plates are used and pipetting and incubation of solutions can be highly automated.

Some of these properties were used also in the Abott Prism described in 1991 by O. S. Khalil for donor screening in blood banks, allowing simultaneous determination of 6 analytes *(30)*. The sample was split between reaction trays in a group of linear tracks. Timing belts moved the trays along the tracks to 9 different operational positions, which could be used for the performance of assay steps. The system was designed for heterogeneous chemiluminescence immunoassays in which the analyte was captured by appropriate coating on microparticles. Hence, not-bound tracer and excess fluid could easily be removed by washing steps and absorbent pads in the trays. Assay protocols for different analytes were designed by choosing assay modules for a suitable assay format (e.g. competitive or sandwich immunoassay) and moving assay modules between the operational positions. As an automated pipettor was integrated in the system all assays were completely automated though a sufficient flexibility was maintained to allow adaption to alternative analytes. A maximum sample throughput of 150/h delivering 900 data/h was achieved.

Automation is not only required for analysis of large numbers of samples, but also for continuous monitoring of analyte concentrations. Whereas in central laboratories automated analysers comprise robots and pipettors controlled by suitable software, on-line monitoring of compounds is achieved by automated flow-through systems based, for example, on the principles of flow injection analyis (FIA). Integration of reaction chambers, mixing devices, various types of valves and pumps delivering constant flows of reagents allow the design of systems in which ELISA-protocols are automatically performed *(31)*. The immunoreaction usually takes place in affinity columns through which samples, tracer and enzyme substrates are pumped. These systems can be extended to multi-analyte systems either by the choice of universal affinity reactions, such as the biotin-avidin-reaction *(32)* or the protein A (or protein G)–IgG-reaction, and subsequent application of different antibodies, which leads to a multiplication of the total analytical procedure, or by parallel arrangement of several affinity columns *(33)*. A possible set-up is shown schematically in Fig. 3. The parallel arrangement of the affinity columns allowed different immunoassays to be performed partly in parallel, whereas the detection occurred serially as only one detector was used. Nevertheless, total assay time was reduced significantly compared with a totally serial performance of several assays. However, increasing the number of analytes increases the complexity of the system as not only an increasing number of reactors has to be integrated in the system, but also the number of tracers increases even if a common label is used, as the part of the tracer which is bound by the antibody is antibody-specific.

