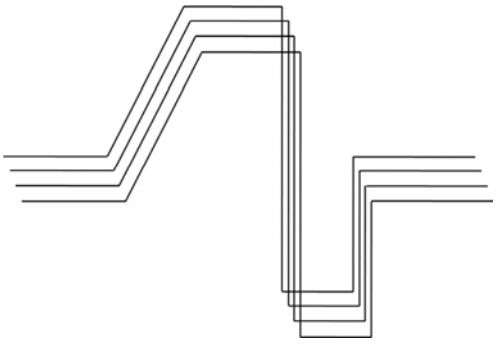
Multiplex biosensor immunoassays for antibiotics in the food chain



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Multiplex biosensor immunoassays for antibiotics in the food chain

Willem Haasnoot

Thesis

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To

Annie,

Annemarie en Caroline

“I hate losing more than I like winning”

Jimmy Connors 1982

Abstract

The use of antibiotics in food-producing animals may result in unwanted residues in food products. The main objective of the present research was to study the development and application of fast and automated multiplex surface plasmon resonance (SPR)-based biosensor immunoassays (BIAs), based on multi-component antibodies and/or combined immunoassays in serially connected flow channels, for the detection of selected antibiotics in the food chain. The scientific challenges to deal with were: the development of multi-sulfonamide monoclonal antibodies (Mabs) against the generic structure of sulfonamides and the evaluation of mutated recombinant antibodies (Rabs) derived thereof, finding of the best BIA format with aminoglycosides as model compounds and solving foreseen matrix and combined immunoassay interferences, and to study the use of antibiotic concentrations in blood serum as predictors for concentrations in edible tissue.

Broiler's blood serum, easy to collect in slaughterhouses, was chosen for the detection of sulfonamides and quinolones which are frequently used in poultry. With a Mab raised against sulfamethazine (21C7), the BIA could detect at least eight sulfonamides in ten times diluted broiler serum with limits of detection (LODs) far below the desired detection limit. Other less performing Mabs were developed against the generic part of sulfonamides. The best Mab-producing hybridoma cell-line (27G3) was used by the University of Turku to develop better performing mutated Rabs and the mutant-based BIA in broiler serum was found to be the most sensitive towards most of the sulfonamides. The assay was fast (5 min per sample), robust (>1000 runs per chip) and the sample preparation was easy (dilution in buffer only). The Rab-based multi-sulfonamide immunoassay was applied to analyze serum samples from broilers treated with sulfamethoxazole and sulfadiazine and the concentrations found were higher than the concentrations found in tissue by LC-MS/MS. This, and the good correlation with tissue concentrations, made this assay suitable to predict levels in edible tissue. A similar result was obtained with the specific BIA for flumequine.

Unique direct BIAs for the detection of aminoglycosides in milk were developed with Mab-coated chips. However, the inhibition assays with aminoglycosides on the chips were found to be more robust. For the simultaneous detection of five aminoglycosides in milk, the sensor chip surfaces in the four serially connected flow channels were covered with four aminoglycosides. In combination with a mixture of four specific antibodies, gentamicin, neomycin, kanamycin and (dihydro) streptomycin could be detected in milk far below the maximum residue limits (MRLs) and within 7 min.

In conclusion, serum and milk are suitable sample materials for the biosensor detection of antibiotics in the food chain. Such assays are fast, robust, automated, easy to handle, and require simple sample preparations (dilutions in antibody-containing buffer). In principle, such assays can be combined with assays for the detection of anti-pathogens, which broadens the application area in a food safety control system. However, the four-channel biosensor systems are too limited and the antibodies too specific for the simultaneous detection of more antibiotics. More extended multiplex systems (e.g. imaging SPR sensors or multiplex flow cytometry-based systems) need to be explored in which the knowledge obtained in the present research will likely be of great value.

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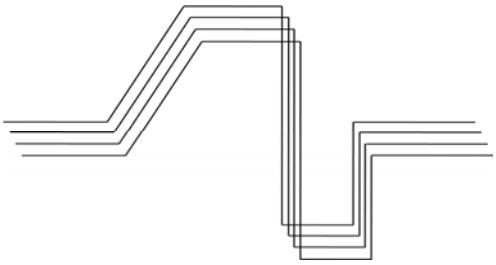
Chapter 1

General introduction



1.1 ANTIBIOTICS IN THE FOOD CHAIN

Antibiotic means “against life” and antibiotics are medicines that kill or cause growth retardation of micro-organisms. In the original definition, an antibiotic was described as any substance produced by a micro-organism that is antagonistic to the growth of other micro-organisms. This excluded synthetic compounds such as the sulfonamides and quinolones. In modern usage, an antibiotic is any chemotherapeutic agent with activity against micro-organisms such as bacteria, fungi or protozoa (Davey, 2000).

Penicillin was the first antibiotic originating from a micro-organism and was discovered by Alexander Fleming in 1929, when he observed by chance that bacterial growth was inhibited by the contaminating mold *Penicillium* (Stryer, 1996). Fleming was encouraged to find that an extract from the mould was not toxic when injected into animals. The search for other antibiotics was stimulated because penicillum was only effective against some infections caused by Gram-positive bacteria (Biotol, 1993). A second success was obtained through the discovery of streptomycin which showed a broad action spectrum and was found to be also effective against Gram-negative bacteria. Nowadays, the variety of antibiotics is enormous and there are hardly any processes in the living cell that are not sensitive to one of these antibiotics. Some antibiotics are still produced and isolated from living organisms, such as the aminoglycosides. Many others are produced via purely synthetic means, such as the quinolones and sulfonamides. Antibiotics can be classified as either bactericidal or bacteriostatic. Bactericidals (such as the quinolones and sulfonamides) kill bacteria directly where bacteriostatics (such as aminoglycosides) target protein synthesis and prevent cell division.

Veterinary drugs are used to treat disease and improve health in animals, analogous to pharmaceuticals in human beings. The use of antibiotics as feed additives is one of the most effective management tools available to meat cattle producers (Gillespie, 2004). Added in low doses to the feed of farm animals, they improve growth performance. Broad-spectrum antibiotics were preferred for use as feed additives. They generally give better results in terms of rate of gain, feed efficiency, and improved animal health. Therefore, antibiotics have extensively been used in animal production worldwide for decades. The International Federation for Animal Health (IFAH) reported in 2007 about a global market value of 17.9 billion US dollars for the animal health industry (http://www.ifahsec.org/media_room/IFAH_annual_report_2007_final.pdf).

However, the potential for widespread use of agricultural antibiotics to provoke development of antibiotic-resistant bacteria has stimulated intense debate. This antibiotic resistance may spread to other microbial populations and infectious diseases that have become resistant to standard anti-microbial treatments present a threat to human and animal health (Sapkota et al., 2007). *Staphylococcus aureus* is one of the

major resistant pathogens. It was the first bacterium in which penicillin resistance was found in 1947, just four years after the drug started being mass-produced. Methicillin was then the antibiotic of choice, but has since been replaced by oxacillin due to significant kidney toxicity. MRSA (methicillin-resistant *Staphylococcus aureus*) was first detected in Britain in 1961 and is now frequently discovered in hospitals. MRSA causes an infection that is resistant to an entire class of penicillin-like antibiotics called beta-lactams, which includes penicillin, amoxicillin, oxacillin, methicillin, and others. MRSA is acknowledged to be a human commensal and pathogen, but it has also been found in cats, dogs and horses, where it can cause the same problems. Owners can transfer the organism to their pets and vice versa, and MRSA in animals is generally believed to be derived from humans. Farm animals are able to infect people with MRSA and the transmit of MRSA from pigs to humans was reported previously (Huijsdens, et al., 2006). Other antibiotic-resistant strains of bacteria known to be foodborne pathogens - including *Salmonella* spp., *E. coli*, and *Campylobacter* spp - have been isolated from farm animals (Doyle, 2006). These resistant bacteria could cause human diseases that are difficult to treat. Dechet et al., (2006) found that a multi-state outbreak of a multi-drug resistant strain of *Salmonella enterica* serotype Typhimurium DT104 was associated with ground beef. Even if the antibiotic-resistant bacteria in meat animals are not human pathogens, they may pass their resistance genes to other pathogenic bacteria.

The serious problems caused by the emergence and spread of antimicrobial resistance was the reason for an EU-wide ban on the use of antibiotics as growth promoters in animal feed which has been effective since 2006 (EC, 2003). According to this regulation, only additives that have passed an authorization procedure may be placed on the market. Authorizations are granted for specific animal species, for specific conditions of use, and for ten-year periods only. This ban was the final step in the phasing out of antibiotics used for non-medicinal purposes. Antibiotics are now only allowed to be added to animal feed for veterinary medicine. However, in many countries outside the EU they are still used widely as feed additives for growth promotion. This EU regulation did not result in a decrease in the use of veterinary medicines. The Dutch Organization of Manufacturers and Importers of Veterinary Medicines reported a total use of 543 tons of antibiotics in 2006, which implies a 7% increase compared with 2005 with a comparable number of farm animals (FIDIN, 2007). In 2007, the total use of veterinary drugs increased again with 9% to 590 tons with the same number of farm animals (FIDIN, 2008). Due to this massive and increasing use of antibiotics in food-producing animals, residues may be found in food (e.g. milk, eggs or meat). This is especially the case when products from animals have been sent for processing within the withdrawal periods - ranging from a few days to a few weeks - of approved therapeutic antibiotics, or when animals have had access to excreted residues in their environment (Kümmerer, 2003).

To guarantee a high level of consumer protection, Community legislation (EC, 1990) requires that the toxicity of potential residues is evaluated before the use of a medicinal substance in food-producing animals is authorized. If considered necessary, maximum residue limits (MRLs) are established and in some cases the use of the relevant substance is prohibited. All pharmacologically active substances are divided into two groups: (i) Group A compounds, which comprise prohibited substances (listed in the Directive 96/22/EC (EC, 1996a) and in Annex IV of the Council Regulation (EC, 1990)); (ii) Group B compounds, which comprise substances with final and provisional MRLs (listed in Annex I and III of the regulation). Veterinary medicinal products can only be authorized or used in food-producing animals if pharmacologically active substances contained therein have been assessed as safe according to this MRL regulation and rules concerning the documentation of use, prescription and distribution have been established (EC, 2001).

Within the EU, residue monitoring plans for antibiotics and other groups of compounds are established from 1996 (EC, 1996b). They include the frequencies and level of sampling, investigation procedures and requirements on the documentation of use. The plans give indications for sanctions in case of non-compliance, requirements for targeted investigations and for the establishment, reporting and the groups of substances to be controlled for each food commodity. Another Commission Decision (EC, 1997) lays down additional rules for milk, eggs, honey, rabbits and game. A summary report for 2006 on the results of the national residue monitoring plans in food of animal origin in all EU member states (SANCO, 2008) described that the percentage of non-compliant results for antibacterials (exceeding the MRL levels) increased from 0.20% in 2005 to 0.30% in 2006 and that antibacterials remain the main problem for meat (bovine, pigs, sheep, goats, poultry, and rabbits), milk and honey.

For the research described in this thesis, antibiotics belonging to three different groups were used as models. Sulfonamides and quinolones (with flumequine as the model compound) were chosen because of their frequent use in poultry. Biosensor immunoassays were developed for their detection in blood serum of broilers, to indicate the level of these compounds in edible tissue. As third category, aminoglycosides and their detection in milk were selected, because they are often used in dairy cattle for the treatment of mastitis.

1.1.1 Sulfonamides

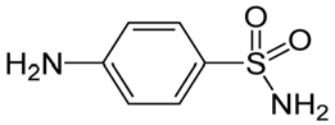


Figure 1. Molecular structure of sulfanilamide.

Sulfonamide drugs were the first synthetic antimicrobial drugs and they contain the sulfonamide group. German bacteriologist and pathologist Gerhard Domagk was awarded the 1939 Nobel Prize for Physiology or Medicine for discovering the antibacterial effects of prontosil red, a dye which contained the active component, sulfanilamide (Figure 1). Once sulfanilamide was recognized as an active antimicrobial agent, scientists synthesized many sulfonamides to test for bactericidal activity.

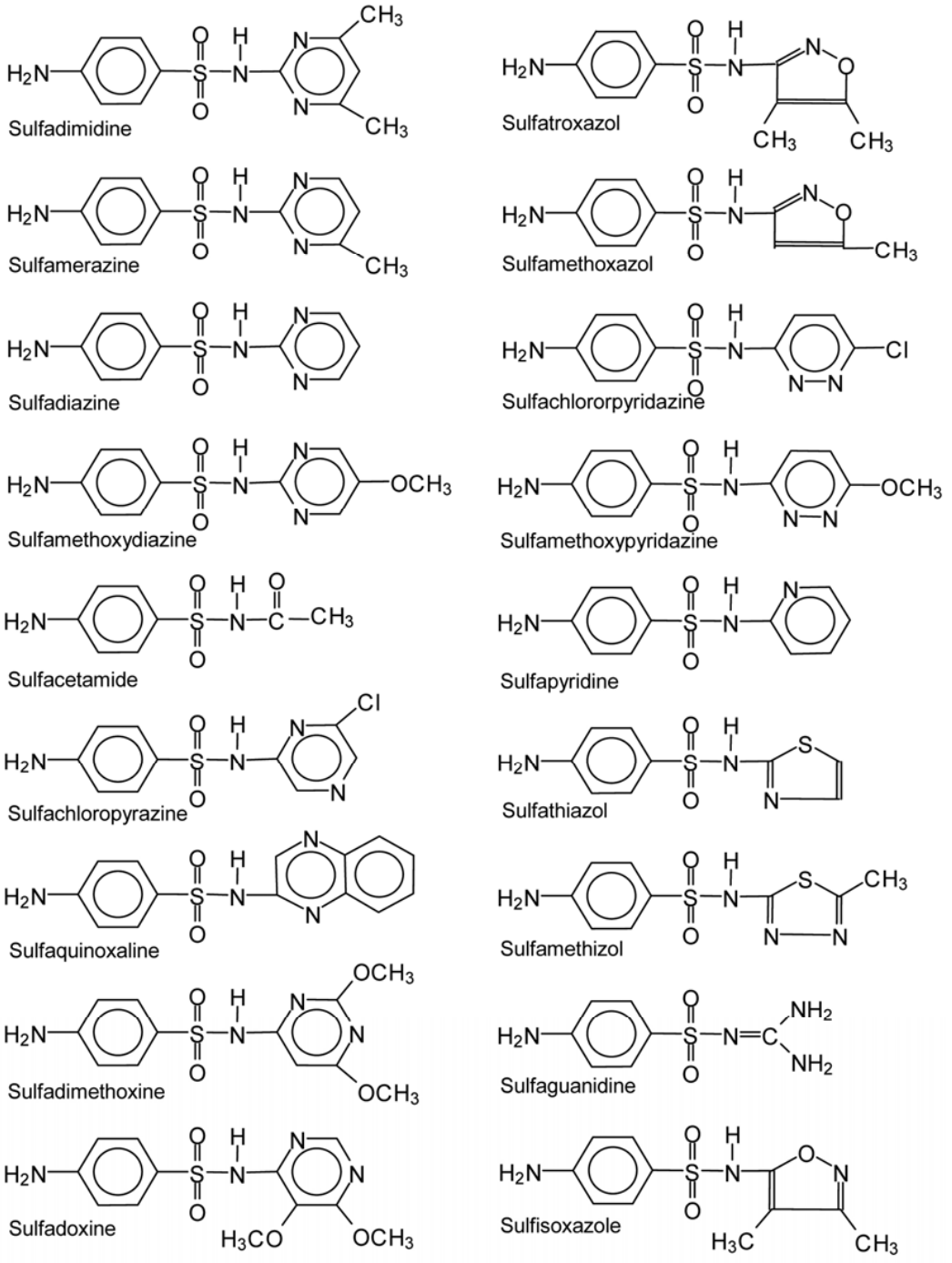


Figure 2. Molecular structures of the different sulfonamides used in this study.

It was later realized that sulfonamides do not actually kill bacteria, they interfere with bacterial growth and replication. Such bacteriostatic sulfa drugs inhibit the enzyme dihydropteroate synthetase (DHPS). DHPS catalyses the conversion of para-aminobenzoate (PABA) to dihydropteroate, a key step in folate synthesis. Folic acid is necessary for the biosynthesis of thymine and the purine bases, the building blocks of DNA and in its absence cells will be unable to divide. Sulfonamides have broad spectrum activity against both Gram-positive and Gram-negative bacteria. In the Netherlands at least nine different sulfonamides have been approved for veterinary applications. For instance, for the medication of poultry, five sulfonamides (sulfamethoxazole, sulfadiazine, sulfamethazine (sulfadimidine), sulfachloro-pyridazine and sulfaquinoxaline) are approved (CBG, 2009) of which the first two are most frequently applied. For the medication of dairy cattle, sulfamethoxazole, sulfadiazine and sulfadoxine are approved (KNMVD, 2007; CBG, 2009). Intensive use of these drugs in animal breeding can lead to unwanted residues in food. To establish safe limits for human consumption, the European Union therefore established an MRL of 100 μg kg^{-1} for the total amount of the parent sulfonamides in muscle, fat, liver and kidney of all food-producing species and bovine, ovine and caprine milk (EC, 1999). In this research, sulfonamides were chosen as model compounds to study the development and performance of group-specific monoclonal antibodies (described in Part I) raised against the generic part of sulfonamides (Figure 1). The development of fast and automated multi-sulfonamide biosensor immunoassays, based on monoclonal and mutated recombinant multi-sulfonamide antibodies, their performance with a range of sulfonamides (Figure 2) and their application in broiler serum as predictor for the levels in tissue are described in Part II.

1.1.2 Aminoglycosides

Aminoglycosides are broad-spectrum antibiotics most commonly used in veterinary drug medicine in the treatment of infections caused by Gram-negative bacteria, such as mastitis (Brander, 1986). The bactericidal ability of aminoglycosides is not fully clear yet, although the main lines of action have been delineated (Shakil et al., 2008). The drug attaches to a bacterial cell wall and is drawn into the cell via channels made up of porin proteins. Once inside the cell, the aminoglycoside attaches to the ribosomes. Ribosomes are the intracellular structures responsible for manufacturing proteins. This attachment either shuts down protein production or causes the cell to produce abnormal, ineffective proteins. The bacterial cell cannot survive with this impediment.

Neomycin is an aminoglycoside antibiotic consisting of 3 components (A, B and C). Neomycin B (see Figure 3I) is the largest component of commercial preparations of neomycin (over 90%). Neomycin is used to treat bacterial infections

of cattle, sheep, pigs, goats and poultry. MRL's for neomycin B were elaborated for cattle, sheep, goats, pigs, chickens, turkeys and ducks of 500 μg kg^{-1} in muscle, liver, fat and eggs. For milk and kidney, the MRL's are 1500 and 5000 μg kg^{-1}, respectively.

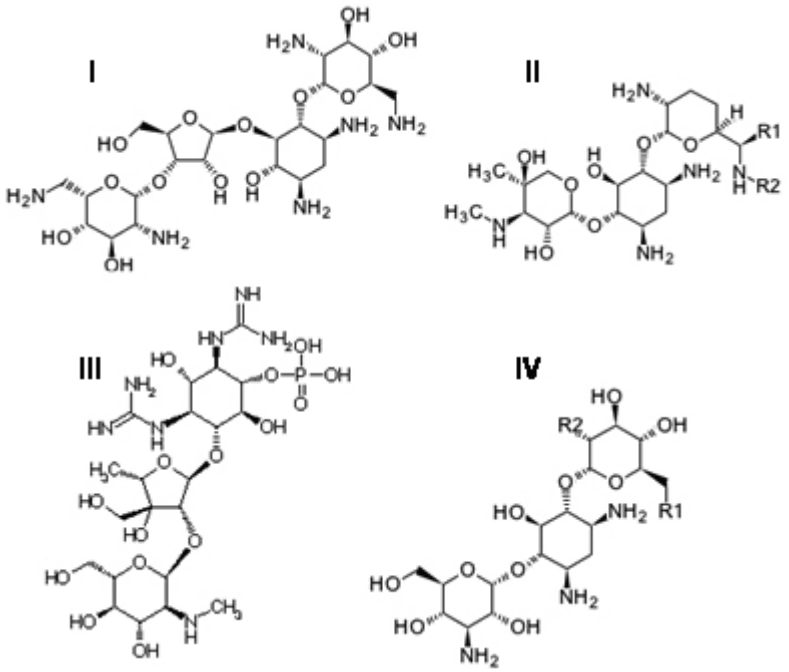


*Figure 3. Molecular structures of the aminoglycosides **I** is neomycin B, **II** is gentamicin C (in C_1 R1 = R2 = CH_3; in C_2 R1 = CH and R2 = H and in C_{1a}, R1 = R2 = H), **III** is streptomycin and **IV** is kanamycin.*

Gentamicin (Figure 3II) is a complex mixture, the main components being gentamicin C_1, C_{1a}, C_2 and C_{2a}. Gentamicin is an aminoglycoside antibiotic indicated for the treatment of a variety of bacterial infections in pigs and cattle and is normally used as the sulfate salt. Gentamicin is included in Annex III of the Council Regulation (EC, 1990) in which MRL's have been established for bovine milk (100 μg kg^{-1}) and bovine or porcine muscle, fat, liver and kidney of 50, 50, 200 and 750 μg kg^{-1}, respectively.

Streptomycin (Figure 3III) and dihydrostreptomycin are aminoglycoside antibiotics that are closely related in structure. Streptomycin, was the first aminoglycoside isolated from *Streptomyces griseus* in the mid-1940s. This antibiotic was very effective against tuberculosis. The use in lactating cows may lead to the presence of residues of these antibiotics in milk. Streptomycin is also used as a pesticide, to combat the growth of bacteria, fungi, and algae. Streptomycin controls bacterial and fungal diseases of certain fruit, vegetables, seed, and ornamental crops, and controls algae in ornamental ponds and aquaria. A major use is in the control of fireblight on apple and pear trees.