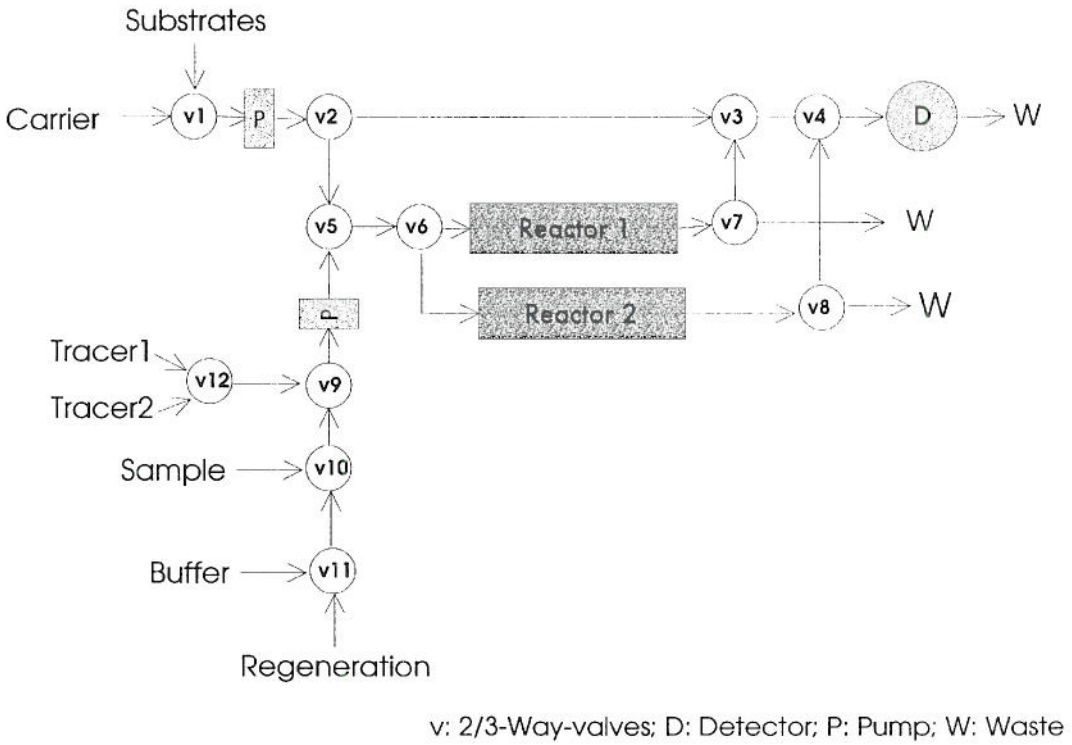

Fig. 3. Schematic diagram of a 2-channel flow injection immunoanalysis system

Manual immunoassay formats

Rapid and simple immunoassays are required for on-site determination of compounds, where neither exact pipetting nor long incubation times of components are possible, and the analysis of single samples has to be performed in contrast to a high sample throughput or quasi-continuous monitoring of compounds. In corresponding assays enzyme tracers usually are replaced for example by coloured or fluorescent tracers, which reduces the number of required reagents and incubation steps, as no enzyme substrates are necessary.

An assay which is comparable to conventional sandwich microtiter plate assays with the detection being based on a fluorescent europium chelate as label was described by Kakabakos *et. al. (34)*. They used monoclonal antibodies to the intact lutropin, the β-subunit of follitropin, the β-subunit of choriogonadotropin and to prolactin, coated microtiter strips separately with these antibodies, isolated the wells and used one of each type in combination on a plastic stick. The stick was incubated in a mixture of the sample with four different biotinylated detection antibodies. After aspiration of this solution a streptavidin-europium-chelate-tracer was added and the fluorescence of each well was quantified on the dry solid phase with laser-excited time-resolved fluorometric measurements.

This assay format does not require special equipment besides the fluorescent detector. However, as the advantage of microtiter plate assays to run a standard curve together with the unknown sample cannot be utilised anymore, R. P. Ekins suggested another format with an in-built standardization *(35,36)*. Immunoassays essentially rely on the measurement of the antibody occupancy by the analyte, which is reflected by the interpretation of data in terms of normalized data, *e.g.* B/B_0 *(1)*. This is achieved either by tracers binding to another binding site of the analyte than the primary antibody (sandwich-format) or by tracers binding to the non-occupied binding sites of the antibodies (competitive assays). Usually the total amount of binding sites is correlated with the signal obtained with a saturating concentration of analyte (sandwich-format) or without analyte (competitive format), respectively. In microspot multi-analyte ratiometric immunoassays the use of two fluorescent labels is suggested *(35,36)*. The first label is bound to the specific antibody coated in microspots on a plastic support, thus indicating the total amount of capture antibody. The second label is conjugated to another antibody (sandwich-format) or to an analyte-analogue (competitive-format). Thus, the ratio of both signals is representative of the occupancy of binding sites by the analyte and allows the determination of the analyte concentration. As only minimum amounts of antibodies are required multi-analyte set-ups are possible. However, the detector suggested was a confocal fluorescence microscope.

A screening system considering also the requirements of a simple and cheap detection was introduced as the Advisor™ drug screening system *(10)*, in which conjugates of drugs of abuse were located in different channels on a disposable element defining the specificity of each channel. The system was designed for the simultaneous screening for five different classes of drugs of abuse (amphetamines, cannabinoids, cocaine metabolites, opiates, phencyclidine). Antibodies and coloured antibody-coated microparticles were added to the sample, and this mixture was placed on the test card. In the absence of drugs the antibodies, conjugates and coloured particles formed agglutinates which could easily be detected due to the colour of the microparticles. Typical pattern of coloured bands indicated the kind of drug found in the sample.

Immunosensor systems

Immunosensor systems are distinguished in indirect and direct sensor systems according to their principle of detection. The so-called indirect sensor systems are based on similar principles as conventional immunoassays, *i.e.* the detection itself is based on the determination of labelled compounds, namely of enzyme tracers or fluorescent tracers. The difference to immunoassays is the immobilization procedure, as in immunosensor systems compounds are usually attached covalently to solid supports, the choice of suitable immobilization matrices and usually the regeneration of the binding sites. Immobilization is done on polymeric or glass beads, which are integrated via affinity columns in automated flow-through sensor systems (see Fig. 3), or on the transducer surface, *e.g.* electrodes *(37,38)* or optical fibres *(39,40)*. In this respect the flow injection analysis systems, which were already described above (Fig. 3), are also to be considered as sensor systems. With the affinity reactants being immobilized directly on the transducer surface the reaction chamber and detector are integrated leading to more compact systems with the potential to further miniaturization. However, reagents, such as tracers and enzyme substrates, are often applied manually. Electrochemical detectors are used in combination with enzyme tracers, with horseradish peroxidase and alkaline phosphatase being suitable enzymes as the amount of enzyme can be determined via enzyme substrates leading to electrochemically active reaction products. Multi-channel systems were based, for example, on systems comparable to microtiter plates, with the electrode being part of the reaction well and the antibodies or analyte being immobilized on the electrode surface *(37)*. Immobilization was done using adsorption on nitrocellulose membranes or covalent binding with cross-linking reagents after silanization of the electrode with aminopropyl triethoxysilane (APTS). The assay principle was a competitive enzyme immunoassay with the antibody or the analyte *(37)* being coupled to the enzyme. Up to now those systems have been used comparable to conventional ELISAs, *i.e.* for the determination of a single analyte (2,4-dichlorophenoxyacetic acid). However, they can be extended, again as conventional microtiter plate ELISAs, to multi-analyte determination. A step further with respect to miniaturized systems was described by Pritchard *et al. (38)*, who used gold working electrodes fabricated by standard photolithographic procedures on silicon wafers as transducers and immobilized antibodies through site-directed activation of photo-biotin via illumination of selected areas through corresponding masks. The immunoassay was performed as a standard sandwich-type ELISA for the simultaneous determination of the two

hormones, follicle stimulating hormone (FSH) and luteinising hormone (LH), using horseradish peroxidase as enzyme label and hydrogen peroxide together with ferrocene monocarboxylic acid as enzyme substrates. The enzyme activity was determined chronoamperometrically with two potentiostats at +150 mv for 20 s.

In the case of fluorescent tracers, affinity reactants were immobilized on optical fibres and binding of the fluorescent compound was monitored. Combination of several single fibres to a fibre bundle allowed multi-analyte determination *(39)*.

The interactions of antigens and antibodies can be monitored directly, on-line and in real-time without the need for tracers by suitable physical transducers, such as piezoelectric crystals monitoring the increase of mass during the formation of the affinity complex *(41)*, electrodes whose capacitance changes due to protein binding *(42)* and optical waveguides allowing observation of protein binding through changes of the refractive index at the surface of the waveguide *(43)*. At present, the optical techniques are the best established and a variety of instruments are commercially available. They are based on different physical effects which are all related to the effective refractive index of the layer surrounding the waveguide through the evanescent wave of a laser beam which is totally reflected at the phase boundary of the optical transducer. Examples are the surface plasmon resonance (SPR), which occurs in thin gold layers on top of a waveguide at a well-defined reflection angle *(44)*, and the incoupling efficiency in planar waveguides at different incoupling angles using grating coupler systems *(43)*. In SPR-systems polarised light is focussed on the phase boundary between the glass substrate and the gold layer and the reflected light is detected with a diode-array allowing resolution of reflection angles down to 0.1°. A four-channel system was introduced by a correspondingly structured flow system allowing the performance of four independent measuring cycles *(45)*. The grating coupler system operating in the reflection mode *(46)* was extended to a multi-channel system by integration of a vertical positioner with stepmotor *(47)*. This allowed the grating coupler to be moved relative to the laser beam. The laser was focussed on a specific part on the grating region of the waveguide and the reflected light bundle was detected by a CCD-camera, delivering a minimum signal at a defined angle. Vertical movement of the waveguide in the flow cell allowed scanning of the waveguide surface and hence analysis of discrete areas on the waveguide surface. The system was designed for the analysis of a single sample with respect to several affinity reactions with the number of channels being limited on the one hand by the optical resolution of the laser beam and the reflected light, and on the other hand by the resolution achieved by the immobilization conditions.