In 2002, the Food Safety Authority of Ireland (FSAI, 2002) requested retailers to remove honey from China and blended honey that contains or may contain honey

from China from the shelves because of the presence of residues of streptomycin. Reybroeck (2003) found streptomycins in a small fraction of honey samples from Belgium beekeepers (4 out of 248 samples (1.6%)) but in imported honey samples available on the Belgium market, streptomycins were frequently found (51 out of 108 (47.2%)). One of the main drawbacks to streptomycin is its toxicity, especially to cells in the inner and middle ear and to the kidney. Furthermore, some strains of tuberculosis have become resistant to treatment with streptomycin. Therefore, medical researchers have put considerable effort into identifying other antibiotics with streptomycin's efficacy, but without its toxicity. The MRL's for streptomycin and dihydrostreptomycin (EC, 1999) are comparable, and for bovine, ovine and porcine muscle, liver and kidney they are 500, 500 and 1000 μg kg^{-1}, respectively. For bovine and ovine fat and milk, the MRL's are 500 and 200 μg kg^{-1}, respectively, and for porcine skin and fat 500 μg kg^{-1}.

Kanamycin (Figure 3IV) is an aminoglycoside antibiotic produced by the growth of *Streptomyces kanamyciticus* and it comprised three components, kanamycin A - the major component usually designated as kanamycin - and kanamycins B and C, the minor congeners. The MRL's for kanamycin A in bovine, ovine, porcine, chicken and rabbit muscle, fat, liver and kidney are 100, 100, 600 and 2500 μg kg^{-1}, respectively and for bovine milk 150 μg kg^{-1} (EC, 1999).

Other aminoglycosides not included in this study are apramycin and paronomycin – as they are not for use in animals from which milk is produced for human consumption - and spectinomycin, for which an MRL in milk of 200 μg kg^{-1} is established. MRL's for spectinomycin in muscle, fat, liver and kidney of all food producing species are 300, 500, 2000 and 5000 μg kg^{-1}, respectively. The MRL's for apramycin in bovine muscle, fat, liver and kidney are 1000, 1000, 10.000 and 20.000 μg kg^{-1} and the MRL's for paronomycin in muscle, liver and kidney of all food producing animals are 500, 1500 and 1500 μg kg^{-1}, respectively.

In this study, the aminoglycosides gentamicin, neomycin, (dihydro)streptomycin and kanamycin were used as model compounds to study different biosensor immunoassay formats (direct, inhibition and combined) for the analysis of milk samples, as described in Part III.

1.1.3 Fluoroquinolones

The quinolones are a family of synthetic broad-spectrum antibiotics, and they are divided into generations based on their antibacterial spectrum (Ball, 2000). The earlier generation agents are, in general, more narrow-spectrum antibiotics than the later ones. Flumequine is a first-generation broad-spectrum antibiotic often used in veterinary medicine for the treatment of enteric infections. The majority of quinolones in clinical use belongs to the subset of fluoroquinolones, which have a fluoro group attached the central ring system (Figure 4). Quinolones and fluoroquinolones are

bactericidal drugs, actively killing bacteria. They inhibit the bacterial DNA gyrase or the topoisomerase IV enzyme, thereby inhibiting DNA replication and transcription. Quinolones can enter cells easily via porins, and therefore are often used to treat intracellular pathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae*.

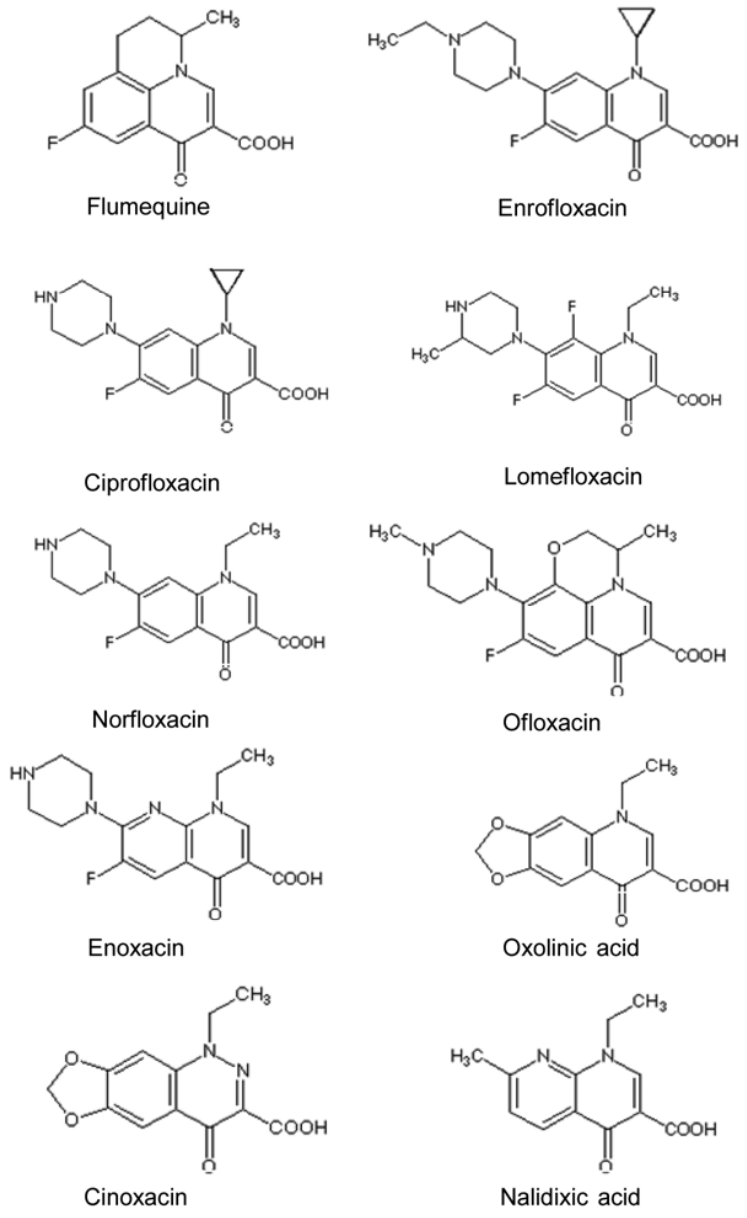


Figure 4. Molecular structures of the (fluoro)quinolones.

For many Gram-negative bacteria DNA gyrase is the target, whereas topoisomerase IV is the target for many Gram-positive bacteria. Eukaryotic cells do not contain DNA gyrase or topoisomerase IV. Fluoroquinolones are well distributed into bone tissue, and so well absorbed that in general they are as effective by the oral route as by

intravenous infusion. They are widely used to treat infections in farmed fish, turkeys, pigs, calves and poultry and monitoring of their residues in food is therefore regulated by law. The illegal and/or overuse of fluoroquinolones in animal production is of particular concern to humans because of their important applications in human medicine, and repeated exposure via food will limit the future effectiveness of these drugs due to the build up of antimicrobial resistance. As shown in Figure 4, flumequine has a deviating structure compared to most of the other quinolones and that is the reason why it was included in this study to develop antibodies and a biosensor immunoassay for this compound (Chapter 9). Outside the scope of this thesis, this assay was combined with another multi-fluoroquinolone immunoassay, to obtain a dual biosensor immunoassay for the simultaneous detection of six fluoroquinolones in chicken muscle (Marchesini, 2007).

1.2 DETECTION OF ANTIBIOTICS IN FOOD

For the detection of antibiotics in food and related products (e.g. blood, urine, renal pelvis fluid, etc.), three techniques are mainly applied: (i) microbial inhibition screening tests; (ii) chromatography-based screening and confirmation methods; and (iii) immuno- or receptor screening assays.

Because of their high cost-effectiveness and broad spectrum characteristics, microbial inhibition methods are preferred for large scale surveillance programs on veterinary drug residues. The methods usually use a medium inoculated with a susceptible bacterium and rely on diffusion of the antimicrobial residue. Inhibition of growth indicates the presence of antimicrobial compounds. Many test systems have been developed based on this principle, and methods using one to seven agar plates have been reported. The fast antimicrobial screening test (FAST) is a one-plate microbial method with *Bacillus megaterium* that requires a minimum of 6 h for development (USDA, 2006). The Premi® test uses a vial containing spores of the thermophile *B. stearothermophilis* in agar, and acts similarly to a single-plate method (Cantwell and O'Keeffe, 2006). This test is more rapid, with development times typically from 3 to 4 h. Recently, a similar test, the kidney inhibition swab (KIS™) test, has been developed. It uses a differently configured vial of *B. stearothermophilis* spores in agar, and also allows results within 3-4 h (Charm Sciences, 2008). These three fast tests were compared for screening antibiotic residues in beef kidney juice and serum (Schneider, 2008) and one of the conclusions was that there is not one rapid screening microbial inhibition assay for antibiotics that is ideal for all analytes. To cover all possibilities, one would have to run a number of different assays or separate plates. Such a five-plates test has been described (Pikkemaat et al., 2008), and comprises various microbes for the group-specific identification of antimicrobial residues in slaughter animals, the so-called Nouws antibiotic test (NAT screening).

The NAT screening combines a simple and efficient sampling and sample processing strategy with a high detection capability because it detects the great majority of antibiotics used in veterinary medicine at or below their maximum residue levels in kidney. However, it requires a rather lengthy incubation time of 16-18 h.

A faster alternative for the microbial inhibition assay is a whole-cell-based bioassay, also named whole-cell biosensor, which has been described for the detection of tetracyclines by Korpela et al. (1998). They constructed a genetically engineered luminescent bacterial strain that contained the regulation unit of tetracycline resistance factor (tetracycline-responsive element) to control the expression of the luciferase operon. This resulted in a tetracycline-dependent light production. The time needed for optimal induction of light emission was 90 min. They found that this kind of sensor cell can be freeze-dried without any loss of sensitivity or overall performance which simplifies the applicability of the assay system. This tetracycline group-specific bioassay was sensitized to meet the EU MRL for tetracycline residues in poultry tissue (100 ng g^{-1}) by adding membrane-permeabilizing and chelating agents and sensitivities of 5 ng g^{-1} for doxycycline, 7.5 ng g^{-1} for chlortetracycline and 25 ng g^{-1} of tetracycline were reached (Virolainen et al., 2008). The assay was performed in 96-well flat bottom microtiter plates within 4 h with little preparation, as lyophilized cells are always ready, and a multitude of samples can be assayed easily in this format. Being at least as sensitive and better suited for high-throughput analysis, whole-cell biosensors have the potential to displace growth inhibition assays as the favored method for tetracycline residue screening. However, such bioassays are not yet described for other antibiotics although the use of the multidrug-binding repressor protein (QacR) from *Staphylococcus aureus* (Schumacher et al., 2001) might offer an interesting approach for the future development of a multi-drug biosensor. Currently, cellular biosensors - based on various microbial species containing reporters that are specifically induced via selected promotors - are widely used in pharmaceutical drug discovery and in environmental biology (Urban, et al., 2007) and for monitoring environmental chemical contaminants (Patel, 2006). This will also further influence the development of new bioassays for the detection of antibiotics in food.

Of the chromatographic techniques, high-performance thin-layer chromatography (HPTLC) has been applied successfully for the qualitative and quantitative detection of multi-residues in food samples even though its use has rapidly decreased during the last decade (Toldrá and Rieg, 2006). The use of high-performance liquid chromatography (HPLC) expanded during the 1990s and the availability of automation facilitated its use as a screening technique. The choice of the detection system is very important to obtain both sufficient selectivity and sensitivity. UV-diode array detection in combination with reversed-phase HPLC has been applied for the detection of many antibiotics in a lot of different food matrices. Nowadays, more than 80% of the analytical techniques for the determination of veterinary drugs use HPLC

in combination with mass spectrometry (MS) (Gentili, 2008). Chromatographic methods are mainly used for the simultaneous detection and quantification of closely related compounds belonging to a single drug class. In combination with an MS/MS technique they fulfill the established criteria (EC, 2002a) for confirmation of identity of drugs (Danaher et al., 2007; Yuan, et al., 2008; Nielen et al., 2008). Recently, ultra-performance liquid chromatography combined with time-of-flight mass spectrometry (UPLC-Tof-MS) was used for the screening and quantification of more than 100 veterinary drugs in milk (Stolker et al., 2008) and in muscle, kidney and liver (Kaufmann et al., 2008). This technique combines high resolution for both LC and MS with high mass accuracy, and as such is very powerful for the multi-compound analysis of veterinary drugs. However, in general, chromatography-based methods are laborious and require trained personnel with high expertise and expensive equipment which make them only suitable for specialized laboratories and less suitable for measurements in the food chain.

According to the General Food Law (EC, 2002b), the food and feed industries are responsible for the safety of their products and their demand for simplified and rapid test methods at critical control points in the entire chain has never been greater. These methods must be able to detect an analyte or class of analytes at the level of interest. Some false positives (false non-compliants) are acceptable, as they will be further submitted for confirmatory analysis. However, the test must avoid or reduce to a minimum the number of false negative results (false compliants), because they will not be further analyzed.

There are different techniques available for the fast screening of veterinary drugs in animal foods. Of the immunochemical methods, the 96-wells microtiter plate enzyme-linked immunosorbent assay (ELISA) is most frequently used and many test kits to detect specific compounds or groups of compounds are commercially available. In general, they are sensitive and easy to use, have a high specificity, require minimal sample preparations, and are therefore suitable for the screening of a large number of samples in a short time (about 2-3 h). These tests can be used within food-producing facilities. For instance, antibiotic-ELISAs are described for the detection of sulfamethazine in swine urine and plasma (Haasnoot et al., 1996), aminoglycosides in milk and kidney (Haasnoot et al., 1999), chloramphenicol in urine, tissue, milk and eggs (Cazemier et al., 1996; Gaudin et al., 2003), nitroimidazoles in eggs and chicken muscle (Huet et al., 2005) and gentamicin in milk (Jen et al., 2005). In Part I of this thesis, the ELISA technology is used during the evaluation of antibodies against different sulfonamides and sulfonamide derivatives aiming for the development of multi-sulfonamide antibodies.

Immunochromatographic or lateral flow tests or dip sticks are much faster antibody-based assays, which can be performed in minutes. In these tests, all ingredients are already present in the test device, and the sample (extract) is needed only to perform

an assay. Therefore, this format is ideal for some food-producing facilities and field applications. Such fast tests are described for the detection of sulfadimidine in calf serum (Verheijen et al., 1998) and (dihydro) streptomycine in raw milk (Verheijen et al., 2000). Based on this technology, Unisensor (Angleur, Belgium) developed a receptor-based assay dipstick format (Twin sensor^{BT}) for the rapid detection of β-lactams and tetracyclines molecules in raw milk. Other examples of commercially available products are the Rapid One Step Assay (ROSA$^{®}$) tests for β-lactams, tetracyclines, enrofloxacin and sulfadimethoxine/sulfamethazine of Charm Sciences Inc. (Lawrence, MA, USA). The commercial availability of these rapid tests is still limited to a few antibiotics. This has been the reason to include the development of such fast multi-component dipsticks for tetracyclines, quinolones, sulfonamides, chloramphenicol and tylosin in the new EU-project entitled "Contaminants in Food and Feed: Inexpensive detection for control of exposure" (CONffIDENCE (http://www.conffidence.eu)).

Another more recent approach to screen animal products for veterinary drugs consists of the application of biomolecule-based biosensors. While bioassays or cellular biosensors utilize the response of whole cells to detect biologically active agents, these biosensor instruments use a biological recognition element (e.g. antibodies, enzymes, lectins, receptors and nucleic acids) in close contact with a signal transduction element (e.g. optical, acoustic, and electrochemical) connected to data acquisition and processing systems (Patel, 2006). Thus, the signal from the biological element is converted to a quantifiable signal, e.g., electrical. Enzymatic biosensors utilize specific enzymes for the capture and catalytic generation of the product, which is then directly determined using a range of transducers (e.g. electrochemical, optical, photothermal, amperometric, and acoustic). In contaminant analysis, enzyme biosensors have largely been used for organophosphorus and carbamate pesticide and herbicide analysis, with fewer applications being reported for antibiotics, e.g. for the detection of penicillins using penicillinase (Kiran et al., 2002).

Antibody-based immunosensors are frequently described with transduction elements based on piezoelectric, electrochemical, and optical components. A piezoelectric immunosensor is a device based on materials such as quartz crystals, which resonate on application of an external alternating electric field. The frequency of the resulting oscillation is a function of the mass of the crystal. This mass increases during interactions of e.g. antibodies to immobilized antigens, and thus yields a directly measurable decrease in the frequency. Such a fast (5 min) inhibition piezoelectric immunosensor is described for the detection of the mycotoxin ochratoxin A in liquid food samples (Hauck et al, 1998).

For the detection of antibiotics, electrochemical and optical immunosensors are most frequently applied. Electrochemical biosensors include potentiometric and amperometric immunosensors. The potentiometric immunosensors are based on the

change in potential that occurs when an antigen in a sample reacts with the corresponding antibody previously immobilized to an electrode. The potential difference between an antibody-immobilized electrode and a reference electrode is a function of the analyte in the sample. Amperometric immunosensors rely on the measurement of current generated when an electroactive species is either oxidized or reduced at an antibody- (or antigen-) coated electrode to which an analyte (or antibody) binds specifically. Zacco et al. (2007) developed a novel electrochemical immunosensing strategy for the detection of sulfonamide antibiotics in milk based on magnetic beads coated with class-specific anti-sulfonamide antibodies and a sulfonamide-peroxidase as tracer.

The interest in optical biosensors for food analysis, with fluorescent, bioluminescent or chemiluminescent labels for detection, as well as the direct (label-free) detection, is increasing. Label-free biosensors do not require the use of reporter elements to generate a signal, which is convenient during assay development and during application by saving washing steps and time (Morrow, 2007). One of the most popular label-free technologies for performing rapid analysis is based on commercially available automated multi-channel surface plasmon resonance (SPR)-based biosensors. Such SPR sensors are used in the present research to investigate different assay formats for the detection of drug residues in food and the principle of the technology and coupling procedures to the biosensor surfaces are described below.

1.3 SPR-BASED BIOSENSORS

Biacore™ SPR-based biosensors (models 3000 and Q (nowadays from GE Healthcare)) were used in this study, and the SPR principle and the essential components (Figure 5) are described in more detail below.

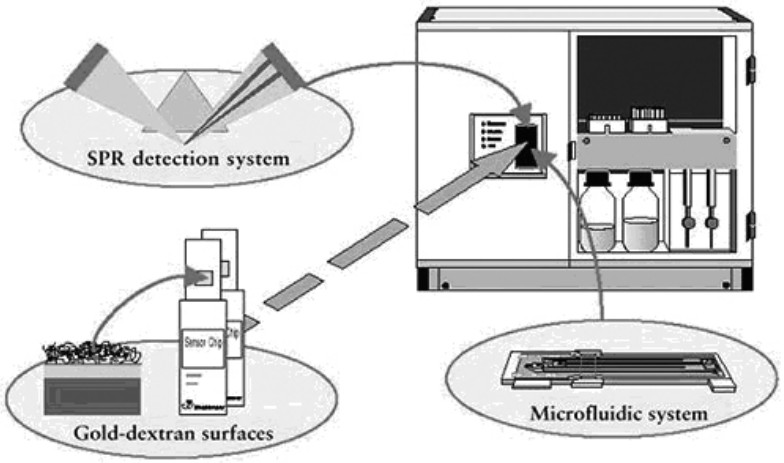


Figure 5. Essential components of the Biacore™ instruments used in this study.

The SPR phenomenon (Figure 6) occurs when monochromatic and p-polarized light (i.e. the electric vector component is parallel to the plane of incidence), under conditions of total internal reflection, strikes an electrically conducting metal (e.g. gold) layer at the interface between media of different refractive index: the glass of a sensor surface (high refractive index) and a buffer (low refractive index). An electric field intensity, known as an evanescent wave (EW), is generated when the light strikes the glass. This EW interacts with, and is absorbed by, free electron clouds in the gold layer, generating electron charge density waves called surface plasmons, and causes a reduction in the intensity of the reflected light at a specific angle of reflection (Figure 6). The incident light angle at which this reduction occurs is called the SPR angle.

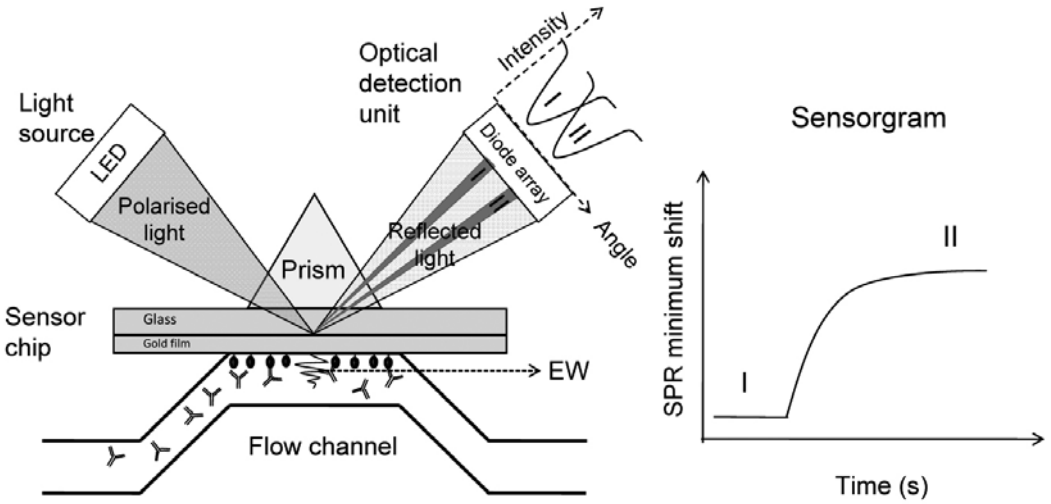


Figure 6. Schematic view of the optical and flow channel setup of a Biacore™ system (reproduced from www.biacore.com)

This angle varies with the refractive index close to the sensor chip surface on the side opposite from the reflected light (in the flow channel). This refractive index changes when molecules are immobilized on and/or bound to the surface. SPR response values are expressed in resonance units (RU) and one RU is 0.0001°. A close correlation between the SPR response and the amount of surface-bound protein has been demonstrated to be close to 1000 RU/ng protein/mm^2 for different proteins using radioactively labeled proteins to enable measurement of absolute surface concentration (Stenberg et al., 1991). The SPR angle changes when immobilized molecules (ligands) interact with molecules (analyte) in a mobile phase running along a flow cell (Figure 6 left panel). If binding occurs to the immobilized ligands, the local refractive index changes and the change of the SPR angle over time results in a sensorgram (Figure 6 right panel). A typical "real life" sensorgram (Haasnoot et al., 2005) of one cycle of analysis obtained with a blank sample (broiler serum) mixed with anti-sulfonamide antibodies is shown in Figure 7. After the increase of the bulk

response of the diluted sample (having a higher refractive index than the running buffer), the binding of the antibodies to a sulfonamide derivative immobilized on the biosensor chip is shown as the increasing response during the injection (association). This is followed by a fast bulk response decrease after the injection and a slow response decrease during the dissociation in which some of the bound antibodies are released. The response obtained just prior to the injection of the regeneration solution is caused by the bound antibodies (without bulk response) and is used for the calculations of sulfonamide concentrations. After the injection of the regeneration solution and stabilizing of the baseline with running buffer, a new cycle can be started. For the concentration analysis applied in this study, calibration curves were obtained by plotting the responses versus concentrations after injections of blank samples to which different concentrations of the analytes were added and comparing these responses with those obtained with the samples of interest.