Conclusions

Immunoanalytical techniques find increasing acceptance in various fields of application, as they allow the specific determination of analytes in even complex samples, such as serum, blood, food, at low concentrations. Moreover, the performance can be simplified so that the assays can be performed on site without the need for expensive instrumentation and sample pretreatment. However, usually more than one analyte has to be determined, multiplying the number of assays to be performed, whereas instrumental chemical analytical methods, such as HPLC or GC, in general are suitable for the analysis of mixture of compounds. Therefore, different approaches to combine several immunoassays into a common assay were investigated and some even resulted in commercially available products. The upper limit of analytes, which can be determined simultaneously, at present is in the order of 5 to 6, which is restricted on the one hand by the needs of the application, and on the other hand by technical limitations. An increasing number of possible analytes usually increases the complexity of the resulting system, including not only hardware but also data acquisition and interpretation and methods such as neural network analysis may become necessary. Moreover, immunoassays are characterized not only by their lower limit of detection but also by their upper limit of detection. The useful analytical range is often in the range of two orders of magnitude. A combination of assays is only reasonable, if all assays can be performed with the same degree of dilution of the sample, *i.e.* the analytical ranges of the assays and the concentrations of the corresponding analytes in the samples must fit.

In spite of these practical limitations, increasing research efforts are put into the development of affinity sensor arrays. Driving forces for these efforts are not the conventional immunoanalytical applications but new fields of applications of affinity reactions. Due to the progress made in combinatorial chemistry, libraries of compounds are available, which are used for screening purposes with respect to binding to the target molecules of interest. The applications range from epitope mapping of antibodies, identification of binding regions on proteins, to interactions between proteins and DNA and hybridisation of single-stranded DNA. Through structured immobilization of a complete library on a sensor surface, all binding events can be monitored simultaneously, thus reducing the required experimental effort significantly. However, suitable immobilization strategies together with adapted detection systems are still under development and only some examples of high-density arrays together with a fluorescent detection are known, mainly developed for medical diagnosis based on genetic disorders *(48,49)*. Spatially resolved immobilization is usually achieved by the use of photoaffinity labels and photolithography.

High-density arrays of oligonucleotides are obtained by light-directed synthesis of the probes directly on the sensor surface leading to the so-called DNA-chips. This approach is not suitable for the immobilization of whole proteins, such as antibodies or F_{ab}- fragments. For these applications immobilization procdures based on photoactive crosslinking reagents were developed, one example being the already above mentioned use of photobiotin *(38)*. Other compounds which can be activated by irradiation, such as benzophenone *(50)* or trifluoromethyl-aryldiazirines *(51)*, were also used for coupling of proteins to solid supports. Spatially resolved de-

tection of the affinity reactions is often achieved with confocal fluorescence microscopes requiring the use of fluorescent tracers. At present, the achievable degree of spatial resolution and lower detection limit of direct sensing systems have not been evaluated yet.

References

1. P. Tijssen: Practice and Theory of Enzyme Immunoassays, in: *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 15, Elsevier, Amsterdam (1985).
2. D. Catty: *Antibodies a Practical Approach*, IRL Press at Oxford University Press (1989).
3. A. M. Campbell: *Monoclonal Antibody and Immunosensor Technology*, Elsevier, Amsterdam (1991).
4. J. R. North, *Trends in Biotechnol. 3* (1985) 180.
5. E. Liddell, I. Weeks: *Antibody Technology*, BIOS Scientific Publishers Ltd. Oxford (1995).
6. R. Sitru-Ware, *Drugs 39* (1990) 203.
7. F. H. Wians, J. Dev, M. M. Powell, J. J. Heald, *Clin. Chem. 32* (1986) 887.
8. C. Barzen, A. Brecht, A. Klotz, G. Gauglitz, *Sensor 97, Kongressband III* (1997) 231.
9. G. Jones, M. Wortberg, B. D. Hammock, D. M. Rocke, *Anal. Chim. Acta 336* (1996) 175.
10. R. G. Parsons, et al. *Clin. Chem. 39* (1993) 1899.
11. M. G. Scott, *Trends in Biotechnol. 3* (1985) 170.
12. U. Bilitewski: *Kombination von enzymatischen und immunologischen Einzeltests zu Testsystemen*, Papierflieger, Clausthal-Zellerfeld (1995).
13. P. Y. K. Cheung, L. M. Kauvar, A. E. Engqvist-Goldstein, S. M. Ambler, A. E. Karu, L. S. Ramos, *Anal. Chim. Acta, 282* (1993) 181.
14. C. Wittmann, R. D. Schmid, S. Löffler, A. Zell, *ACS Symp. Ser. 657* (1997) 343.
15. M. Wortberg, G. Jones, S. B. Kreissig, D. M. Rocke, S. J. Gee, B. D. Hammock, *Anal. Chim. Acta, 319* (1996) 291.
16. J. Vuori, S. Rasi, T. Takala, K. Väänäen, *Clin. Chem. 37* (1991) 2092.
17. F. J. Hayes, H. B. Halsall, W. R. Heineman, *Anal. Chem. 66* (1994) 1860.
18. I. Hemmilä, S. Holtinen, K. Pettersson, T. Lövgren, *Clin. Chem. 33* (1987) 2281.
19. C. Blake, M. N. Al-Bassam, B. J. Gould, V. Marks, J. W. Bridges, C. Riley, *Clin. Chem. 28* (1982) 1469–1473.
20. J. M. Buckwalter, X. Guo, M. E. Meyerhoff, *Anal. Chim. Acta, 298* (1994) 11.
21. R. Vikho, L. Koskinen, *Clin. Chem. 27* (1981) 1744.
22. Z. Yu, J. M. Carter, L. H. Sigal, S. Stein, *J. Immunol. Methods, 198* (1996) 25.
23. M. Vanderlaan, B. E. Watkins, L. Stanker, *Environ. Sci. Technol. 22* (1988) 247.
24. T. Giersch, *J. Agric. Food Chem. 41* (1993) 1006.
25. C. Wittmann, B. Hock, *Food Agric. Immunol. 1* (1989) 211.
26. E. P. Meulenberg, W. H. Mulder, P. G. Stoks, *Environ. Sci. Technol. 29* (1995) 553.
27. G. Jones, M. Wortberg, S. B. Kreissig, D. S. Bunch, S. J. Gee, B. D. Hammock, D. M. Rocke, *J. Immunol. Methods, 177* (1994) 1.
28. T. Giersch, B. Hock, *Food Agric. Immunol. 2* (1990) 85.
29. G. Jones, M. Wortberg, S. B. Kreissig, B. D. Hammock, D. M. Rocke, *Anal. Chim. Acta, 313* (1995) 197.
30. O. S. Khalil, et al., *Clin. Chem. 37* (1991) 1540.
31. P. M. Krämer, R. Kast, U. Bilitewski, S. Bannierink, U. Brüss, *ACS Symp. Ser. 657* (1997) 133.
32. M. Stiene, U. Bilitewski, *Analyst, 122* (1997) 155.
33. R. Kast, U. Bilitewski, *German Russian Summer School, Anal. Biotechnol.* (1996).
34. S. E. Kakabakos, T. K. Christopoulos, E. P. Diamandis, *Clin. Chem. 38* (1992) 338.
35. R. Ekins, F. Chu, E. Briggart, *Anal. Chim. Acta, 227* (1989) 73.
36. R. P. Ekins, F. Chu, *TIBTECH, 12* (1994) 89.
37. P. Skladal, T. Kalab, *Anal. Chim. Acta, 316* (1995) 73.
38. D. J. Pritchard, H. Morgan, J. M. Cooper, *Anal. Chim. Acta, 310* (1995) 251.
39. D. Bock, A. Hartmann, S. Kaul, M. Martin, V. Schulz, J. Wulfrum, S. Seeger, *SPIE 2629* (1993) 96.
40. F. F. Bier, W. Stöcklein, M. Böcher, U. Bilitewski, R. D. Schmid, *Sensors and Actuators B 7* (1992) 509.
41. J. Ngeh-Ngwainbi, A. A. Suleiman, G. G. Guilbault, *Biosens. & Bioelectr. 5* (1990) 13.
42. A. Gebbert, M. Alvarez-Icaza, W. Stöcklein, R. D. Schmid, *Anal. Chem. 64* (1992) 997.
43. K. Tiefenthaler, *Adv. Biosensors, 2* (1992) 261.
44. U. Jönsson, M. Malmqvist, *Adv. Biosensors, 2* (1992) 291.
45. R. Karlsson, R. Stahlberg, *Anal. Biochem. 228* (1995) 274.
46. A. Brandenburg, R. Polzius, F. F. Bier, U. Bilitewski, E. Wagner, *Sensors and Actuators B, 30* (1996) 55.
47. J. Henkel, A. Brandenburg, P. Klein-Gunnewigk, U. Bilitewski, *4th World Congress on Biosensors* (1996) 145.
48. J. G. Hacia, L. C. Brody, M. S. Chee, S. P. A. Fodor, F. S. Collins, *Nature Genetics, 14* (1996) 441.
49. R. J. Lipshutz, D. Morris, M. Chee, E. Hubbell, M. J. Kozal, N. Shah, N. Shen, R. Yang, S. P. A. Fodor, *BioTechniques, 19* (1995) 442.
50. L. F. Rozsnyai, D. R. Benson, S. P. A. Fodor, P. G. Schultz, *Angew. Chem. Int. Ed. Engl. 31* (1992) 759.
51. H. Sigrist, H. Gao, B. Wegmueller, *Bio/Technology 10* (1992) 1026.