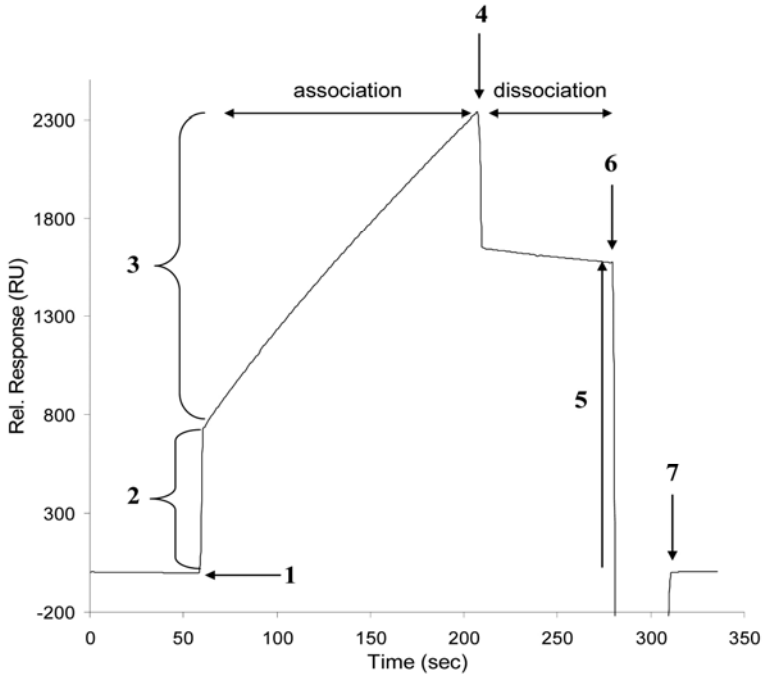


Figure 7. Sensorgram obtained in the Biacore 3000 after the injection of 50 µl of diluted broiler serum (20 µl serum diluted with 100 µl of diluted anti-sulfonamide antibodies) followed by a 20 µl injection of 0.1 M hydrochloric acid for the regeneration: (1) start sample injection (at this moment the relative response is set to 0); (2) bulk response; (3) binding of the mutant; (4) stop sample injection; (5) response used for calculation; (6) start injection regeneration solution; and (7) stop injection regeneration solution.

Although not applied in the present study, an important application of SPR biosensors is the possibility to perform kinetic analysis on interaction plots. The association and the dissociation phases are often used to obtain kinetic information from monitoring interactions in real time, yielding values for association and dissociation rate constants and/or equilibrium constants.

The reaction between the immobilized ligand (L) and the analyte (A) can be described by:

(1) $$L + A \underset{k_d}{\overset{k_a}{\rightleftharpoons}} LA$$

where k_a is the association rate constant [$M^{-1}s^{-1}$] describing the rate of complex formation, i.e. the number of LA formed per second (typical range 1 × 10^{-3} – 1 × 10^{7}) and k_d is the dissociation rate constant [s^{-1}] describing the stability of the complex.

The association rate is

(2) $$\frac{d[LA]}{dt} = k_a[L][A]$$

The dissociation rate is

(3) $$\frac{d[LA]}{dt} = -k_d[LA]$$

At equilibrium the rate of association equals the rate of dissociation, and provide K_A.

(4) $$k_a[L][A] = k_d[LA]$$

(5) $$K_A = \frac{[LA]}{[L][A]} = \frac{k_a}{k_d}$$

where K_A is the equilibrium association constant [M^{-1}] describing the association tendency and a high K_A is a high affinity (typical range 1 × 10^{5} – 1 × 10^{12}).

Analogously, the equilibrium dissociation constant K_D can be defined:

(6) $$K_D = \frac{[L][A]}{[LA]} = \frac{k_d}{k_a}$$

where K_D is the equilibrium dissociation constant [M] describing the dissociation tendency and a high K_D is a low affinity (typical range 1 × 10^{-5} – 1 × 10^{-12}).

In the association phase of the interaction, the observed rate of formation is the sum of reaction equations 2 and 3 and the net rate of binding is:

(7) $$\frac{d[LA]}{dt} = k_a[L][A] - k_d[LA]$$

In the biosensor, the formation of complex (*d[LA]*) is observed as an increase in response *R* (measured in resonance units RU). The concentration of free analyte *(A)* is

equal to the bulk analyte concentration, since analyte is constantly replenished during the sample injection. The available concentration of the ligand attached to the sensor surface (L) may be expressed in RU as the difference between the maximum analyte binding capacity R_{max} and the amount of complex formed at equilibrium (R_{eq}). The observed rate of formation (dR/dt) is zero at equilibrium.
Submitting these terms gives:

(8) $$\frac{dR}{dt} = k_a C(R_{max} - R_{eq}) - k_d R_{eq} = 0$$

where C is the concentration of analyte in the sample.

This equation can be rearranged to:

(9) $$\frac{R_{eq}}{C} = K_A R_{max} - K_A R_{eq}$$

A plot of R_{eq}/C (y-axis) versus R_{eq} (x-axis) results in a straight line with a slope of $-K_A$ and an y-intercept of $K_A R_{max}$. Therefore, the association constant K_A can be determined by linear regression from transformed data points at equilibrium.
This represents the pseudo-first order kinetics observed for binding of analyte to surface-attached ligand with 1:1 stoichiometry. Similar model equations may be applied to more complex interaction models.

The size of the change in SPR signal is directly proportional to the mass being immobilized and bound. For a given number of ligand sites, a higher molecular weight (MW) analyte will give a proportionally larger response. Conversely, for a given level of ligand immobilization in RU, a higher molecular weight ligand will provide fewer binding sites on the sensor surface. Therefore, a low molecular weight analyte will give a low response, particularly when the ligand response is low (equation 10).

(10) $$R_{max} = \frac{analyte\ MW}{ligand\ MW} \times ligand\ response \times valence$$

where valence is the number of analyte molecules that can bind to one ligand molecule

With current instrumentation (Biacore 3000), analytes as small as 200 daltons can be detected directly, although the response is low (see Chapter 7). As shown in Table 1, the theoretical maximum binding capacity (R_{max}) of a direct immunoassay for streptomycin (MW 581), in which the chip surface is coated with Mab (corresponding to 15000 RU) with an MW of 150000, is 116 RU, which corresponds with the R_{max} obtained in Chapter 7 of this thesis. Much higher responses can be obtained in the inhibition assay format, in which streptomycin is coated on the chip surface (direct

or as an ovalbumin-streptomycin conjugate) and the bound antibodies are responsible for the response (Table 1).

Table 1. Theoretical maximum RU's for combinations of ligand and analyte molecular weights (MW).

Ligand RU	Ligand MW	Analyte MW	Valence	R_{max}
15000	150000	581**	2	116
1000	48000*	150000	5***	15600
100	581**	150000	1	25800

*MW of ovalbumin-streptomycin; **MW of streptomycin ***Estimated number of streptomycin molecules per protein molecule available for antibody binding

For a more detailed description of SPR as an analytical technique, see Markey (2000). The SPR detection system in the Biacore biosensors uses the light from a near-infrared light-emitting diode (LED), with a peak intensity wavelength of 760 nm, which is focused through a prism on to the sensor chip surface in a wedge-shaped beam, giving a fixed range of incident light angles. Light reflected from the sensor chip is monitored by four separate linear arrays of light-sensitive diodes, one for each flow channel, covering the range of incident light angles. The spacing between diodes corresponds to a difference of 0.1° in incident light angle. Computer algorithms automatically calculate the angle at which minimum reflection occurs (the SPR angle) with a resolution of about 10^{-5}°.

The fluidic system consists of two liquid delivery pumps (one for maintaining a constant flow of liquid over the sensor chip surface and the other for handling samples in the autosampler), the autosampler, the microfluidic system (Integrated μ-Fluidic Cartridge (IFC), containing liquid delivery channels, sample loop and valves), 4 detector flow cells formed by the IFC pressing against the sensor chip and microprocessors (for controlling pumps, autosampler and IFC valves). The IFC (Figure 8) consists of a series of channels and pneumatic valves encased on a plastic housing and serves to control delivery of liquid to the sensor chip surface. It is connected to the buffer supply through the connector block. This has an injection port for loading samples from the autosampler into the IFC, which connects directly with the detector flow cells. The two biosensor systems used within this study (Biacore Q and 3000) have four flow channels (Fcs) each and are fully automated with a capacity of 192 samples (two 96-wells microtiter plates). The Biacore Q is dedicated to the qualitative or quantitative determination of analytes in food related products and can be used in combination with specially developed Qflex® Kits of which two (for sulfamethazine and for sulfonamides) were used in this study (Chapter 5). A disadvantage of the Biacore Q is that only one of the four Fcs can be used at the same time. In the Biacore 3000, the four Fcs can be serially connected and simultaneously

detected (Figure 8).

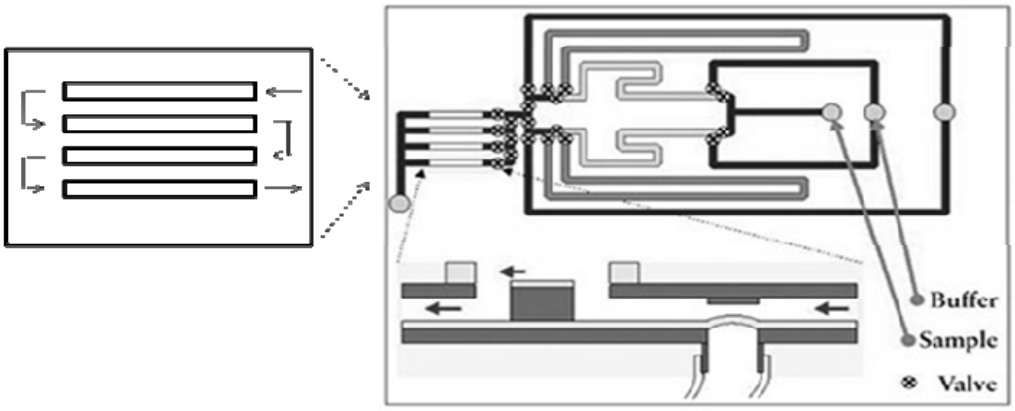


Figure 8. Schematic illustration of the IFC and a pneumatic valve (right panel) and the serial flow cell configuration of the Biacore 3000 (left panel). Software-controlled valves at each outlet and at the entry to each flow cell enable the user to select a combination of active flow cells during the experiment.

This gives the opportunity to run multi-assay or multiplex analysis, as was proven with the simultaneous detection of five aminoglycosides using a mixture of four specific antibodies and four different aminoglycosides immobilized into the four Fcs (Chapter 8). According to the manufacturer, the Biacore 3000 is the most sensitive instrument of their SPR biosensors and suitable for the direct detection of small molecules, as was shown in this study for the direct detection of aminoglycosides (Chapter 7).

The sensor chip (Figure 9) is the signal transducer and is a glass slide with a thin layer of gold deposited on one side. Gold is chosen for its combination of chemical inertness and good SPR response. The gold film is covered with a covalently bound matrix (attached through a linker layer) onto which biomolecules can be immobilized. The removable sensor chip is inserted into the cassette port on the detector unit, and docks into the instrument to form one side of the detector flow cell. Different sensor chips are available for different purposes. All, however, use the same principle (Figure 9).

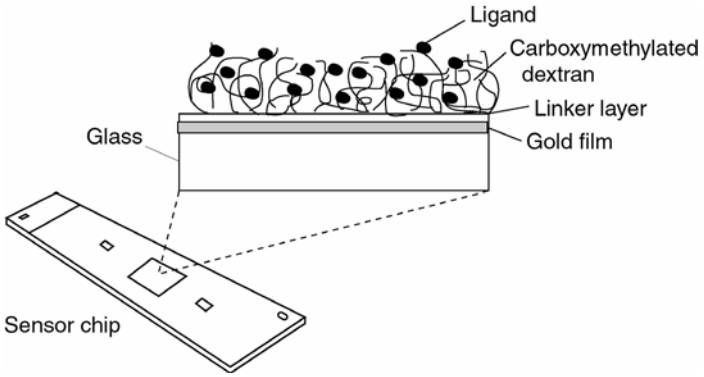


Figure 9. The surface of a sensor chip consists of three layers: glass, a thin gold film and a dextran layer, to which (bio)molecules can be immobilized.

The most generally applicable chip is the CM5 chip with a surface matrix of non-crosslinked carboxymethyl dextran covalently attached to the gold via a self-assembled monolayer of ω-hydroxyalkanethiol. Dextran is a linear polymer of glucose units, which exhibits very low non-specific adsorption of biomolecules. The dextran on the sensor chip surface is swollen in aqueous media, providing an extensively solvated hydrogel of 100 nm thickness and it is carboxymethylated to a substitution degree of approximately one carboxyl group per glucose residue. This modification serves two primary purposes. It provides a chemical possibility for covalent immobilization of biomolecules and it places a net negative charge on the dextran at physiological pH values, so that positively charged biomolecules (e.g. proteins at pH values below their isoelectric point (IP)) are attracted electrostatically to the dextran layer under conditions of low ionic strength. This concentrates biomolecules in the matrix, and allows effective covalent immobilization even from solutions with low bulk concentration. The carboxymethyl groups also enhance the hydrophilicity of the dextran matrix. The matrix is chemically stable in most buffers used in biomolecular interaction studies and can be exposed to extremes of pH for short periods without deterioration. The matrix enhances the ligand immobilisation capacity of the surface from typically 1-5 ng/mm^2 on untreated gold to up to 50 ng/mm^2 in the matrix. The carboxymethylated matrix is amenable to a range of generally applicable immobilization chemistries via -NH_2, -SH, -CHO, -OH or -COOH groups (see Figure 10 and O'Shannessy et al., 1992).

The amine coupling is the most common conjugation method in which the surface is activated with a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), to give reactive succinimide esters. The ligand (in a buffer giving a positive charge to the ligand) is passed over the surface and the esters react rapidly with amino groups or other nucleophilic groups. Any sterically unrestricted amine groups can react with the surface. Most proteins contain several of such amine groups via lysine residues, so that an efficient and random attachment can be achieved, which usually yields many bioconjugates that are unaffected with regard to the overall biological activity of the ligand.

Thiol coupling utilizes exchange reactions between thiol and active disulfide groups (Figure 10). The active disulfide moiety may be introduced either on the dextran matrix (to exchange with a thiol group on the ligand, referred to as the ligand thiol approach) or on the ligand molecule (to exchange with a thiol group introduced on the dextran matrix, referred to as the surface thiol approach). A recommended reagent for introducing active disulfide groups is 2-(2-pyridinyldithio) ethaneamine (PDEA). The amine group in PDEA can be used to attach the molecule to activated carboxyl groups on either the surface or the ligand. Thiol coupling can be a valuable approach if the ligand is inactivated by amine coupling in the analyte binding site. The thiol approach can also help to immobilize ligands in a defined orientation, since the number of

potential attachment sites is often less than with amine coupling, and in many cases is reduced to one single site.

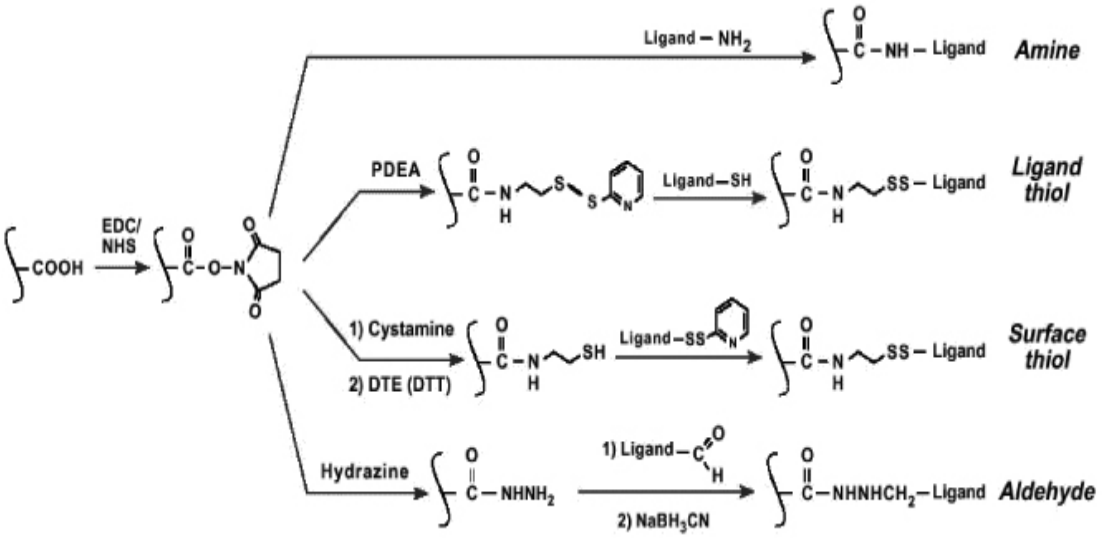


Figure 10. Chemistries for immobilizing (bio)molecules onto the surface of CM-series (carboxymethylated dextran) sensor chips.

Ligands containing aldehyde groups (either native or introduced by oxidation of cis-diols) can be immobilized after activating the surface with hydrazine or carbohydrazine. Alternative approaches for immobilizing glycoproteins and other glycoconjugates include aldehyde-amine coupling, thiol-ene click chemistry, and the use of unnatural amino acids to allow specific conjugation reactions that are orthogonal to the reactivity of all naturally occurring amino acids, e.g. via azide-alkyne click chemistry (Hermanson, 2008).

Although the CM5 biosensor chip is used most frequently, other sensor chips are available for special purposes. The CM4 sensor chip has a lower degree of carboxymethylation than CM5 (less negatively charged) resulting in reduced nonspecific binding. The CM3 sensor chip, with shorter carboxymethylated dextran than CM5, which works well with whole cells. The C1 chip has a flat carboxymethylated surface and no dextran matrix and works well for whole cells and viruses. The AU chip contains bare gold. The NTA (nitrilotriacetic acid) chip is designed to bind histidine-tagged molecules. The SA chip which is a CM5 chip with covalently immobilized streptavidin for capturing biotinylated molecules. The HPA chip is essentially a "blank" chip that lacks the carboxymethyl dextran of the CM5 chip. The surface is composed of hydrophobic alkanethiol groups. This chip can be used to mimic conditions of an ELISA (hydrophobic adsorption of protein), or to lay down lipid monolayers of various compositions. The latter can be used to study protein-lipid interactions or protein-protein interactions using a membrane-associated protein/receptor.

For drug residue detection, the CM5 biosensor chip is most frequently applied (Johansson, 2004). In the present study, antibodies, antibiotic-protein conjugates, antibiotic-derivatives and antibiotics themselves were immobilized on CM5 biosensor chips using the routine NHS-based amine coupling chemistry. For the immobilization of macromolecules, such as antibodies and drug-protein conjugates (Part III), electrostatic attraction is used for concentrating the ligand at the surface. The carboxymethylated surface of the sensor chip has a pKa of around 4. The surface is fully charged at pH values above 7. Below pH 7, the charge is gradually reduced, with roughly 30% of the groups charged at a pH of 3.5. To achieve efficient pre-concentration of the ligand, the pH of the buffer used during the immobilization should be higher than 3.5 and lower than the IP of the ligand. Due to this pre-concentration, relatively low ligand concentrations (of the order of 20 – 50 μg ml^{-1}) can be applied. Such electrostatic pre-concentrations are usually ineffective for small molecules and high concentrations (typically 5-10 mM) are used during the immobilizations to compensate for this effect (Part II). Due to contamination problems in the IFC, the direct immobilization of small molecules into the different flow channels (Fcs) is not recommended. Sensor chip whole surfaces were coupled with small molecules outside the instrument. For the immobilizations to the surfaces in the different Fcs, lower concentrations of protein-conjugates were used (Haasnoot et al., 2000b). Nowadays, a device (Surface Prep Unit) is available for the Biacore 3000 that makes it possible to immobilize small molecules on the chip surface in the different Fcs avoiding the use and contamination of the IFC. This device was used for the immobilization of the different aminoglycosides in the different Fcs (Chapter 8). In the case of the immobilization of carboxylated-sulfonamide derivatives (Chapter 5) or flumequine via its carboxyl group (Chapter 9), ethylenediamine (EDA) was used during the two-steps coupling procedure in which the carboxylated chips surface was reacted with EDA to create an amino-group containing surface which reacted with the esterified carboxylated ligands (O'Shannessy et al., 1992).

1.4 ANTIBODIES

1.4.1. Antibody classes

The central ingredient of any immunoassay (IA) is the antibody molecule. Antibodies belong to the family of macromolecules known as immunoglobulins, which are present at 12-15 mg ml^{-1} in blood serum, amounting to nearly one fifth of its total protein content (Harlow and Lane, 1988). Immunoglobulins appear as a very diverse group of proteins, also named globulins, that share key structural and functional features. Functionally, they can be characterized by their ability to bind both to antigens and to specialized cells or proteins of the immune system. Structurally, antibodies are composed of one or more copies of a characteristic unit that can

typically be visualized as forming a Y shape (Figure 11). Each Y contains four polypeptides – two identical copies of a polypeptide known as the heavy chain and two identical copies of a polypeptide called the light chain. There are two forms of light chains and a single antibody will have light chain subunits of either lambda (λ) or kappa (κ) variety, but not both types in the same molecule. In mammals five immunoglobulin classes (IgG, IgM, IgA, IgD and IgE) are distinguished by the type of heavy chains found in the molecule. Where IgG molecules have heavy chains known as γ-chains, IgMs have μ-chains, IgAs have α-chains, IgEs have ε-chains, and IgDs have δ-chains. The sequences of the IgG heavy chains have also shown that there are four subclasses of γ-chains known as IgG_1, IgG_{2a}, IgG_{2b} and IgG_3. The differences in the heavy-chain polypeptides, primarily in the Fc fragment (for the **f**ragment that **c**rystallizes), allow these proteins to function in different types of immune responses and at particular stages of the maturation of the immune response. Different classes of antibodies may also vary in the number of Y-like units that join to form the complete protein. IgM antibodies have five Y-shaped units and IgA antibodies have one, two or three units. The sites for association between the different Y units, the J-chain - a very acidic polypeptide of MW 15000 that is very rich in carbohydrate -, are also found in the Fc region.

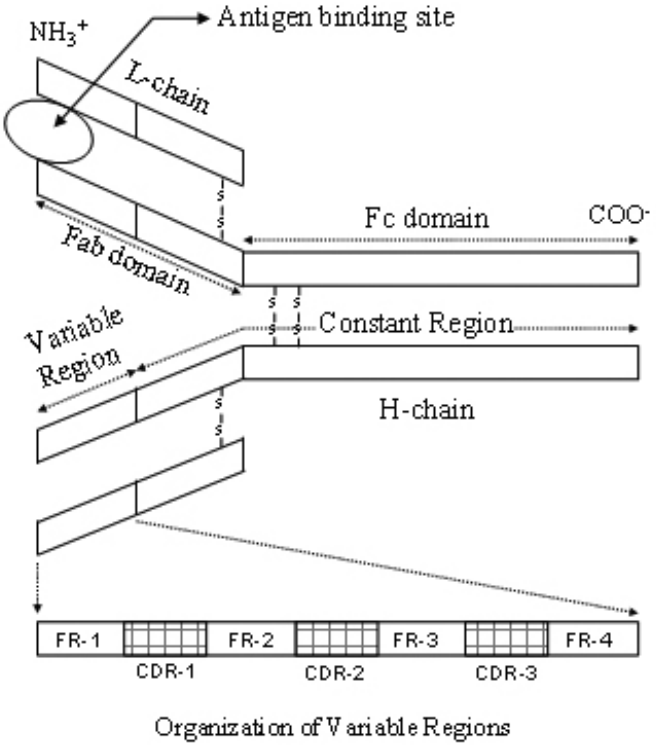


Figure 11. Schematic representation of a typical immunoglobulin-G molecule. The organization of the variable portions of the heavy and light-chains depicting the framework regions (FR) and the complementarity determining regions (CDRs) is shown in the lower portion of the figure.

In mammals, IgM constitutes about 10% of the antibodies. It is produced early in the

immune response and reacts quite strongly with an antigen due to the combined strength of the multiple bond interaction, which is called the avidity (Tijssen, P., 1985). The multivalent IgM has an avidity of 10^2-10^4 times higher than the affinity of the isolated sites (its Fab fragments). However, the pentameric IgM is particularly sensitive to mild reduction by thiol reagents at neutral pH (splitting into monomers) and losing its high avidity.

IgG represents 70% of the serum immunoglobulins and constitutes the majority of the secondary immune response to most antigens. IgG molecules have three domains (two Fab domains and one Fc domain; see Figure 11). The Fab domains (**f**ragment that carries the **a**ntigen **b**inding site) - forming the arms of the Y shape - are identical, which makes IgG molecules bivalent. The domain that is involved in immune regulation is the Fc fragment, and this forms the base of the Y structure. The two heavy (H-chain) polypeptides (each 440 amino acids [approximately 55,000 Daltons]) in the Y-structure are identical. The two light (L)-chains (each about 220 amino acids (about 25,000 Daltons)) are also identical. One L-chain associates with the amino-terminal region of one H-chain to form an antigen-binding site. The carboxy-terminal regions of the two H-chains fold together to make the Fc domain. The four polypeptide chains are held together by disulphide bridges and noncovalent bonds. L-chains can be divided into two regions, the variable (V) and the constant (C) region (each about 110 amino acids in length) and H-chains can be divided into four regions (1 V and 3 C regions). The V regions (located at the amino end) of one H-chain and one L-chain combine to form one antigen binding site. The V regions of both chains are organized into three hyper-variable or complementarity determining regions (CDRs) separated by four framework regions (FR) (Figure 11, lower panel). The greatest amino acid sequence variation occurs within the CDRs whereas the framework regions are less variable. The association of the CDRs, three from the H- and three from the L-chains, form the antigen-binding site. The heterogeneity in the V-regions of both L- and H-chains confer the capability to respond to a very large number of antigens. The diversity of the mammalian immune response is maximally 10^5-10^6 different antibodies.

1.4.2 Antibody-antigen interaction

The ability of an antibody molecule to specifically bind an antigen, or ligand molecule, is controlled by structural and chemical interactions that occur within the antigen-binding site. The antigen-antibody interaction is a reversible interaction dominated by thermodynamic control (Equation 5), and does not involve formation of covalent bonds. The binding is the result of a variety of interactions including hydrophobic, ionic, hydrogen bonds, π-π electron interaction, and van der Waals forces. Typically, the binding energy (relative affinity) of the antibody is larger with an increasing number of specific chemical interactions that occur between an analyte

and the amino acid residues in the antigen binding site. Thus the specificity and sensitivity of an immunoassay are controlled by the precise nature of the antigen-antibody binding process and small changes in antigen structure can profoundly affect the strength (or affinity) of the antibody-antigen interaction.

The affinity of an antibody describes the amount of antibody-antigen complex that will be found at equilibrium. High-affinity antibodies (i.e. $K_A = 10^{12}$ mol^{-1}) will bind larger amounts of antigen in a shorter period of time than low-affinity antibodies (i.e. $K_A = 10^4$ mol^{-1}). High-affinity antibodies perform better in all immunochemical techniques. Whereas the affinity of an antibody reflects its binding energy to a single epitope, avidity reflects the overall binding intensity between antibodies and a multivalent antigen presenting multiple epitopes. Avidity is determined by the affinity of the antibody for the epitope, the number of antibody binding sites, and the geometry of the resulting antibody-antigen complexes. Avidity is also assay specific, and differs when the same antibodies are used in different techniques (Lipman et al., 2005).

1.4.3 Antibodies towards haptens

The principal function of the immune system is to protect the organism from infectious organisms and from their toxic products. The immune system contains more than 10^9 antibody-producing cells (lymphocytes) distributed throughout the body. They travel through the blood and lymphatic systems, pausing and accumulating in specialized structures known as lymphoid organs, which in mammals are the lymph nodes and spleen. Many types of lymphocytes with different functions have been identified. The immune system has two basic components that respond to a challenge of a foreign substance: a cellular response mediated by T lymphocytes and a humoral response mediated by antibodies secreted by B lymphocytes (Figure 12).

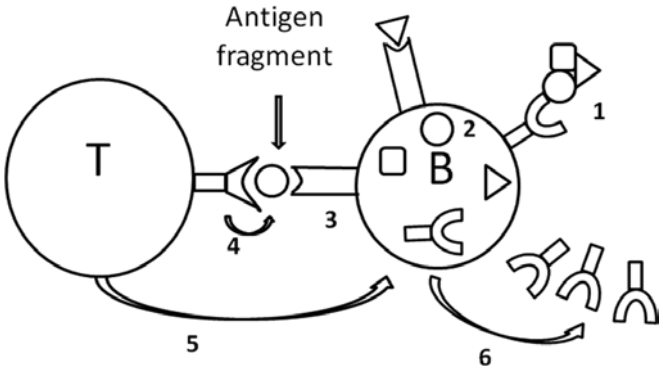


Figure 12. General simplified graphic of the process of antibody production showing the necessary physical link between the cells involved. B cell is a cell that secretes antibodies, T cell activates B cells and antigen is the substance recognized by the immune system. Interactions in the graphic:1. antigen is bound, 2. antigen is degraded in the cell, 3. B cell presents an antigen fragment, 4.T cell recognizes the antigen fragment, 5. T cell activates the B cell to produce antibodies, 6. the B cell secretes antibodies (Harlow and Lane, 1988).

The B cells recognize antigens through cell-surface immunoglobulins that bind to discrete chemical and structural epitopes on the antigen molecule. Each B cell possesses surface immunoglobulins of a single type (i.e., is monoclonal) and has a binding capacity that is directed against a discrete epitopic target. Most of the cellular functions of the immune system can be described by grouping lymphocytes into three basic types – B cells, cytotoxic T cells and helper T cells. All three carry cell-surface receptors that can bind antigens. B cells secrete antibodies, and carry a modified form of the same antibody on their surface, where it acts as a receptor for antigens. Cytotoxic T cells lyse foreign or infected cells, and they bind to these target cells through their surface antigen receptor, known as the T-cell receptor. Helper T cells play a key regulatory role in controlling the response of B cells and cytotoxic T cells, and they also have T-cell receptors on their surface. Two fundamental requirements must be met by a molecule to be immunogenic: (i) it should be foreign to activate the defense mechanism; and, (ii) it must be of a certain complexity to react with the different components of the immune system necessary to induce the immune response (immunogenicity). In general, for a compound to elicit a primary antibody response and a strong secondary response, it must contain an epitope that can bind to the cell-to-cell communication between B cells and helper T cells. This is achieved by providing a physical link between these two cells (Figure 12). When an antigen binds to the antibodies on the surface of the B cell, this antigen/antibody complex is taken up by the B cell and processed by proteolysis into peptides. These fragments migrate to the surface, where they bind to receptors known as class II proteins. This complex attracts a matching helper T cell, which releases lymphokines and activates the B cell. As the activated B cell then begins to divide, its offspring (plasma cell) secretes millions of copies of the antibody that recognizes this antigen in the blood. The physical link between the B and T cells is formed by the antigen fragment and is an essential step in the differentiation of a B cell into a plasma cell. The site for class II-T-cell receptor binding does not need to be related structurally to the epitope for surface antibody binding, but the two sites must occur on the same molecule or physical complex. This requirement for both an epitope and a class II-T-cell receptor binding site imposes a minimum size limit on an immunogen, and molecules of less than about 3000-5000 Daltons are generally not good immunogens. Compounds smaller than this can often bind to surface antibodies on the B cell but may not have suitable sites for the simultaneous binding of a class II protein and a T-cell receptor. Physical coupling of small molecules to larger immunogenic molecules overcomes this problem by providing the missing class II-T-cell receptor binding sites and allows the induction of a strong antibody response against the small molecules. Small molecules showing this behavior are defined as haptens. The large molecules that render them immunogenic are defined as carriers.

1.4.4 Conjugation of haptens

Haptens are coupled to proteins for the preparation of immunogenic hapten-carrier complexes. A foreign protein administered *in vivo* by any one of a number of potential routes nearly ensures the stimulation of an immune response. In addition, protein carriers are chosen for their solubility and functional groups that could facilitate easy conjugation with a hapten molecule. The most common carrier proteins are keyhole limpet hemocyanin (KLH; MW 4.5 x 10^5 to 1.3 x 10^7), bovine serum albumin (BSA; MW 67,000), the highly positive charged aminoethylated (or cationized) BSA (cBSA - prepared by modification of its carboxylate groups with ethylene diamine to increase the immunogenicity compared to BSA), thyroglobulin (MW 660,000) and ovalbumin (OVA; MW 43,000).

The coupling chemistry used to prepare an immunogen from a hapten and carrier protein is an important consideration for the successful production and correct specificity of the resultant antibodies. The choice of linking methodology is governed by the functional groups present on both the carrier and the hapten, as well as on the orientation of the hapten desired for appropriate presentation to the immune system (Hermanson,1996).

Antisera (blood serum of immunized animals) to sulfadimidine and other sulfonamides have been raised using hapten conjugates prepared by diazotization of the aromatic amino group (Ram et al., 1991; Renson et al., 1993) or by introducing spacers such as glutaraldehyde (Fleeker and Lovett, 1985; Dixon-Holland and Katz, 1988; Märtlbauer et al., 1992) or a succinyl group (Jackman, 1992; Haasnoot et al., 1996). However, antibodies developed by these procedures were very specific in relation to other sulfonamides. In this research, this was confirmed during the evaluation of polyclonal antibodies raised against eight different sulfonamides, coupled to carrier proteins via different procedures (succinylation, glutaraldehyde coupling, epoxy activation, periodate cleavage and diazo-coupling), which were all very specific (Chapter 3).

Ideally, it would be more efficient to develop a more general immunoassay that could detect a number of different sulfonamides (a multi-sulfonamide assay). Sheth and Sporns (1991) were the first who reported about the development of such an ELISA based on polyclonal antibodies. They chemically linked a sulfathiazole derivative (TS) to proteins in such a way that the aromatic amino group, common to all sulfonamides was distal to the protein (Figure 13). TS was coupled to the carrier proteins via the carboxyl group which was esterified with *N*-hydroxysuccinimide (NHS) and reacted with the amines of the carrier proteins. A similar approach was described by Assil et al. (1992) who used another sulfonamide derivative (PS, see Figure 13). In this study, the NHS coupling of TS and PS to the carrier proteins KLH and BSA was used prior to the immunization of mice. Pabs were obtained and Mabs were developed and they were both tested in an ELISA format (Part I).

The aminoglycoside dihydrostreptomycin (DHS) was coupled to the carrier proteins via two reactions (Chapter 7). The one-step glutaraldehyde coupling (Hermanson, 1996) was performed at two pH values (7.3 and 8.5) because of observed differences in solubility of the proteins. Glutaraldehyde is a homobifunctional cross-linking reagent that reacts with primary amine groups of the proteins and the aminoglycoside to form Schiff bases, which can be reduced to form stable secondary amine linkages. The other procedure used succinic anhydride pre-activated carrier proteins in order to introduce additional coupling sites and to reduce the chance of creating inter-protein cross-links. Subsequently, the conjugate reaction between the protein carboxyl groups and the amino groups of the aminoglycoside was performed with the carbodiimide reaction (EDC) forming an amide bond.
The mixed anhydride procedure (Tijssen, 1985) was followed for the coupling of flumequine to the carrier proteins (Chapter 9). Here, the carboxyl groups of flumequine were converted to acid anhydrides that react smoothly with the amino groups of proteins to again form amide bonds.

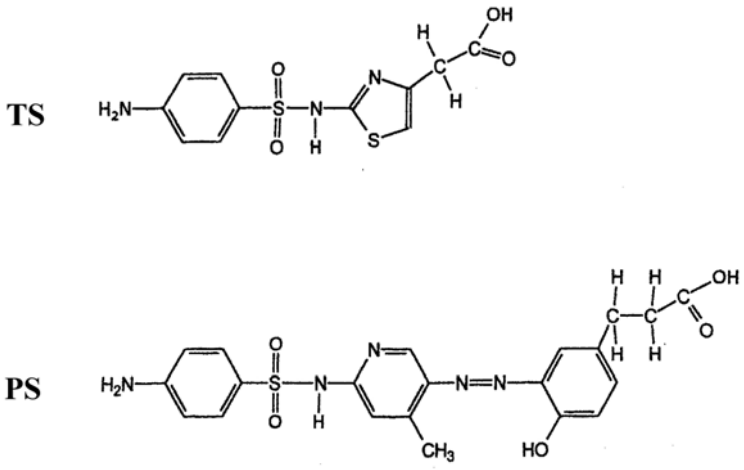


Figure 13. Molecular structures of the immunogens used for the development of generic Pabs and Mabs). TS = N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide; PS = N1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]sulfanilamide.

1.4.5 Immunization

In accordance with the Amsterdam protocol on animal protection and welfare of 1997 (http://eur-lex.europa.eu/en/treaties/dat/11997D/htm/11997D.html (approached on 2009-03-30)) and a recent proposal of the European Commission, of 5 November 2008 to revise Directive 86/609/EEC (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm (approached on 2009-03-30)), animal experiments must be replaced with alternatives wherever possible, the number of animals used should be minimized and suffering of animals must be avoided or kept to a minimum. The animal experiments applied in this study were subject to strict legal controls and

submitted for examination to an Animal Experiments Committee that checked whether the purpose of the experiment is proportionate to the distress caused to the animal.

Immunizing and bleeding of animals for the production of polyclonal sera or to act as donors for hybridoma fusions (Chapter 1.4.6) is a technically straightforward procedure. To obtain good antibody responses, healthy and well-cared-for animals are essential. To inject and bleed animals safely and painlessly requires skill, patience, and practical training. In this study, the facilities at the Laboratory of Hormonology (Marloie, Belgium) were used for the immunization and bleeding of rabbits to obtain polyclonal antisera. For the immunizations and bleedings of mice and the subsequent removal of the spleens for the production of monoclonal antibodies, the facilities of the Centre for Small Experimental Animals (Wageningen, the Netherlands) were used.

The production of antibodies relies on the in vivo humoral response of the animal (Harlow and Lane, 1988). After the primary injection of antigen, an increase in B cells bearing surface antibodies specific for the inoculated antigens is first detected after 5-6 days. Antibodies are usually detected in the serum from around 7 days after the injection and persists at a low level for a few days, typically reaching peak titer around day 10. Primary responses often are very weak and the sera contain a substantial proportion of IgMs. A minimum delay of 2 or 3 weeks is required before reintroducing the antigen. The number of B cells bearing antigen-specific cell-surface antibodies increases exponentially after the second injection and high levels of antibody persist for about 2-4 weeks after the second injection. The responses to the subsequent injections, given every 4-6 weeks, mirror that of the secondary injection. Higher titers of antibody are reached, and the nature (more IgG) and quality (higher affinity) of the antibodies changes during these injections which is known as maturation of the immune response. Levels of 1 mg ml^{-1} of antigen-specific IgG are possible, which is about 10% of the total IgG content of serum.

During these immunizations, adjuvants (e.g. Freund's or Specol) are used to improve or enhance an immune response to the antigen. There are two types of Freund's adjuvants: Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA). FCA is a water-in-oil emulsion that localizes antigen for release periods up to 6 months. It is formulated with mineral oil, the surfactant mannide monoleate and heat-killed *Mycobacterium tuberculosis, Mycobacterium butyricum* or their extracts. This potent adjuvant stimulates both cell-mediated and humoral immunity with preferential induction of antibodies against epitopes of denatured proteins. Although FCA has historically been the most widely used adjuvant, it is one of the more toxic options due to the use of non-metabolizable mineral oil, while it also induces granulomatous reactions. Its use is limited to laboratory animals and it should be used only with weak antigens. In addition, it should not be used more than once in a single animal, since

multiple FCA inoculations can cause severe systemic reactions and decreased immune response. FIA has the same formulation as FCA but does not contain mycobacterium or its components. FIA usually is limited to booster doses of antigen since it is normally much less effective than FCA for primary antibody production. FCA and FIA are normally mixed with equal parts of antigen preparations to form stable emulsions.
Specol is a water-in-oil adjuvant composed of defined and purified light mineral oil (Stills, 2005). It has been suggested as an alternative to Freund's adjuvant for hyperactivation of the immune response in rabbits (Leenaars et al., 1994). Specol can be used for antigens of low immunogenicity and can be administered equally effectively by the subcutaneous or intraperitoneal routes.
In the present research, rabbits (n=12) and mice (n=10) were immunized using Freund's adjuvants or specol (Chapters 2, 3, 7 ,9).

1.4.6 Polyclonal, monoclonal and recombinant antibodies.

In immunoassays for residue analysis in food, polyclonal (Pabs), monoclonal (Mabs) and occasionally recombinant antibodies (Rabs) are used. All three antibody-types were applied during this research and some relevant background is described below.

Pabs are antibodies that are derived from different B cell lines each with one specificity i.e. the clonotype. An immunized animal produces generally a random number of clonotypes and its antiserum becomes polyclonal. It has been estimated that 10-40 × 10^6 distinct clonotypes can theoretically be generated by a mouse which contains 2 × 10^8 B cells (Tijssen, 1985). About 1 out of every 10000 clonotypes seems to recognize a given epitope with varying degrees of affinity. Thus, in theory, several thousand clonotypes could be produced by an animal against a given epitope. In practice, however, only a random few (up to about ten) B-cell clones are activated and only few distinct antibodies against an epitope are generated out of this large repertoire of randomly formed specificities (Briles and Davie, 1980). It is therefore practically impossible to make reproducible Pabs against any epitope. Even antisera from the same animal taken at different times differ in their properties. Pabs are typically produced by immunization of a suitable mammal, such as a mouse, rat, guinea pig, hamster, rabbit, goat, sheep, donkey or horse. However, rabbits are the most commonly used laboratory animals for this purpose. They are easy to keep and handle, can be safely and repeatedly bled, and the antibodies they produce are well characterized and easily purified. With careful management, a few hundred ml of serum can be obtained from one rabbit through the course of an immunization regime and from different bleedings. The concentration of specific antibody in polyclonal sera is typically 50 to 200 μg ml^{-1}, and the range of total IgG concentration in sera is between 5 and 20 mg ml^{-1} (Lipman et al., 2005). Goats, sheep or horses are generally

used when large quantities of antisera are required. When using rabbits, young adult animals (2.5–3.0 kg) should be used for the primary immunization because of the vigorous antibody response. Immune function peaks at puberty and primary responses to new antigens decline with age. Female rabbits are generally preferred because they are more docile and are reported to mount a more vigorous immune response than males. At least two animals per antigen should be used to reduce the change of potential total failure resulting from non-responsiveness to antigens of individual animals. Some investigators favor chickens because they transfer high quantities of IgY (equivalent to mammalian IgG antibodies) into the egg yolk, and harvesting antibodies from eggs eliminates the need for the invasive bleeding procedure (Albrecht et al. (1996); Pichler et al., (2004); Schneider et al. (2004)).
So far, in residue assays, Pabs are applied most frequently. The main problem with polyclonal antisera is the batch quantity and the batch-to-batch variation. Therefore the amount of tests is limited. However, with for instance a batch of 25 ml of a polyclonal antiserum and a usually optimum dilution of 40,000 times (total volume = 1,000,000 ml of which 2.5 ml is used per microtiter plate), 400,000 microtiter plate tests kits can be prepared. For most of the drug residue test kits, this is much more than the world-wide market needs for 50 or more years. In special circumstances, e.g. for high volume (on-line) testing, test strips, immunoaffinity chromatography (IAC) and for “best-sellers”, more antibodies are necessary. Increased volumes of Pabs can be obtained by mixing different batches of (rabbit) antisera obtained after different boosters or from different rabbits. However, in most cases, the quality of antisera improves after several boosters (antibody maturation) and mixing with lower-quality batches might reduce the test performance. Other possibilities are the immunization of larger animals (goat, sheep, donkey, etc.) or the use of chicken eggs.
In the present study, Pabs were developed in rabbits against sulfonamides (Part I) and flumequine (Chapter 9).