Simultano određivanje nekoliko spojeva imunokemijskim postupcima

Sažetak

Imunoanaliza se zasniva na raspoznavanju antigena s pomoću protutijela, a određivanje se provodi odgovarajućim obilježenim spojevima ili izravno koristeći sustav afinitetnih senzora. Različiti antigeni mogu se razlučiti samo s različitim protutijelima, što zahtijeva razvitak postupaka ili odgovarajućih senzora koji omogućavaju interakciju svakog pojedinog antigena s odgovarajućim protutijelom. Najuobičajeniji je pristup inkubacija uzorka sa smjesom odgovarajućih protutijela zajedno s obilježenim antigenom. Za svaki ispitani antigen treba koristiti drukčiji biljeg kako bi se provela specifična reakcija obilježenoga antigena s odgovarajućim protutijelom. Ovaj je pristup, međutim, ograničen brojem biljega koji bi se mogli razlikovati unutar jednog određivanja što se provodi pod identičnim uvjetima. Alternativno, različite antigen-protutijelo reakcije mogu se prostorno odijeliti tijekom analitičkog postupka, što omogućava primjenu samo jedne vrste biljega za sve ispitivane antigene. Određivanje različitih antigena postignuto je odgovarajućim prostornim odvajanjem transdukcije signala. Stoga je broj antigena, koji se mogu simultano odrediti, ograničen stupnjem prostorne rezolucije što se postiže povezivanjem s određenim enzimskim sustavom, dopuštenom kompleksnosti dobivenog sustava i stupnjem prostorne odvojenosti uređaja za transdukciju signala.