Mabs. The preparation of a homogeneous population of antibodies was achieved with the development of the technology for hybridoma production. Antibodies that are produced by hybridomas are known as monoclonal antibodies (Mabs). In the animal, antibodies are synthesized primarily by plasma cells (B lymphocytes), which cannot be grown in tissue culture and cannot be used as an in vitro source of antibodies. Köhler and Milstein (1975), developed a technique that allows the growth of clonal populations of cells secreting antibodies with a defined specificity. In this technique an antibody-secreting cell, isolated from an accumulating organ of an immunized animal (e.g. from the spleen), is mixed with a myeloma cell, a type of B-cell tumor, centrifuged to generate good cell-to-cell contact, and fused with polyethylene glycol (PEG) as a fusing agent. These hybrid cells or hybridomas can be prepared by fusing myelomas and antibody-producing cells isolated from different species, but the

number of viable hybridomas increases dramatically when closely related species are used. Even in the most efficient hybridoma fusions, only about 1% of the starting cells are fused, and only about 1 in 10^5 form viable hybrids. This leaves a large number of unfused cells still in the culture. The cells from the immunized animal do not continue to grow in tissue culture, and so do not disturb further work. However, the unfused myeloma cells are well adapted to tissue culture and must be killed, which is achieved by drug selection (e.g. aminopterin) in which the synthesis of nucleotides is blocked. The hybridomas can be maintained *in vitro* and will continue to secrete antibodies with a defined specificity. Approximately one week after the fusion, colonies of hybrid cells will be ready to screen. During the screening, samples of tissue culture media are tested for the presence of the desired antibodies using for instance antibody capture assays with antigen-coated 96-wells microtiter plates (Chapters 2 and 7). After a positive well has been identified, the cells are cloned. The original positive well will often contain more than one clone of the hybridoma cells and single-cell cloning has to be performed. In cloning by limiting dilution (Goding, 1986), cells are distributed over a number of wells at a dilution so that some wells should not show cellular growth. By repeating this process two or three times (recloning), single-cell clones are obtained and medium volumes can be expanded. The antibody concentration in the medium is about 2-50 μg/ml (Tijssen, 1985). Standard procedures for the preparation, purification and characterization of Mabs are described in laboratory manuals (e.g. Harlow and Lane, 1988, Campbell, 1984, and Goding, 1986). The usefulness of Mabs stems from three characteristics: their specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities. The production of Mabs allows the isolation of reagents with a unique, chosen specificity. Because all of the antibodies produced by descendants of one hybridoma cell are identical, Mabs are powerful reagents for testing for the presence of a desired epitope. In addition, one unique advantage of hybridoma production is that impure antigens can be used to produce specific antibodies. The limitations of hybridoma technology include the extensive commitment of time, labor and expense, the requirement for animal use and specialized cell culture facilities, and the expertise needed to prepare and screen large number of hybridomas to select the best ones (Lipman et al., 2005).

Compared with the development of Pabs, the developments of Mabs is laborious and this technology is applicable only for tests that are intended for a large (worldwide) and stable market (i.e. pesticide, mycotoxin and beta-lactam assays). Pabs can be generated much more rapidly, at less expense, and with less technical skills than is required to produce Mabs. One can reasonably expect to obtain Pabs within several months of initiating immunizations, whereas the generation of hybridomas and subsequent production of Mabs can take up to a year or longer in some cases (Lipman et al., 2005), with no guarantee for a better performance. Märtlbauer et al. (1994) compared Mabs and Pabs for the detection of antibiotics and sulfonamides and with

most Pabs (five out of six) a better sensitivity was obtained.
In the present study, Mabs were developed against sulfonamide-derivatives (Part I) and dihydrostreptomycine (Chapter 7). Kohen et al. (2000) raised another Mab against sulfamethazine with the purpose to produce anti-idiotype antibodies (recognizing the binding site of the Mab). This Mab was also identified as an antibody recognizing several important sulfonamides (Haasnoot et al., 2000b), and was used in this research to develop a fast multi-sulfonamide biosensor immunoassay for the analysis of broiler sera (Chapter 4).

Rabs. Until the late 1980s, the production of antibodies relied primarily on animal immunisation. From then, the development of molecular methods for the expression of recombinant antibody fragment in bacteria and techniques for production and screening of combinatorial libraries has opened a wide range of opportunities for the selection of recombinant antibodies and their engineering (Maynard and Georgiou, 2000). Several *in vitro* methods have been developed for the production of antibodies (Bradbury et al., 2003), but the most commonly used technology is phage display (Brichta et al., 2005). Phage display refers to the display of functional foreign peptides, proteins or antibody fragments on the surface of a bacteriophage. This is accomplished by fusion of the DNA coding sequences of the protein to be displayed into the phage genome to the gene encoding one of the phage surface proteins. Surface display of the antibodies allows affinity selection of antibodies by exposing the phage library to immobilized antigen molecules. The captured phage particles are eluted from the antigen, amplified by infecting *Escherichia coli* host cells and used in a subsequent round of affinity selection. After the final round of affinity selection, phage particles are amplified in order to prepare and characterize their displayed antibodies individually and the monoclonal phage population with the desired binding specificities can be isolated.
With regard to antigenic specificity, different types of phage libraries are used for selection of specific recombinant antibodies: (1) a specific library sourced from immunized animals, (2) a single-pot (general) library created with no specificity against a particular analyte which avoids the use of laboratory animals (Willats, 2002). The most popular recombinant antibody format is the single-chain variable fragment (scFv), containing the variable domains of the heavy and light chain (Figure 11) linked to a single protein by a polypeptide linker. It contains a complete binding site and it is the smallest effective antibody fragment (26-27 kDa). Spinks et al. (1996) used this technique to study the development of broad-specificity (generic) antibodies to beta-lactams and aminoglycosides. Other Rab applications are described for pesticides (Kramer, Hock, 1996 and 2003) and potato glycoalkaloids (Kamps-Holtzapple, Stanker, 1996). In comparison to Pabs and Mabs, Rabs, using the phage display technique, can be prepared faster, in more automatic process and with reduced

consumption of laboratory animals (Brichta et al., 2005).
The multi-sulfonamide Mab 27G3 producing hybridoma cell line (Chapter 2) was used by Korpimäki et al. (2002, 2003 and 2004a,b) for the development and modification of multi-sulfonamide scFv molecules. Random mutant libraries were created with error-prone PCR over the whole scFv coding region. Several of the obtained mutants had significantly altered binding properties and improved broad specificity. In a competitive time-resolved fluoroimmunoassay, one of the first selected mutants (A.3.5) showed an improved sensitivity towards several sulfonamides compared with the wild-type Mab (Korpimäki et al., 2002). However, they displayed a low sensitivity towards important sulfonamides, such as sulfamethazine, sulfadimethoxine and sulfaquinoxaline. In the following study these authors generated new antibody libraries by recombining the previously enriched libraries with DNA-shuffling and introducing new mutations with error-prone PCR and oligonucleotide-directed mutagenesis (Korpimäki et al., 2003). In the time-resolved fluoroimmunoassay, the selected mutant from that study (M.3.4) showed an improved sensitivity for all 13 sulfonamides tested, and even sulfamethazine, sulfadimethoxine and sulfaquinoxaline could be measured below the 100 ng/ml level (Korpimäki et al, 2004b). Both Rabs (A.3.5 and M.3.4) were used in the present study for the development and evaluation of multi-sulfonamide inhibition biosensor immunoassays (Chapters 5 and 6).

1.5 PERFORMANCE OF ANALYTICAL METHODS

With the publication of Commission Decision 2002/657/EC from 12 August 2002 (EC, 2002a), new defined analytical parameters came into force which, until then, were to a large extent unknown to the analytical community. The decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant and it replaced terms as limit of detection, limit of determination etc. The detection capability (CCβ) was defined as the smallest content of a substance that may be detected, identified and/or quantified in a sample with an error probability of β. The minimum required performance limit (MRPL) was defined as the minimum content of an analyte in a sample which at least has to be detected and confirmed. According to the definitions described in this decision, the biosensor immunoassays (BIAs) used in this study are quantitative screening methods because they determine, by means of calibration curves, the amount of substances expressed as numerical values of appropriate units (ng ml^{-1} or g^{-1}). Furthermore, the BIAs are classified as screening methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput, and are used to sift large numbers of samples for potential

non-compliant results and should be designed to avoid false compliant results. Only those analytical techniques for which it can be demonstrated that they are validated and have a false compliant rate of <5% at the level of interest shall be used for screening purposes in conformity with Directive 96/23/EC (EC, 1996b). In the case of a suspected non-compliant result, this result should be confirmed by a confirmatory method which provides information on the chemical structure of the analyte. In the case of substances for which no permitted limit has been established, the CCα can be established for quantitative and qualitative assays by analyzing at least 20 blank materials per matrix to be able to calculate the signal-to-noise ratio and three times the signal-to-noise ratio can be used as the decision limit. In the case of substances with an established permitted limit such as an MRL (section 1.1), CCα can be established by analyzing at least 20 blank materials per matrix fortified with the analyte at the permitted limit. The concentration at the permitted limit plus 1.64 times the corresponding standard deviation equals the decision limit. The detection capability (CCβ) can be established by analyzing 20 blank samples spiked with analyte(s) at the decision limit. The value of CCα plus 1.64 times the standard deviation of the within-laboratory reproducibility of the measured content equals the detection capability. In Chapter 9 of this thesis, these new parameters were established for the detection of flumequine in tissue and serum.

1.6 AIMS AND SCOPE

Within this research, SPR-based biosensors from Biacore were used to develop and study the application of multiplex biosensor immunoassays (using multi-component antibodies and/or combined immunoassays with serially connected flow channels) in the animal food chain for the detection of antibiotics classified within three different groups; (i) sulfonamides, (ii) aminoglycosides and (iii) quinolones. Different types of antibodies (polyclonal, monoclonal and recombinant mutants) were compared in different assay formats (direct and inhibition) and combined (multiplexed) assays were developed and tested with different sample materials (milk, serum and tissue). Sulfonamides were chosen as model compounds to study the development and performance of group-specific or multi-component antibodies (Part I and II).

The scientific challenges of this part of the study were: (i) the development, selection and improvement of multi-sulfonamide antibodies raised against the generic structure of sulfonamides, (ii) the study of the performance of the different antibodies and optimize their application for the multi-sulfonamide detection in the optical biosensors, and (iii) the development of a well-based answer to the questions whether biosensor immunoassays can be used to analyze blood serum for the prediction of the levels in tissue, and whether such assays can be combined with serological assays

for anti-pathogens. To this aim, two sulfonamide derivatives were prepared and linked to carrier proteins in such a way that the common sulfonamide group was distal to the proteins (Chapter 2). The performances of Mabs and/or Pabs against these derivatives were compared with those of compound-specific Pabs in ELISA (Chapter 3). Multi-sulfonamide biosensor immunoassays for the fast detection of sulfonamides in chicken sera were developed and are described in Part II. These assays used multi-sulfonamide monoclonal antibodies (Chapter 4) and recombinant mutated antibodies (Chapter 5). Mutant antibodies were applied for the biosensor detection of sulfonamides in sera of treated broilers and, to predict the levels in tissue, results were compared with tissue levels as determined by LC-MS (Chapter 6).

Mabs against aminoglycosides were used to study the performance of different biosensor immunoassay formats for the analysis of milk samples (Part III). In this part of the study the scientific challenges were: (i) the investigation of different assay formats (direct, inhibition and combined (multiplexed)) and discovery of the best assay format for the multiplex detection of antibiotics in milk, (ii) the development of the easiest and still effective sample preparation procedure to deal with the foreseen matrix interferences from milk, and (iii) the clarifications of the interactions and interferences of combined immunoassays in the multiplexed format. In the direct assay format, the antibodies were immobilized onto the sensor chip surface and the binding of aminoglycosides was measured directly and compared with the inhibition assay format, using chips coupled with antibiotic-protein conjugates in which solutions for the observed matrix effects from milk were studied (Chapter 7). The inhibition assay format, with chips directly immobilized with the aminoglycosides, was applied in an assay in which four flow channels were serially connected to develop a multiplex immunoassay for the simultaneous detection of five aminoglycosides in milk (Chapter 8). For the detection of fluoroquinolones, a multi-quinolones antiserum was developed previously. However, flumequine has a deviating structure and the cross-reactivity in the multi-quinolones assay towards flumequine was low. For that reason, a biosensor immunoassay for flumequine in serum and muscle was developed in Chapter 9. In a subsequent study (outside the scope of this thesis), this assay was combined with the multi-quinolones assay to obtain a dual-assay format for the detection of all interesting fluoroquinolones in muscle (Marchesini et al., 2007).

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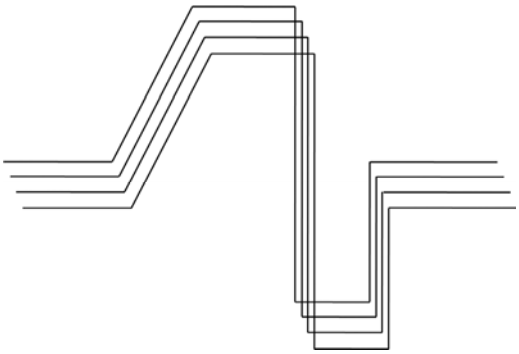
Part I

From compound-specific to group-specific antibodies for sulfonamides



Chapter 2

Monoclonal antibodies against a sulfathiazole derivative for the immunochemical detection of sulfonamides

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ABSTRACT

To prepare monoclonal antibodies (Mabs) against the generic part of sulfonamides, a sulfathiazole derivative was chemically linked to carrier proteins in such a way that the aromatic amino group, common to all sulfonamides, was distal to the proteins. Four mice were immunized with the sulfathiazole-protein derivatives. The spleen cells of one of the mice were fused with myeloma cells to produce hybridomas of which the supernatants were screened in an indirect ELISA (iELISA) for the presence of sulfathiazole antibodies. After cloning, positive supernatants were tested in a competitive iELISA (ciELISA) for inhibition with 18 sulfonamides. This resulted in four different Mabs (all IgG1 kappa light chain) which recognized several sulfonamides. By use of the best Mab (27G3) and an optimized ciELISA protocol, eight structurally different sulfonamides showed 50% inhibition at concentrations less than 100 ng ml^{-1} or 5 ng/well. However, other relevant sulfonamides (such as sulfadimidine, sulfatroxazole and sulfachloropyrazine) were detected at a high level only with this Mab. This means that the ciELISA (with the best Mab) showed a broad specificity for sulfonamides but the sensitivity towards the different sulfonamides varied too much to call it a generic sulfonamide ELISA.

INTRODUCTION

The sulfonamides are a group of antimicrobial agents applied in veterinary as well as human medicine for the treatment and prophylaxis of bacterial infections (Saschenbrecker & Fish, 1980). As a result, foods derived from animals treated with sulfonamides may be contaminated with these drugs. Maximum residue limits (MRLs) for sulfonamides have been established in many countries. In Canada, the European Union and the USA, an MRL of 100 μg kg^{-1} is set for total sulfonamides in edible tissues (Food and Drug Regulations, 1991; EU Regulation, 1999); in Japan, an MRL of 20 μg kg^{-1} is applied for total sulfonamides. In the Netherlands, at least nine sulfonamides are allowed to be used in veterinary medicine and, ideally, control methods should detect the presence of the total sulfonamides.

Today, Integral Production Chain Systems demand faster on-site (farmhouses) and/or on-line (slaughterhouses) test systems. Immunoassays are capable of detecting low amounts of residues in many samples in a short time. Such a high-volume ELISA system for slaughterhouse screening of sulfadimidine (= sulfamethazine) in swine plasma/serum (approximately 2400 serum/plasma samples in an 8 h working day) has been described (Ram *et al.*, 1991). Antisera to sulfadimidine and other sulfonamides have been raised using hapten conjugates prepared by diazotization of the aromatic amino group (Ram *et al.*, 1991; Renson *et al.*, 1993) or by introducing spacers such as glutaraldehyde (Fleeker & Lovett, 1985; Dixon-Holland & Katz, 1988; Märtlbauer *et al.*, 1992) or a succinyl group (Jackman, 1992; Haasnoot *et al.*, 1996). However, antibodies developed by these procedures are very specific in relation to other sulfonamides. Ideally, it would be more efficient to develop an immunoassay that could detect a number of different sulfonamides. Sheth & Sporns (1991) were the first who reported about the development of such an ELISA based on polyclonal antibodies (Pabs). They chemically linked a sulfathiazole derivative to proteins in such a way that the aromatic amino group, common to all sulfonamides, was distal to the protein. A subset of the Pabs developed against this hapten was competitive with different sulfonamides. By use of the best competitive ELISA protocol, nine structurally different sulfonamides showed 50% inhibition at concentrations of less than 5 μg ml^{-1}. Assil *et al.* (1992) used a similar approach to develop such an ELISA for longer sulfonamides with *N*1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide linked to proteins as the immunization agent. By the application of an affinity-purified fraction of the polyclonal serum, they produced an ELISA which showed 50% inhibition with seven sulfonamides at concentrations less than 10 μg ml^{-1}.

Muldoon *et al.* (1999) were the first who reported about the development of generic monoclonal antibodies (Mabs) against sulfonamides and use a *N*-sulfanilyl-4-aminobenzoic acid hapten-protein conjugate as the immunogen. The sensitivities of

their best Mab, referred to as Sulfa-1, for sulfanitran, sulfapyridine and sulfathiazole (expressed as IC_{50} values), were 1.41, 22.8 and 322 ng ml^{-1}. However, the sensitivities towards other sulfonamides ranged from 3 to 100 μg ml^{-1}. The detection of sulfonamides at the μg ml^{-1} level is not very useful for the screening of sample materials at the MRL levels (20–100 ng ml^{-1}/g^{-1}).

In this study, we report about the results of experiments in which we followed the approach of Sheth and Sporns (1991) and used a sulfathiazole derivative coupled to carrier proteins [keyhole limpet haemocyanin (KLH) and bovine serum albumin (BSA)] as immunogens. Instead of Pabs, as applied by Sheth & Sporns (1991), we investigated the possibility of producing generic Mabs to develop an ELISA in which several sulfonamides could be detected at the ng ml^{-1} level.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), ovalbumin (OVA), *N*-hydroxysuccinimide, polyethylene glycol 1500 (PEG 1500, 50%), ammonium bicarbonate, dimethyl sulfoxide (DMSO), sulfadimidine (= sulfamethazine), sulfamethoxazole, sulfamethoxypyridazine, sulfamerazine, sulfaguanidine, sulfadimethoxine, sulfadiazine, sulfachloropyridazine, sulfacetamide, sulfapyridine, sulfathiazole, sulfamethizole and sulfisoxazole were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). Sulfadoxine, sulfachloropyrazine, sulfaquinoxaline and Visking dialysis tube were supplied by Serva (Heidelberg, Germany) and sulfamethoxydiazine by Bayer (Leverkusen, Germany). Sulfatroxazole was a gift from Leo Pharmaceutical Products (Weesp, the Netherlands). (2-Amino-4-thiazolyl)-acetic acid, *N*-acetylsufanilyl chloride and 1,3-dicyclohexylcarbodiimide were obtained from Aldrich Chemie (Zwijndrecht, the Netherlands). Magnesium sulfate, methanol, sodium hydrogen carbonate, sodium chloride, ethyl acetate, urea, glycerol, phosphoric acid, pyridine, iso-propanol, chlorotrimethylsilane and dimethylformamide were supplied by Merck (Darmstadt, Germany).

Rabbit anti-mouse immunoglobulins-horseradish peroxidase (RAM-HRP) was purchased from DAKO (Denmark). ELISA microtiter plates, tissue culture plates (96- and 24-wells) and culture flasks (5 and 20 ml) were obtained from Costar (Cambridge, MA, USA). Dulbecco modified Eagle's medium (DMEM) and hybridocult were obtained from Bio Whittaker (Verviers, Belgium) and Fetal Clone I (FCI) was from HyClone (Logan, Utah, USA). Hypoxanthine/thymidine (HT; 50x) and aminopterin (A; 100x) were obtained from Life Technologies (Breda, the Netherlands).

Solutions of tetramethylbenzidine (TMB) peroxidase substrate and peroxide were obtained from Kirkegaard and Perry Labs (Gaithersburg, MD, USA). Specol was

obtained from DLO Institute for Animal Science and Health (ID-DLO, Lelystad, the Netherlands). The BCA protein assay reagents were obtained from Pierce (Rockford, ILL, USA).

Preparation of the sulfathiazole derivative

The sulfathiazole derivative *N*1[4-(carboxymethyl)-2-thiazolyl] sulfanilamide (TS) was prepared according to a modified procedure previously described by Sheth and Sporns (1991).

(2-Amino-4-thiazolyl)-acetic acid methyl ester

A solution of (2-amino-4-thiazolyl)-acetic acid (7.8 g) in dry methanol (100 ml) was treated at 0 °C with 20 ml of chlorotrimethylsilane and thereafter refluxed for 2 days. Solvents were removed on a Rotavapor and the residue was dissolved in ethyl acetate (250 ml). The solution was washed four times with 50 ml of an aqueous saturated solution of sodium hydrogen carbonate, until the pH of the carbonate solution was > 7, followed by a two times washing with 30 ml of an aqueous saturated solution of sodium chloride. The solution was dried by the addition of magnesium sulfate which was removed by filtration and the solvent evaporated under reduced pressure on a Rotavapor. The solid residue was recrystallized from a mixture of ethyl acetate (30 ml) and methanol (5 ml) to give 2.5 g of (2-amino-4-thiazolyl)-acetic acid methyl ester. Thin layer chromatography (TLC) on silica gel 60 plate (Merck, Germany) with ethyl acetate/ether/ethanol (65/30/5) as eluent resulted in one spot and the melting point was determined as 122–125 °C. The product was confirmed by NMR: ^{1}H NMR (DMSO-d_6, 200 MHz): δ 3.46 (d, J = 0.4 Hz, 2H); 3.60 (s, 3H); 6.32 (s, 1H); 6.92 (br s, 2H). ^{13}C NMR (DMSO-d_6, 50 MHz); δ 36.8 (t); 51.6 (q); 103.2 (d); 144.3 (s); 168.3 (s); 170.6 (s).

N4-Acetyl-N1-[4-[(methoxycarbonyl)methyl]-2-thiazolyl]-sulfanilamide

To a solution of (2 amino-4-thiazolyl)-acetic acid methyl ester (1.27 g) in dry pyridine (5 ml) was added *N*-acetylsulfanilyl chloride (1.8 g) at 0 °C. This mixture was stirred thereafter for 24 h at room temperature. To this mixture, 2 ml of ethanol was added and the pH was adjusted to 3 by the addition of 4 M hydrochloric acid. The precipitate, which was formed after 10–20 min, was filtered off and dried under vacuum which resulted in 2.1 g of product with a melting point of 225 °C. *N*4-Acetyl-*N*1-[4-[(methoxycarbonyl)methyl]-2-thiazolyl]-sulfanilamide was confirmed by NMR: ^{1}H NMR (DMSO-d_6, 200 MHz): δ 2.05 (s, 3H); 3.61 (s, 3H); 3.62 (d, J = 8.4 Hz, 2H); 6.60 (s, 1H); 7,65 (s, 4H); 10.28 (s, 2H). ^{13}C NMR (DMSO-d_6, 50 MHz): δ 24.1 (q); 32.7 (t); 52.1 (q); 105.6 (d); 118.4 (d); 127.0 (d); 130.7 (s); 136.7 (s); 142.5 (s); 168.3 (s); 168.9 (s); 169.1 (s).

N*1-*[4-(Carboxymethyl)-2-thiazolyl]sulfanilamide (TS)

*N*4-Acetyl-*N*1-[4-[(methoxycarbonyl)methyl]-2-thiazolyl]-sulfanilamide (1 g) was dissolved in 25 ml of 2 M sodium hydroxide. This solution was refluxed for 5 h and after cooling to room temperature, the pH was lowered to 4 by the addition of 4 M hydrochloric acid. The solution was extracted three times with 25 ml of ethyl acetate. The ethyl acetate was dried over magnesium sulfate and evaporated which resulted in 0.2 g of the product. The product was confirmed by NMR: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.43 (d, J = 7.2Hz, 2H); 6.50 (s, 1H); 6.57 (d, J = 8.4Hz, 2H); 7.42 (d, J = 8.4 Hz, 2H).

Coupling of TS to proteins

Preparation of N-hydroxysuccinimide (NHS) ester

TS (62 mg), *N*-hydroxysuccinimide (34 mg) and 1,3-dicyclohexyl carbodiimide (44 mg) were reacted overnight in 1.5 ml of dry dimethylformamide at 4 °C. The dicyclohexylurea precipitate was removed by filtration and the clear solution was used for coupling to proteins.

Coupling to proteins

Protein (KLH, BSA and OVA) solutions (1 ml of 5 mg ml^{-1} of PBS) were cooled in an ice bath, 0.5 ml of the NHS ester solution was added and the pH was adjusted to 7.6. The mixture was stirred for 21 h at 4 °C, thereafter transferred to a dialysis tube and dialyzed against 8 M urea (1 l) followed by dialyzing against 0.5 M ammonium bicarbonate (4 l) and finally against 0.25 M ammonium bicarbonate (4 l). The protein concentrations (0.41, 0.28 and 2.19 mg ml^{-1} for BSA, KLH and OVA, respectively) were determined with the BCA test. The protein solutions were lyophilized and stored at –20 °C before use.

Production of Mabs

Immunization of mice

Four female mice (Balb/c Ola Hsd), 10–12 weeks old, were immunized subcutaneously (sc) with 50 μg of BSA- or KLH-hapten conjugates in 100 μl of PBS emulsified with 100 μl of Specol. Booster injections (sc) with 25 μg of the immunogen in 100 μl of PBS emulsified with 100 μl of Specol were given at 2-week intervals. Blood was taken from each mouse prior to the first immunization (pre-immune) and 1 week after each booster injection. The sera were tested by an indirect ELISA for antibodies against TS coupled to OVA.

Fusion and cloning

The four mice were primed intraperitoneally with 25 μg of immunogen in 200 μl PBS 4–5 days before spleen cell isolation. One mouse was selected for the production of hybridomas and the spleen cells of the other mice were stored in liquid nitrogen. Hybridoma cells secreting Mabs to the sulfathiazole derivative were prepared using murine myeloma P3/NS-1/1-Ag4–1 (NS-1), non-secreting mouse myeloma (American Type Culture Collection, Rockville, MA, USA) as the fusion partner. The myeloma cells were fused with splenocytes prepared from mouse 98008 at a ratio of 1:2 using PEG 1500 precipitation. Cells were suspended in HAT selection medium [culture medium (DMEM, supplemented with 8% FCI + 1% hybridocult + 0.5% P/S) + 1% HT + 1% A] and plated in 96-well tissue cell culture plates at 100000 cells/well. After one week, the medium was changed to HT medium (culture medium + 1% HT) and five days thereafter, by culture medium. From day 12 to 14, hybridoma supernatants were tested for antibodies to the sulfathiazole derivative using the indirect ELISA (iELISA). Cultures containing cells secreting antibodies to the conjugate were further tested in the iELISA for antibodies recognizing free sulfonamides. Positive cultures were immediately cloned by limiting dilution (average 0.5 cells/well; Goding, 1986) until stable (2–3 clonings). The Mab-producing clones of interest were transferred from the culture in the 96-well plate to 0.5 ml of culture medium in a 24-well plate. After the 24-well culture became dense, the culture was transferred to 5 or 20 ml culture flasks. Colonies were propagated, frozen (overnight at –80 °C in a freezing container filled with isopropyl alcohol) in culture media containing 12% dimethyl sulfoxide and stored in nitrogen. The immunoglobulin subclass of Mabs secreted by each clone was determined by the Mouse Monoclonal Antibody Isotyping Kit (IsoStrip) of Boehringer Mannheim (Germany).

Indirect ELISA (iELISA)

Determination of antibody titers

TS-OVA in coating buffer (50 ng/100 μl/well) was used as the solid-phase immobilized agent. After overnight incubation at 4 °C or 1 h at room temperature, the wells were emptied and blocked with 0.1% OVA in coating buffer (50 mmol l^{-1} sodium carbonate; pH 9.6) for at least 1 h at room temperature. The plate was emptied and stored in 25% glycerol in coating buffer. Prior to use, the plate was washed four times with washing buffer. To each well, 100 μl of a serial dilution of serum or culture supernatant were added. The plate was incubated at room temperature for 1 h and washed four times with washing buffer. Subsequently, 100 μl of rabbit anti-mouse IgG-HRP (RAM-HRP) in a 1:2500 dilution in PBST were added to each well and incubated for 1 h at room temperature. After washing the plate, the bound peroxidase was assessed by adding 100 μl of a tetramethylbenzidine peroxidase

substrate system (a freshly prepared mixture of solution of TMB peroxidase substrate and peroxidase; 1/1; v/v). After incubation in the dark for 20–30 min at room temperature, the reaction was stopped by adding 100 µl aliquots of 1 mol l^{-1} phosphoric acid and the colored product of the peroxidase reaction was measured at 450 nm using an Argus 400 microplate reader (Canberra Packard, Downers Grove, IL, USA). Serum titers were defined as the final dilution which resulted in an absorbance of 2.0.

Competitive iELISA (ciELISA) for characterization of antibody specificity

After washing the coated and blocked plate (see determination of antibody titers), 50 µl of a standard solution of sulfonamides was added to the well followed by 50 µl of diluted serum or culture supernatant dilutions. The plates were treated further as described before. In total 18 sulfonamides were used for competition (see Figure 1). In the case of testing culture supernatants, the individual sulfonamides were added to the ELISA in amounts of 0.01, 0.1, 1 and 10 µg ml^{-1} which corresponds to 0.5, 5, 50 and 500 ng $well^{-1}$. In the case of competition in blood serum, standard solutions of 1000 µg ml^{-1} (5000 ng $well^{-1}$) were included also.

Optimization experiments for the ciELISA

A calibration curve of sulfathiazole (0.1–100 ng ml^{-1}) was used during the optimization experiments of the ciELISA with the Mabs. Three concentrations of coating (TS-OVA) of the microtiter plate (5, 25 and 50 ng of protein 100 $µl^{-1}$ $well^{-1}$) were compared. The concentration of the Mabs was varied. The conditions of the first incubation were varied in time (0.5, 1, 2 and 4 h and overnight) and in temperature (4–6 °C, room temperature and 37 °C). These incubations were performed with and without mild shaking. The concentration of HRP-labeled second antibody was varied and the conditions of the second incubation were varied as with those applied for the first incubation.

RESULTS

Synthesis of sulfathiazol derivative

The synthetic route for the sulfathiazol derivative (TS) is shown in Figure 2. TS was prepared according to a modified procedure previously described by Sheth & Sporns (1991). The (2 amino-4-thiazolyl)-acetic acid methyl ester was prepared in another way by using a chlorotrimethylsilane mediated esterification procedure. The so-prepared ester had a melting point of 122–125 °C. Sheth & Sporns reported a melting point of 169–171 °C. They probably measured the melting point of the hydrochloric acid salt.

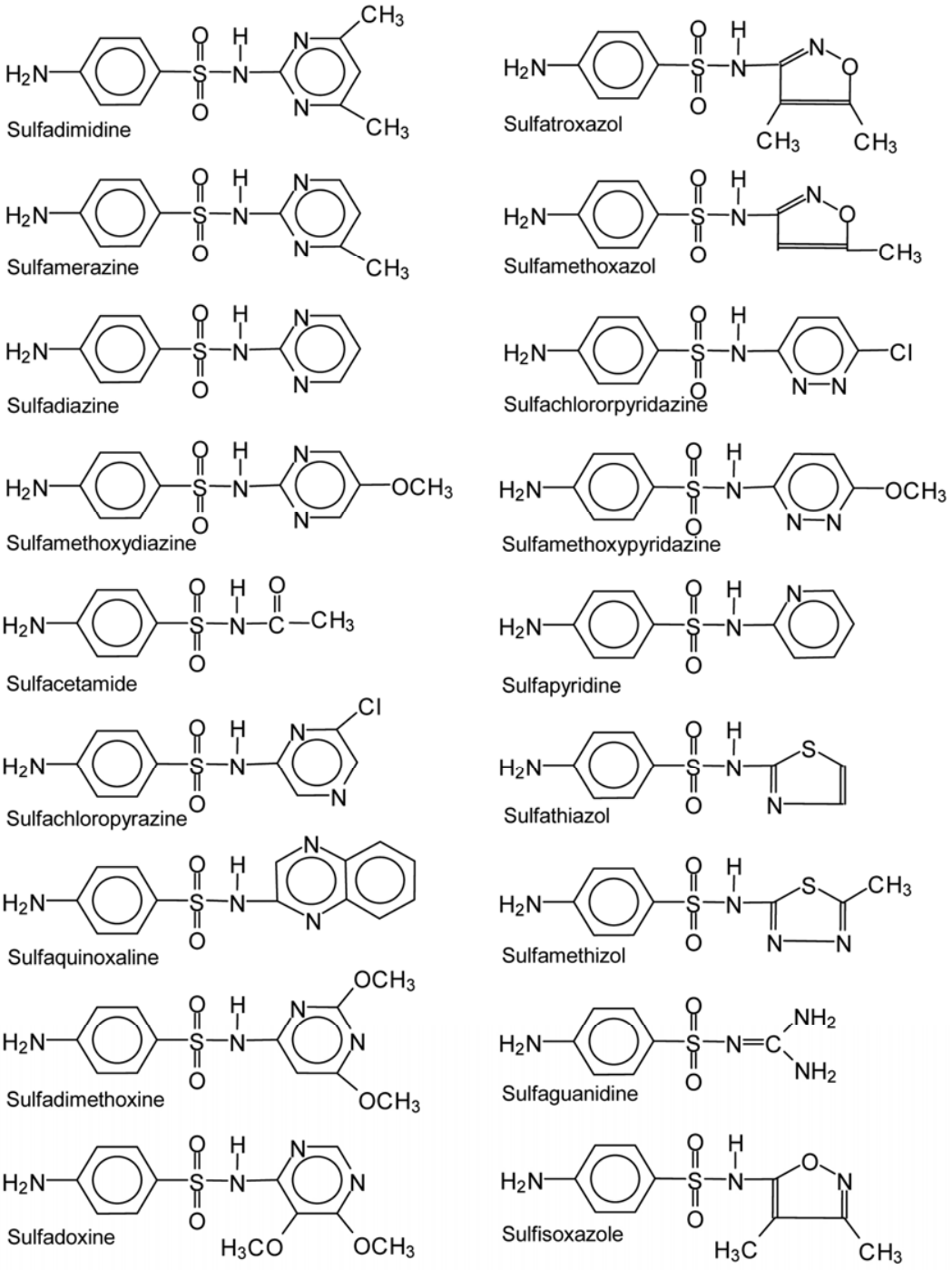

Figure 1. Molecular structures of the sulfonamides used in the competition experiments.

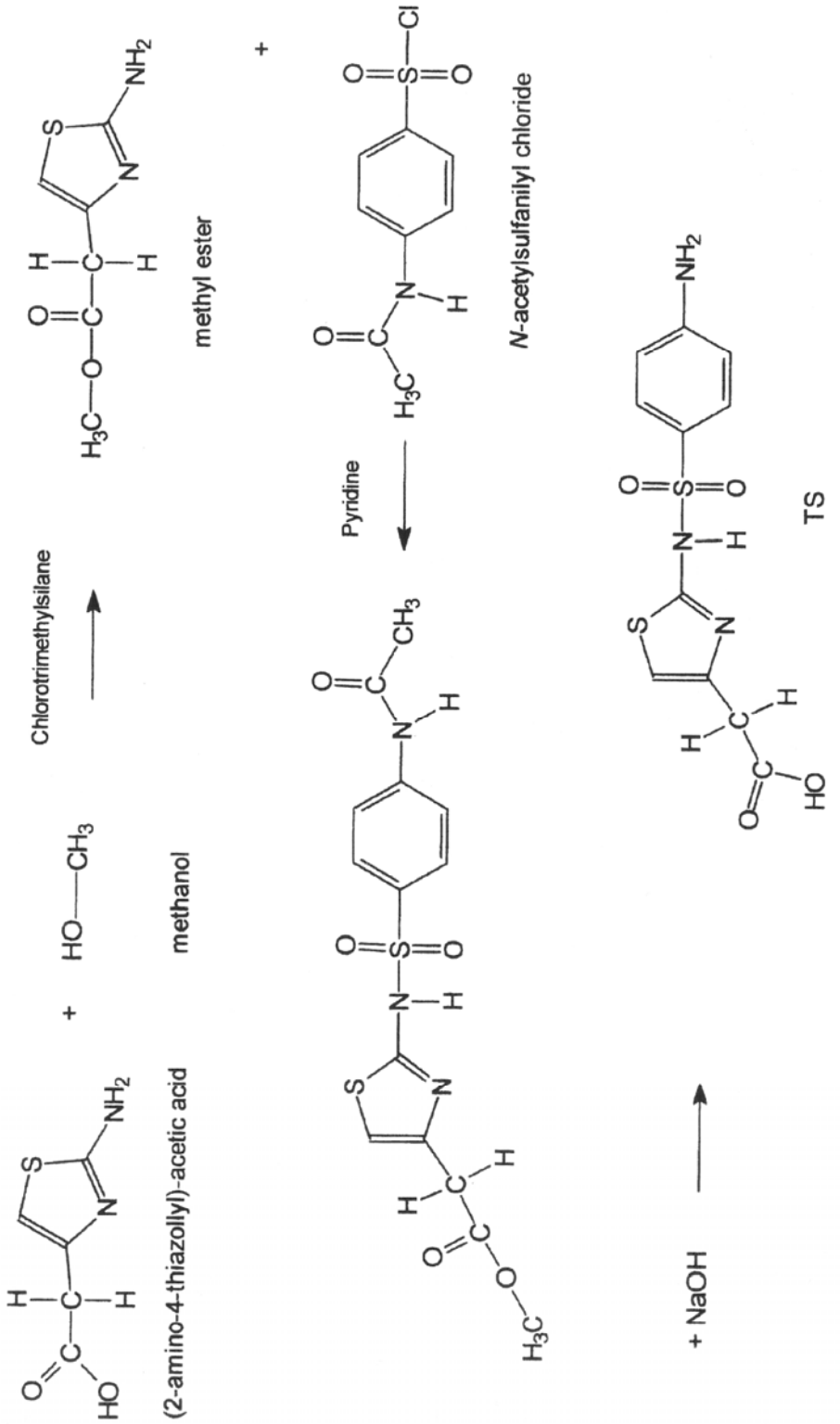

Figure 2. Synthetic route to the sulfathiazole derivative N1[4-(carboxymethyl)-2-thiazolyl]-sulfanilamide (TS).

For the synthesis of *N*4-acetyl-*N*1-[4-[(methoxycarbonyl)methyl]-2-thiazolyl]-sulfanilamide, Sheth & Sporns (1991) refluxed the reactants in pyridine for 1.5 h and extracted the product in hot water. In our hands this resulted in tarry products. Therefore, we performed the synthesis at room temperature by stirring for 24 h and the product formed was precipitated at pH 3 and filtered.

The TS–protein ratios were estimated according to Erlanger *et al.* (1957) and by the presence of free aromatic amino groups measured by the modified Bratton Marshall (BM) test as was described by Garden & Sporns (1994). For the BSA conjugates, the ratio determined according to Erlanger *et al.* was 15 mol of hapten mol^{-1} of protein and 33 mol mol^{-1} with the BM reaction.

For the KLH conjugate, the ratio was 276 mol mol^{-1} (Erlanger) and 203 mol mol^{-1} (BM) (estimated molecular weight of KLH 3000 kDa).

Determination of antisera titer and specificity

Antisera from the four mice (98005–98008) immunized with KLH- and BSA-hapten recognized the hapten conjugated to OVA.

Table 1. The concentration of sulfonamide (μg ml^{-1}) required to result in a 50% inhibition using the bleeding sera of the four mice in the ciELISA

Sulfonamide	**Mouse 98005**	**Mouse 98006**	**Mouse 98007**	**Mouse 98008**
Sulfathiazole	0.9	0.2	1.5	0.6
Sulfamethizole	0.9	0.4	4	0.5
Sulfachloropyridazine	2	0.8	5	2
Sulfamethoxypyridazine	3	5	7	3
Sulfamethoxazole	15	15	50	8
Sulfadimethoxine	50	5	>100	50
Sulfapyridine	50	9	50	20
Sulfamethoxydiazine	90	5	>100	>100
Sulfamerazine	90	10	90	50
Sulfadiazine	100	8	>100	50
Sulfaquinoxaline	100	15	>100	>100
Sulfadoxine	>100	>100	>100	>100
Sulfachloropyrazine	>100	80	>100	>100
Sulfisoxazole	>100	>100	>100	>100
Sulfatroxazole	>100	>100	>100	>100
Sulfaguanidine	>100	>100	>100	>100
Sulfadimidine	>100	>100	>100	>100
Sulfacetamide	>100	>100	>100	>100

No reaction was observed when pre-immune sera were reacted with OVA hapten or when sera from immunized mice were tested against ovalbumin. Thus, a specific reaction to the hapten was apparent. For mice immunized with KLH-TS (98005 and 98006) the titers ranged from 1/3000 (mouse 98005) and 1/9000 (mouse 98006) after the first injection to 1/200000 (98005) and 1/400000 (98006) after the fourth injection. For mice immunized with BSA-TS (98007 and 98008) the titers ranged from 1/3500 (98007) and 1/5000 (98008) after the first injection to 1/50000 (98008) after the third injection and 1/150000 (98007) after the fourth injection.

The optimum final dilutions of the bleeding sera were determined as 1/400000 (98005), 1/800000 (98006), 1/400000 (98007) and 1/150000 (98008). Using these optimum final dilutions, the bleeding sera of the four mice were tested in the ciELISA for competition with 18 sulfonamides (see Table 1). With all sera, free sulfathiazole and sulfamethizole competed at the lowest concentration (0.2–4 μg ml^{-1} at 50% inhibition), followed by sulfachloropyridazine (0.8–5 μg ml^{-1}) and sulfamethoxy-pyridazine (3–7 μg ml^{-1}). Of the mice immunized with KLH-TS, the bleeding serum of mouse 98006 showed competition at the lowest concentration. Of the mice immunized with BSA-TS, the bleeding serum of mouse 98008 showed competition at the lowest concentration. The spleen cells of mouse 98008 were used for the fusion experiment.

Production of Mabs

After two booster injections, one of the mice (98008) was primed with a final booster injection. Four days later, spleen cells were isolated. One month later, and with an extra booster injection, the same procedure was performed with the other three mice (98005, 98006 and 98007) of which the spleen cells were stored in liquid nitrogen. The fresh spleen cells of mouse 98008 were used for fusion with myeloma cells. Screening of 550 wells in the iELISA resulted in 21 wells with culture supernatant reactive to the OVA-TS coated plate. These supernatants were screened with the iELISA for optimum dilution and with the ciELISA for competition with sulfathiazole. Competition was found in five of these supernatants (3A1; 4E10; 14G11; 26E5 and 27G3). These hybridoma cells were cloned by limiting dilutions (2–3 times). This procedure resulted in four stable clones (27G3; 14G11; 26E5 and 4E10) and supernatants of 20 ml culture flasks were tested for optimum dilution. The supernatants of clones 27G3, 14G11, 4E10 and 26E5 could be used in final dilutions of 1/12000, 1/10000, 1/10000 and 1/750, respectively. This means that three of the clones produce high amounts of Mabs and one is a less producer.

The subclass of the Mabs present in the four supernatants was determined by the Mouse Monoclonal Antibody Isotyping Kit and all were identified as IgG1 with kappa light chain. Of the four Mabs, 27G3 showed the best sensitivity towards

sulfathiazole and was used in experiments to increase the sensitivity of the ciELISA using a sulfathiazole calibration curve.

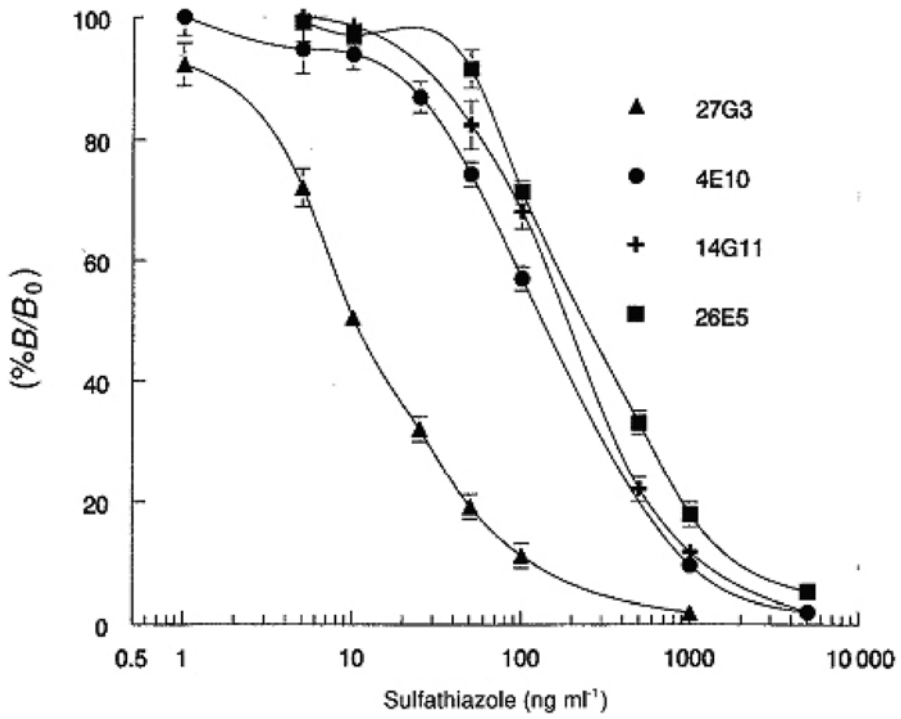

Figure 3. Sulfathiazole calibration curves (n = 4) obtained with the four Mabs in the optimized ciELISA (B0 = maximum absorbance and B = absorbance of standards). Error bars are ± standard deviation.

Decreasing the amount of immobilized OVA-TS (2 and 10 times) did not result in an improved sensitivity. The conditions of the first incubation (temperature, time and with or without mild shaking) were varied. The best results were obtained with short incubation times (1 h) at 4–6 °C under mild shaking conditions. Then the conditions of the second incubation step were varied. Here the optimum conditions were 1 h at 4–6 °C under mild shaking conditions. Under these optimized conditions, the sensitivity of the ciELISA for sulfathiazole improved six times. Using the other three Mabs under optimum incubation conditions, the sensitivity of the ciELISA towards sulfathiazole increased also. However Mab 27G3 showed the best sensitivity with a working range (20–80% inhibition) between 3.5 and 50 ng ml^{-1} (see Figure 3).

In the optimized ciELISAs, the competition with 18 sulfonamides was tested and the results are shown in Table 2. Mab 14G11 is most sensitive for sulfathiazole, sulfamethoxypyridazine, sulfamethizole and sulfachloropyridazine. The other sulfonamides showed much less competition (< 2–6% cross-reactivity compared to sulfathiazole). Mab 26E5 is the most sensitive for competition with sulfisoxazole and sulfamethizole followed by sulfaquinoxaline, sulfathiazole and sulfadimethoxine. Mab 4E10 is the most sensitive for competition with sulfamethizole and six other sulfonamides showed 50% inhibition at a concentration <100 ng ml^{-1}. Of the four

Mabs, 27G3 is the most sensitive towards sulfathiazole and seven other sulfonamides showed 50% inhibition below 100 ng ml^{-1} and this Mab was selected as the best of the four Mabs.

Table 2. The concentration of sulfonamide (ng ml^{-1}) required to result in a 50% inhibition using the Mabs obtained from mouse 98008 in the ciELISA and as described by Sheth & Sporns (1991) using a sub-population of a polyclonal antiserum (Pab)

Sulfonamide	**Mab 27G3**	**Mab 4E10**	**Mab 14G11**	**Mab 26E5**	**Pab***
Sulfathiazole	10	130	180	250	5000
Sulfamethizole	0.6	2	250	80	1300
Sulfachloropyridazine	4	4	300	1500	–
Sulfamethoxypyridazine	15	13	210	1500	–
Sulfamethoxazole	150	30	3000	3000	3300
Sulfadimethoxine	250	100	> 10000	500	4700
Sulfapyridine	30	150	3000	> 10000	1300
Sulfamethoxydiazine	30	30	> 10000	> 10000	–
Sulfamerazine	500	250	> 10000	> 10000	> 25000
Sulfadiazine	80	80	> 10000	> 10000	1600
Sulfaquinoxaline	1200	3000	> 10000	200	–
Sulfadoxine	30	800	> 10000	> 10000	–
Sulfachloropyrazine	1800	8000	> 10000	> 10000	–
Sulfisoxazole	250	80	> 10000	50	–
Sulfatroxazole	7000	2000	> 10000	3000	–
Sulfaguanidine	500	> 10000	> 10000	> 10000	–
Sulfadimidine	8000	7000	> 10000	> 10000	> 25000
Sulfacetamide	1800	> 10000	> 10000	> 10000	18 000

–not tested; * as described by Sheth & Sporns (1991)

DISCUSSION

Sheth & Sporns (1991) produced Pabs in rabbits which were immunized with TS conjugated to LPH and to BSA. Indirect ELISA's performed on collected sera, using TS conjugated OVA as the coating, resulted in high titers in each pair of rabbits. Serum dilutions of 1/200000 gave three times background absorbance. In our study, the bleeding sera of the four mice resulted also in high titers. Serum dilutions of 1/50000 to 1/400000 still resulted in an absorbance of 2.0. This means that the immunogens (TS-BSA and TS-KLH) are well recognized by the mice.

Sheth & Sporns (1991) tested their rabbit sera in a ciELISA with high concentrations (25 μg ml^{-1}) of the different sulfonamides and no significant decrease in absorbance

was noted. Free sulfonamides at high concentrations could not compete with bound hapten and they concluded that most of the antibodies had greater affinity for the TS-protein than for free TS and other sulfonamides.

In our study, using the bleeding sera of the four mice, we noticed a significant decrease of absorbance due to the competition with sulfathiazole, sulfamethizole, sulfachloropyridazine, sulfamethoxypyridazine and sulfamethoxazole at the 25 μg ml^{-1} level or lower (see Table 1).

As expected, sulfathiazole (part of the immunogen) and sulfamethizole (with an extra nitrogen and methyl group only) showed competition at the lowest concentration.

Sheth & Sporns (1991) selected a sub-population of antibodies with specificity for the aromatic amino portion of sulfonamides by the use of a deviating sulfonamide derivative coupled to proteins as the coating in the ELISA. This procedure resulted in a ciELISA in which nine structurally different sulfonamides showed 50% inhibition at concentrations ranging from 1.3 to 22 μg ml^{-1} (see Table 2). Assil *et al.* (1992) used a similar approach, with another immunogen, but applied an affinity-purified fraction of the polyclonal serum to obtain an ELISA which showed 50% inhibition with seven sulfonamides at concentrations less than 10 μg ml^{-1}.

In our study, the production of generic Mabs against a sulfathiazole derivative, four Mabs were obtained and one of them (27G3, see Table 2) showed 50% inhibition below the 10 μg ml^{-1} (= 10000 ng ml^{-1}) level with all sulfonamides tested. Eight of these sulfonamides showed 50% inhibition below the 100 ng ml^{-1} level.

In general, we see major improvements with respect to the sensitivity and cross-reactivity by the use of two of the Mabs (27G3 and 4E10; see Table 2) compared with our Pabs (see Table 1) and those described by Sheth & Sporns (1991; see Table 2). However, even with our best Mab (27G3), some essential sulfonamides (like sulfadimidine, sulfatroxazole, sulfaquinoxaline and sulfachloropyrazine) are detected at a high level only. Therefore, although one of the Mabs showed a broad specificity and a nice sensitivity towards several sulfonamides, the ciELISA was not considered to be generic towards all sulfonamides. On the basis of the results obtained in this study, the authors think that the production of a generic competitive assay, based on just one Mab, for the detection of sulfonamides will be very difficult. Future experiments will be focused on the application of this Mab in immunoaffinity chromatography to investigate its use as a generic sulfonamide antibody in non-competitive systems.

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Chapter 3

Sulfonamide antibodies: from specific polyclonals to generic monoclonals

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ABSTRACT

Polyclonal antibodies (Pabs) against eight different sulfonamides were raised in rabbits. The aromatic amino group, common to all sulfonamides, was used for linking the different sulfonamides to the carrier proteins (bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH)) and enzyme (horseradish peroxidase (HRP)), using different coupling procedures. The competitive direct ELISAs (cdELISAs) developed with these antisera and HRP-conjugates showed high sensitivity (0.2–8.0 ng ml^{-1} at 50% inhibition) and high specificity. The performances of these antibodies were compared with Pabs raised in mice against two sulfonamide derivatives (*N*1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide (TS) and *N*1-[4-methyl-5-[2-(4-carboxy-ethyl-1-hydroxyphenyl)]-azo-2-pyridyl]sulfanilamide (PS)) linked to proteins (BSA and KLH) in such a way that the common aromatic amino group was distal to the protein. In competitive indirect ELISAs (ciELISAs), these Pabs recognized several structurally different sulfonamides. The Pabs from mice immunized with TS–BSA reacted with sulfonamides containing thiazolyl, thiadiazolyl, pyridazinyl and isoxazolyl groups. The Pabs from mice immunized with PS–KLH reacted with sulfonamides containing pyrimidinyl, pyridazinyl, quinoxalinyl and pyridinyl groups. The spleen cells of the mice were fused with myeloma cells to obtain monoclonal antibodies (Mabs) producing hybridomas. So far, with only one of the mice (immunized with TS–BSA), this resulted in four different Mabs which recognized several sulfonamides. By use of the best Mabs (27G3 and 4E10) and an optimized ciELISA protocol, eight structurally different sulfonamides showed 50% inhibition at concentrations less than 100 ng ml^{-1} or 5 ng/well. However, other relevant sulfonamides (such as sulfadimidine, sulfatroxazole and sulfachloropyrazine) were detected at a high level only.

INTRODUCTION

Sulfonamides are antimicrobial agents applied in veterinary as well as human medicine for the treatment and prophylaxis of bacterial infections. As a result of veterinary application, foods derived from animals treated with sulfonamides may be contaminated with these drugs. In the Netherlands and the USA (1998), at least nine different sulfonamides are approved for veterinary application and maximum residue limits (MRLs) in edible tissues have been established for total sulfonamides (100 μg kg^{-1} in Canada, the USA and the European Union and 20 μg kg^{-1} in Japan).

Sulfonamides are derivatives of sulfanilamide (*p*-aminobenzene sulfonic acid). They can be described as *N*1- or *N*4-substituted sulfanilamides depending on whether the substitution is on the amido or aromatic amino group, respectively. Substitution in the benzene ring of sulfonamides usually yields inactive compounds such as the main metabolites (*N*4-acetylated sulfonamides). Substitutions at the amido group with heterocyclic aromatic nuclei yields compounds with varying degrees of anti-microbial activity.

Immunoassays are capable of detecting low amounts of residues in many samples in a short time. For most immunoassays, antisera to sulfonamides have been raised using hapten conjugates prepared by diazotization of the aromatic amino group (Ram *et al.*, 1991, Renson *et al.*, 1993) or by introducing spacers such as glutaraldehyde (Fleeker & Lovett, 1985; Dixon-Holland & Katz, 1988; Märtlbauer *et al.*, 1992) or a succinyl group (Jackman, 1992; Haasnoot *et al.*, 1996) which resulted in specific assays.

Another approach is to raise antibodies against the aromatic amino group which is common to all sulfonamides. Sheth and Sporns (1991) were the first who reported about the development of such antibodies. They chemically linked a sulfathiazole derivative (*N*1-[4-(carboxymethyl)-2-thiazolyl] sulfanilamide) to proteins in such a way that the aromatic amino group was distal to the protein. Polyclonal antibodies (Pabs) were raised in rabbits and by the use of a subset of the antibodies, nine structurally different sulfonamides showed 50% inhibition at concentrations of less than 5 μg ml^{-1}. Assil *et al.* (1992) used a similar approach to develop such an ELISA for longer sulfonamides with (*N*1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2- pyridyl]-sulfanilamide) linked to proteins as the immunisation agent. By the application of an affinity-purified fraction of the polyclonal antiserum, they produced an ELISA which showed 50% inhibition with seven sulfonamides at concentrations less than 10 μg ml^{-1}.

Muldoon *et al.* (1999) used an *N*-sulfanilyl-4 aminobenzoic acid–protein conjugate to develop cross-reactive antibodies to sulfonamide antibiotics. Most of the antibodies that were detected in the blood of immunized mice ($n = 5$) recognized the free hapten but only the antibodies from one mouse recognized other free sulfonamides also. The spleen cells of this mouse were used for hybridoma production which resulted in one

monoclonal antibody (Mab)-producing clone. This Mab recognized eight sulfonamide drugs at levels below 1 μg/well or 10 μg ml^{-1}.

Haasnoot *et al.* (2000) followed the approach of Sheth and Sporns (1991) to prepare Mabs against the aromatic amino group of sulfonamides. By use of the best Mab in a competitive indirect ELISA (ciELISA), all sulfonamides tested (n = 18) showed 50% inhibition at concentrations less than 0.5 μg/well or 10 μg ml^{-1} and eight of them at concentrations less than 5 ng/well or 0.1 μg ml^{-1}. However, other relevant sulfonamides were detected at a high level only. Therefore, to date, true generic antibodies against sulfonamides are not available and to detect sulfonamides of interest, either specific antibodies (poly- or monoclonal) have to be used or new generic antibodies have to be developed.

In this study, the development of competitive direct ELISAs (cdELISA), using Pabs raised against eight different sulfonamide-conjugates, is described. The performances of these ELISAs (sensitivity and cross-reactivity towards 18 sulfonamides (see Figure 1)) are compared with those obtained by ciELISAs in which Pabs raised against two different sulfonamide derivatives (prepared according to Sheth & Sporns (1991) and Assil *et al.* (1992)) are used. The performances of the different Pab-based ELISAs are compared with those obtained with the previously developed 'generic' Mabs (Haasnoot *et al.*, 2000) and suggestions for improvements are given.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), ovalbumin (OVA), succinic anhydride, polyethylene glycol 1500 (PEG 1500, 50%), dimethyl sulfoxide (DMSO), 1-ethyl-3[3-(dimethyl amino)propyl] carbodiimide (EDC), Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvant (FIA), sodium azide, sulfadimidine (= sulfamethazine), sulfamethoxazole, sulfamethoxypyridazine, sulfamerazine, sulfaguanidine, sulfadimethoxine, sulfadiazine, sulfachloropyridazine, sulfacetamide, sulfanilamide, sulfapyridine, sulfathiazole, sulfamethizole and sulfisoxazole were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). Sulfadoxine, sulfachloropyrazine, sulfaquinoxaline, sodium periodate, sodium borohydride and Visking dialysis tubing (36/32) were obtained from Serva (Heidelberg, Germany) and sulfamethoxydiazine from Bayer (Leverkusen, Germany). Sulfatroxazole was a gift from Leo Pharmaceutical Products (Weesp, the Netherlands). Dioxane, Tween-20, pyridine, sodium hydroxide, di-sodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, potassium dihydrogen phosphate, sodium chloride, sodium carbonate, sodium hydrogen carbonate, ethanol, saccharose, tris(hydroxymethyl)-aminomethane (Tris), glutaraldehyde, phosphoric

acid, hydrochloric acid, sodium acetate, glycerol and sodium nitrite were obtained from Merck (Darmstadt, Germany).
Horseradish peroxidase (HRP) was obtained from Boehringer (Mannheim, Germany). Solutions of tetramethylbenzidine (TMB) peroxidase substrate and peroxide were obtained from Kirkegaard and Perry Labs (Gaithersburg, MD, USA). Goat anti-rabbit IgG (GAR) was obtained from Caltag Laboratories (Burlingame, CA, USA) and rabbit anti-mouse immunoglobulins–horseradish peroxidase (RAM-HRP) from DAKO (Denmark). Ammonium sulfamate was supplied by Fluka Chemie (Zwijndrecht, the Netherlands). 1,4-Butanediol diglycidyl ether, deuterium oxide and sodium deuterium oxide were supplied by Acros Chimica (‘s Hertogenbosch, the Netherlands).
Centriprep concentrators (30) were supplied by Amicon, Inc. (Beverly, MA, USA). Specol was obtained from DLO Institute for Animal Science and Health (ID-DLO, Lelystad, the Netherlands). Dulbecco modified Eagle’s medium (DMEM) and hybridocult were obtained from Bio Whittaker (Verviers, Belgium) and Fetal Clone I (FCI) was from HyClone (Logan, Utah, USA). Hypoxanthine/thymidine (HT; 50x) and aminopterin (A; 100x) were obtained from Life Technologies (Breda, the Netherlands). ELISA microtiter plates, tissue culture plates (96- and 24-wells) and culture flasks (5 and 20 ml) were obtained from Costar (Cambridge, MA, USA).

Linking of sulfonamides to proteins via the aromatic amino group

Several procedures for the coupling of sulfonamides, via the aromatic amine group, to the carrier proteins (BSA and KLH) and the enzyme (HRP) were applied.

Succinylation
Succinylated sulfonamides were coupled to carrier proteins and HRP according to a previous described procedure for the preparation of sulfamethazine–protein conjugates (Haasnoot *et al.*, 1996). A mixture of the sulfonamide (1 g) and succinic anhydride (0.5 g) was refluxed for 1.5 h in anhydrous ethanol containing 10 µl of anhydrous pyridine. The mixture was then evaporated to dryness and refluxed for 30 min in 10.5 ml of a mixture of ethanol and water (6:4.5; v:v) and again evaporated to dryness. The residue was dissolved in 10.5 ml of a mixture of ethanol and water (6:4.5; v:v) and filtered through a sintered glass filter. The filtrate was allowed to crystallize overnight at –20 °C. The crystals were collected by filtration (0.45 µM filter; Millipore) and washed with a cold (–10 to –20 °C) mixture of ethanol: water (6:4.5; v:v). The collected crystals were dried and stored at –20 °C until used.

Coupling to BSA. The succinylated sulfonamide (50 mg) was dissolved in 10 ml of a mixture of Tris buffer (0.05 M; pH 7.5) and dioxane (1:1; v:v). 1-Ethyl-3[3-

(dimethylamino)- propyl] carbodiimide (EDC (40 mg dissolved in 0.5 ml water)) was added. This solution was added to a BSA solution (10 mg dissolved in 0.5 ml of phosphate buffer (0.5 mM)) and mixed for 1 h on a magnetic stirrer. The mixture was dialyzed against phosphate buffer (5 mM, pH 7) for 3 days (two changes of buffer per day). The sulfonamide–hemisuccinate–BSA was stored at –20 °C until used.

Coupling to HRP. The succinylated sulfonamide (5 mg) was dissolved in 0.5 ml of the mixture of the Tris buffer and dioxane (1:1; v:v) and 25 mg of EDC, dissolved in 0.5 ml of water, was slowly added. This solution was added to a HRP solution (58 mg dissolved in 3 ml of a mixture of PBS and water (1:2; v:v). The mixture was stirred on a magnetic stirrer for 3 h and dialyzed against PBS for 3 days (two changes of buffer per day). The dialyzed solution was stored at –20 °C until used.

Glutaraldehyde coupling

Sulfonamides were coupled to BSA via glutaraldehyde as described by Dixon-Holland & Katz (1988). The sulfonamide (350 mg) and BSA (600 mg) were dissolved in 75 ml of 2:1 solution of phosphate buffer (pH 7.2) and dioxane. To the mixture, 0.35 ml of 25% glutaraldehyde was added and stirred for 3 h at room temperature. The mixture was dialyzed for 6 days against PBS with buffer changes twice a day. The dialyzed solution was lyophilized and stored under desiccation at 4 °C until used.

Epoxy (bisoxirane) activation

Sulfonamides were coupled to epoxy activated BSA and HRP according to a previously described procedure (Lommen *et al.*, 1995).

Coupling to BSA. BSA (360 mg) was dissolved in D_2O (6 ml), 1,4-butanediol diglycidyl ether (93 mg) was added and the pH was adjusted to 10.9 with 0.5 M NaOD. After incubation (24 h at 27 °C) the reaction was stopped by the addition of NaH_2PO_4 (100 mg). The free 1,4-butanediol glycidyl ether was removed by applying the following procedure three times; dilution with phosphate buffer followed by concentration with a Centriprep (end volume 6 ml). To this epoxy activated BSA solution (6 ml), Na_2CO_3 (63 mg) was added. To 0.6 ml of this epoxy-activated BSA, 0.06 mM of a sulfonamide was added and the pH was adjusted to 10.8 ± 0.1 with sodium hydroxide (0.1 M) and, after a four days reaction at 27–30 °C, the solution was dialyzed against PBS for three days. The dialyzed solution was stored at –20 °C until used.

Coupling to HRP. HRP (200 mg) was dissolved in D_2O (4.5 ml), 0.5 ml of a solution of 1,4-butanediol diglycidyl ether (20 mg ml^{-1} D_2O) and the pH was adjusted to 10.9 with 0.5 M NaOD. After 24 h at 27 °C, the reaction was stopped by the addition of

NaH_2PO_4 (20 mg). The free 1,4-butanediol glycidyl ether was removed (see coupling to BSA). Sulfonamides were dissolved in 0.1 M Na_2CO_3 (10 mg ml^{-1}) and 0.5 ml of the sulfonamide solution was mixed with 0.5 ml of the epoxy-activated HRP solution. For reaction conditions and purification, the same procedure as described for the coupling to BSA was applied.

Periodate cleavage

For the coupling of some sulfonamides to HRP, the method described by Thomson and Sporns (1995) was followed. HRP (4 mg) was dissolved in 1.0 ml water; 0.2 ml of 0.1 M aqueous sodium periodate were added and the mixture stirred for 20 min at room temperature, transferred to dialysis tubing and dialyzed overnight against 4 l of 1 mM sodium acetate buffer (pH 4.4) at 4 °C. To dialysis tubing contents were added 40 µl of 200 mM carbonate buffer (pH 9.5), one drop of 1 M sodium hydroxide and 10 mg of the sulfonamide and the reaction mixture was stirred for 2 h at 4 °C. Sodium borohydride (4 mg ml^{-1}, 100 µl) was added and the mixture stirred for 2 h at 4 °C. The reaction mixture was dialyzed against three changes of PBS. The contents of the dialysis tubing were recovered and frozen at –20 °C.

Diazo-coupling

For the coupling of some sulfonamides to KLH, the diazo derivative coupling as described by Renson *et al.* (1993) was applied. The sulfonamide (10 mg) was dissolved in 2 ml hydrochloric acid (3.5 M) and 0.6 ml of a solution of sodium nitrite (10 mg ml^{-1}) was added dropwise to this mixture with stirring in the dark at 4°C. After 30 min, the reaction was stopped by addition of 0.75 ml of a solution of ammonium sulfamate (33 mg ml^{-1}). To a solution of KLH (10 mg in 6 ml PBS) 0.1 ml of the diazo derivative solution was slowly added and the pH was adjusted to 9.5 ± 0.5 by the addition of a few drops of sodium hydroxide (1 M). The reaction mixture was left for 6 h at 4 °C and unreacted material was removed by dialysis against PBS. The dialyzed solution was stored at –20 °C until used.

Linking of sulfonamide derivatives to proteins

Two sulfonamide derivatives were coupled to proteins in such a way that the common aromatic amino group was distal to the proteins.

Coupling of a sulfathiazole derivative

The synthesis of the sulfathiazole derivative *N*1[4-(carboxymethyl)-2-thiazolyl]-sulfanilamide (TS) and the coupling to proteins (KLH, BSA and OVA) was described before (Haasnoot *et al.*, 2000).

Coupling of N1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide (PS)

For the synthesis of PS and the coupling to proteins (KLH, BSA and OVA), the procedures as described by Assil *et al.* (1992) were applied.

Immunization

Rabbits

The sulfonamide–protein conjugates (0.75 ml of solutions of 0.5 mg ml^{-1} PBS (0.4 mg of conjugate)) were mixed with 0.75 ml of Freund's complete adjuvant. The emulsions were injected subcutaneously (s.c.) into New Zealand White rabbits. After 4 weeks, the rabbits were subcutaneously injected every 6 weeks with 0.4 mg of conjugate in Freund's incomplete adjuvant (booster injection) and blood samples were taken 1 and 2 weeks after the respective immunizations. The collected sera were stored at –20 °C.

Mice

Female mice (Balb/c Ola Hsd), 10–12 weeks old, were immunized (s.c.) with 50 μg of BSA- or KLH-TS or -PS conjugates in 100 μl of PBS emulsified with 100 μl of Specol. Booster injections (sc) with 25 μg of the immunogens in 100 μl of PBS emulsified with 100 μl of Specol were fiven at 2-week intervals. Blood was taken from each mouse prior to the first immunization (pre-immune) and 1 week after each booster injection. The sera were stored at –20 °C until used for testing for antibodies against TS or PS coupled to OVA by an indirect ELISA.

Production of Mabs

The mice were primed intraperitoneally with 25 μg of immunogen in 200 μl PBS 4–5 days before spleen cell isolation. Hybridoma cells secreting Mabs were prepared using murine myeloma P3/NS-1/1-Ag4–1 (NS-1), non-secreting mouse myeloma (American Type Culture Collerction, Rockville, MD, USA) as the fusion partner. The myeloma cells were fused with splenocytes at a ratio of 1:2 using PEG 1500 precipitation. Cells were suspended in HAT selection medium (culture medium [DMEM, supplemented with 8% FCI + 1% hybridocult + 0.5% P/S] + 1% HT + 1% A) and plated in 96-wells tissue culture plates at 100000 cells/well. After one week, the medium was changed to HT medium (culture medium + 1% HT) and five days thereafter, by culture medium. From days 12–14, hybridoma supernatants were tested for antibodies to TS or PS (conjugated to OVA) using the indirect ELISA. Cultures containing cells secreting antibodies to the conjugates were further tested in the competitive indirect ELISA for antibodies recognizing free sulfonamides. Positive

cultures were immediately cloned by limiting dilution (average 0.5 cells/well) until stable (2–3 clonings).
The Mab-producing clones of interest were transferred from the culture in the 96-well plate to 0.5 ml of culture medium in a 24-well plate. After the 24-well culture became dense, the culture was transferred to 5 or 20 ml culture flasks. Cultures were centrifuged and the supernatants stored at –20 °C until used. Colonies of interest were propagated, frozen (overnight at –80 °C in a freezing container filled with 2-propanol) in culture medium containing 12% dimethyl sulfoxide and stored in nitrogen.

ELISAs

(Competitive) direct ELISA
For the validation of the rabbit sera (antisera titers) and to determine the optimum dilution of the HRP-conjugates the direct ELISA was applied. Thereafter, when optimum dilutions of the reagents were known, the competitive direct ELISA was applied to determine the sensitivity and cross-reactivity towards other sulfonamides.

Direct ELISA (dELISA). Microtiter plates were coated overnight at 4–6 °C with 100 µl aliquots of goat anti-rabbit IgG (5 µg IgG ml^{-1}) in coating buffer (35 mmol l^{-1} sodium hydrogen carbonate + 15 mmol l^{-1} sodium carbonate (pH 9.6)). Plates were washed four times with PBS (pH 7.2) containing 0.05% Tween-20 (PBST) with a microplate washer (Wellwash Model 4 microplate washer, Denley Instruments, Billinghurst, Sussex, UK). For the determination of antibody titers, aliquots (50 µl) of serial dilutions (10^1 till 10^6) in PBST of the raw antiserum were pipetted (in columns 1–6 or 7–12 for another serum) in the microtiter plates. Aliquots (50 µl) of serial dilutions (10^1 till 10^6) in PBST of a sulfonamide–HRP conjugate were pipetted (in rows B–G). To rows A and H, 50 µl of PBST were added. The plate was incubated for 1 h at 4–6 °C and washed four times with PBST. The bound peroxidase was assessed by adding 100 µl of a TMB peroxidase substrate system (a freshly prepared mixture of solutions of TMB peroxidase substrate and peroxide; 1:1; v:v). After incubation in the dark for 20 min at room temperature, the reaction was stopped by adding 100 µl aliquots of 1 mol l^{-1} phosphoric acid and the colored product of the peroxidase reaction was measured at 450 nm using an Argus 400 microplate reader (Canberra Packard, Downers Grove, IL, USA). The antiserum titer was defined as the maximum dilution which resulted in an absorbance of 1.0 at the most possible dilution of the sulfonamide–HRP conjugate.

cdELISA. To the wells of the microtiter plate, 50 µl aliquots of buffer or standard solutions of sulfonamides were added followed by 25 µl quantities of diluted

antiserum and sulfonamide–HRP conjugate. The plates were treated further as prescribed above.

(Competitive) indirect ELISA

TS– or PS–OVA in coating buffer (50 ng/100 µl/well) was used as the solid-phase immobilized agents. After overnight incubation at 4 °C or 1 h at room temperature, the wells were emptied and blocked with 0.1% OVA in coating buffer. The plate was emptied and stored in 25% glycerol in coating buffer until used. Prior to use, the plate was washed four times with washing buffer.

Determination of antibody titers. To each well, 100 µl of a serial dilution of serum or culture supernatant was added. The plate was incubated at room temperature for 1 h and washed four times with washing buffer. Subsequently, 100 µl aliquots of rabbit anti-mouse IgG–HRP (RAM–HRP) in a 1:2500 dilution in PBST were added to each well and the plate incubated for 1 h at room temperature. After washing the plate, the bound peroxidase was assessed by adding 100 µl of a TMB peroxidase substrate system (a freshly prepared mixture of a solution of TMB peroxidase substrate and peroxidase; 1:1; v:v). After incubation in the dark for 20–30 min at room temperature, the reaction was stopped by adding 100 µl aliquots of 1 mol l^{-1} phosphoric acid and the coloured product was measured at 450 nm using an Argus 400 microplate reader (Canberra Packard, Downers Grove, IL, USA). Serum titers were defined as the final dilution which resulted in an absorbance of 1.0.

ciELISA. After washing the coated and blocked plate, 50 µl of a standard solution of sulfonamides (in PBST) was added to the well followed by 50 µl of a diluted serum or culture supernatant dilutions. The plates were treated further as described above.

RESULTS

ELISAs based on specific Pabs

The method described by Jackman (1992), using succinic anhydride to convert the aromatic amine group of the sulfonamide into a carboxylate group with a four-carbon spacer, worked well with sulfadimidine. This carboxylate group is used to couple the sulfonamide to the amine groups of the carrier proteins. As described before (Haasnoot *et al.*, 1996), the same procedure was used to produce the enzyme-conjugate. Other enzyme-conjugates (periodate cleavage and epoxy activation) were tested but a difference in ELISA performance was not observed. The different batches of antisera obtained after the 19 booster injections of rabbit 464 were analyzed for

optimum dilution and a high variation was observed. The titers increased in time from 20000 (after the first booster) till 200000 (after the 12th booster) and thereafter the titers fluctuated between 100000 and 200000. To obtain a larger batch of antiserum of constant quality, the different antisera obtained after the several booster injections were mixed to one batch of about 700 ml. In the cdELISA, this batch was used in a final dilution of 1:40000 which resulted in a calibration curve for sulfadimidine with 50% inhibition at 0.5 ng ml^{-1}. The ELISA showed cross-reactivity towards sulfamerazine (16%) and sulfadimethoxine (0.12%) and no cross-reactivity (< 0.1%) with the other sulfonamides (see Table 1).

Succinylated sulfadiazine coupled to BSA did result in an antiserum (rabbit 466) with a low titer. Another rabbit (474) was immunized with a BSA conjugate in which sulfadiazine was coupled via glutaraldehyde and this resulted in low titers also. The same rabbit was used for immunization with sulfadiazine coupled to epoxy activated BSA and an increase in titer (between 30 000 and 140 000) was observed. The antisera obtained after the seven booster injections were combined to obtain a larger batch of antiserum (280 ml). The performance of this batch in the cdELISA was poor (low maximum signal and bad sensitivity).

New immunizations (rabbit code MH3) with the diazo derivative of sulfadiazine coupled to KLH were more successful. The antiserum titer of the different blood samples obtained after the booster injections started at 20000 and rose to 320000 after the fifth booster injection and then varied from 50000 to 200000. The serum obtained after the fifth booster injection was used in the cdELISA in combination with sulfadiazine–HRP prepared according to the epoxy activation procedure and this resulted in a calibration curve with a 50% inhibition at 0.5 ng ml^{-1}. This ELISA showed cross-reactivity towards sulfathiazole (10%) and low cross-reactivity towards five other sulfonamides (see Table 1).

For the development of the sulfadoxine-ELISA, sulfadoxine was coupled to BSA via glutaraldehyde coupling. This immunogen (in rabbit 478) resulted in antisera with low titers and after the ninth booster the same rabbit was immunized with sulfadoxine coupled to epoxy-activated BSA. This resulted in an increase in antibody titer (up to 200000). A batch of antiserum (280 ml) was prepared of serum obtained after seven booster injections. This batch was used in the ELISA (final dilution of 1:4000) in combination with sulfadoxine–HRP (periodate coupling). This resulted in a calibration curve for sulfadoxine with 50% inhibition at 3.3 ng ml^{-1} and the ELISA showed no cross-reactivity towards other sulfonamides (see Table 1).

Table 1. The concentration of sulfonamide (ng ml^{-1}) required to result in a 50% inhibition (IC_{50}) in the different cdELISAs based on Pabs (% cross-reactivity).

Sulfonamide	cdELISA							
	Sulfa-dimidine	Sulfa-diazine	Sulfa-doxine	Sulfa-dimethoxine	Sulfa-troxazole	Sulfa-chloropyrazine	Sulfa-methoxazole	Sulfi-soxazole
Sulfadimidine	**0.5 (100)**	>10000 (<0.1)	>10000 (<0.1)	2800 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)
Sulfadiazine	800 (<0.1)	**0.5 (100)**	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	3200 (<0.1)	>10000 (<0.1)
Sulfadoxine	>10000 (<0.1)	>10000 (<0.1)	**3.3 (100)**	>10000 (<0.1)	480 (0.6)	>10000 (<0.1)	>10000 (<0.1)	50 (0.4)
Sulfadimethoxine	400 (0.12)	>10000 (<0.1)	>10000 (<0.1)	**1.1 (100)**	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	8000 (<0.1)
Sulfatroxazole	>10000 (<0.1)	5000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	**3.0 (100)**	>10000 (<0.1)	1200 (0.1)	0.9 (20)
Sulfachloropyrazine	2000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	**8.0 (100)**	>10000 (<0.1)	6000 (<0.1)
Sulfamethoxazole	>10000 (<0.1)	100 (0.5)	>10000 (<0.1)	>10000 (<0.1)	280 (1.1)	>10000 (<0.1)	**1.2 (100)**	100 (0.2)
Sulfisoxazole	>10000 (<0.1)	1700 (<0.1)	>10000 (<0.1)	5000 (<0.1)	45 (6.7)	>10000 (<0.1)	1200 (0.1)	**0.2 (100)**
Sulfathiazole	7000 (<0.1)	5 (10)	>10000 (<0.1)	>10000 (<0.1)	4000 (<0.1)	>10000 (<0.1)	90 (1.3)	>10000 (<0.1)
Sulfachloropyridazine	>10000 (<0.1)	500 (0.1)	>10000 (<0.1)	4000 (<0.1)	35 (8.6)	>10000 (<0.1)	330 (0.4)	5 (3.8)
Sulfamerazine	3 (16)	20 (2.5)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)
Sulfapyridine	3000 (<0.1)	50 (1.0)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	6000 (<0.1)	>10000 (<0.1)
Sulfaquinoxaline	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	8000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)
Sulfamethoxypyridazine	6000 (<0.1)	10000 (<0.1)	>10000 (<0.1)	3200 (<0.1)	2100 (0.1)	>10000 (<0.1)	10000 (<0.1)	3000 (<0.1)
Sulfaguanidine	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)
Sulfamethoxydiazine	>10000 (<0.1)	1000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	2000 (<0.1)
Sulfamethizole	>10000 (<0.1)	200 (0.2)	>10000 (<0.1)	10000 (<0.1)	1200 (0.2)	>10000 (<0.1)	1.2 (100)	30 (0.6)
Sulfacetamide	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)	>10000 (<0.1)

For the development of the sulfadimethoxine-ELISA, a rabbit (no. 477) was first injected nine times with sulfadimethoxine–BSA prepared according to the glutaraldehyde coupling. However, the titers were low and from the ninth booster injection another immunogen (sulfadimethoxine coupled to epoxy activated BSA) was used. An increase in antisera titers was observed (1:75000 to 1:250000). The antisera obtained after the seven booster injections were combined to obtain a batch with a volume of 280 ml. This batch could be used in the ELISA in a final dilution of 1:5000. In combination with sulfadimethoxine–HRP (prepared according the epoxy activation procedure) this resulted in an ELISA with a calibration curve for sulfadimethoxine with 50% inhibition at 1.1 ng ml^{-1}. The ELISA showed no cross-reactivity towards the other sulfonamides tested (see Table 1).

For the development of the sulfachloropyrazine-ELISA, the immunogen was prepared according to the epoxy activation method. The serum of rabbit 503 obtained after the fifth booster was used in the sulfachloropyrazine-ELISA (final dilution 1:2400) in combination with the HRP-conjugate prepared according to the epoxy activation procedure. This resulted in a calibration curve for sulfachloropyrazine which showed 50% inhibition at 8.0 ng ml^{-1} and cross-reactivity towards other sulfonamides was not observed (see Table 1).

For the preparation of antisera against sulfatroxazole and sulfamethoxazole, rabbits (nos 496 and 502) were injected with BSA-conjugates prepared according to the epoxy coupling procedure. The response towards these immunogens was low and after increasing the amount of injected immunogen (from 0.45 to 0.9 mg/injection) the response slowly increased. For both ELISA's, the sera obtained after the 10th booster injections were used (final dilution 1:1000) in combination with the HRP-conjugate prepared according to the periodate method. The calibration curve for sulfatroxazole showed 50% inhibition at 3.0 ng ml^{-1}. Cross-reactivities were seen for sulfachloropyridazine (8.6%) and sulfisoxazole (6.7%) and low cross-reactivities (0.1–1.1%) for some other sulfonamides (see Table 1). The calibration curve for sulfamethoxazole showed 50% inhibition at 1.2 ng ml^{-1} and this sulfamethoxazole-ELISA showed a 100% cross-reactivity with sulfamethizole and low cross-reactivity (0.1–1.3%) towards some other sulfonamides (see Table 1).

Sulfisoxazole was coupled to KLH according to the diazo-coupling procedure and the serum obtained after the tenth booster was used in the ELISA (final dilution 1:10 000) in combination with sulfisoxazole–HRP (periodate coupling). This resulted in an ELISA which showed 50% inhibition for sulfisoxazole at 0.2 ng ml^{-1}. This ELISA showed cross-reactivity towards sulfatroxazole (20%) and low cross-reactivity towards some other sulfonamides (0.2–3.8%, see Table 1).

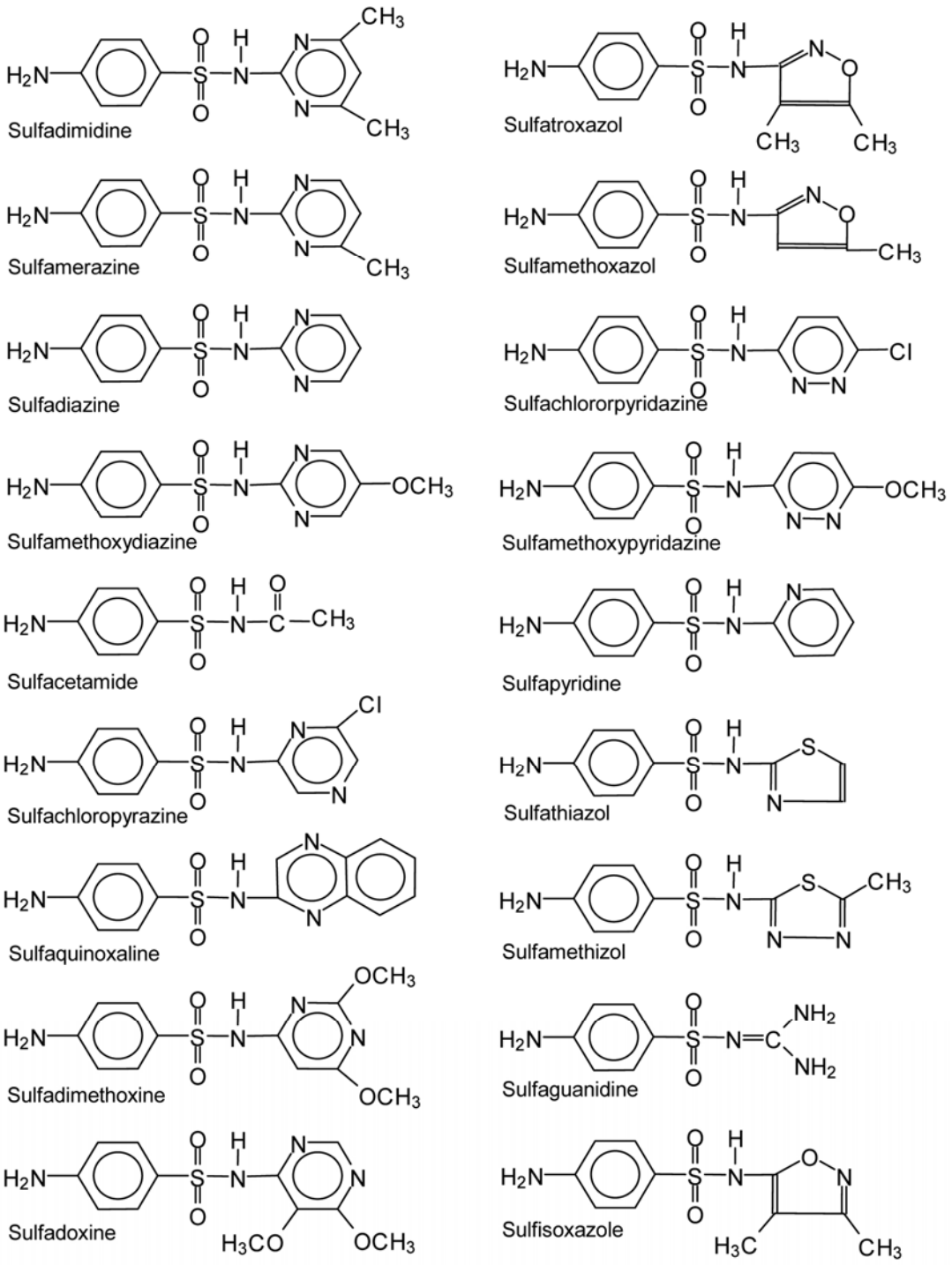

Figure 1. Molecular structures of the 18 sulfonamides used for cross-reactivity studies.

ELISAs based on generic Pabs

The sulfonamide derivatives TS and PS (see Figure 2) were coupled to BSA and KLH and these immunogens, with the aromatic amine group distal from the proteins, were used to immunize eight mice. Mice 98005 and 98006 were immunized with TS

coupled to KLH and mice 98007 and 98008 with TS coupled to BSA. Mice 98012 and 98013 were immunized with PS coupled to KLH and mice 98014 and 98015 with PS coupled to BSA. For the determination of antibody titers, the ciELISAs were used. Antisera from the mice immunized with TS-immunogens (98005–98008) recognized the hapten conjugated to OVA and no reaction was observed when pre-immune sera were added or when sera from the immunized mice were tested against OVA. Thus, a specific reaction to the hapten was apparent and the antisera showed high titers (130000–1200000 after the third or fourth booster injection) with both carrier proteins. This means that TS coupled to the proteins is very immunogenic.

The sera obtained after immunization with PS–protein conjugates showed acceptable titers with KLH as the carrier (120000 and 230000 after the fourth injection) and low titers with BSA as the carrier (3000 and 4000 after the fourth injection).

The six bleeding sera from the mice with acceptable titers were used in the ciELISAs in which 18 different sulfonamides (see Figure 1) were added at the 10 ppm level. The sera from mice 98005–98008 all showed comparable cross-reactivities towards the 18 sulfonamides. The cross-reactivities of the sera from mice 98012 and 98013 were also similar.

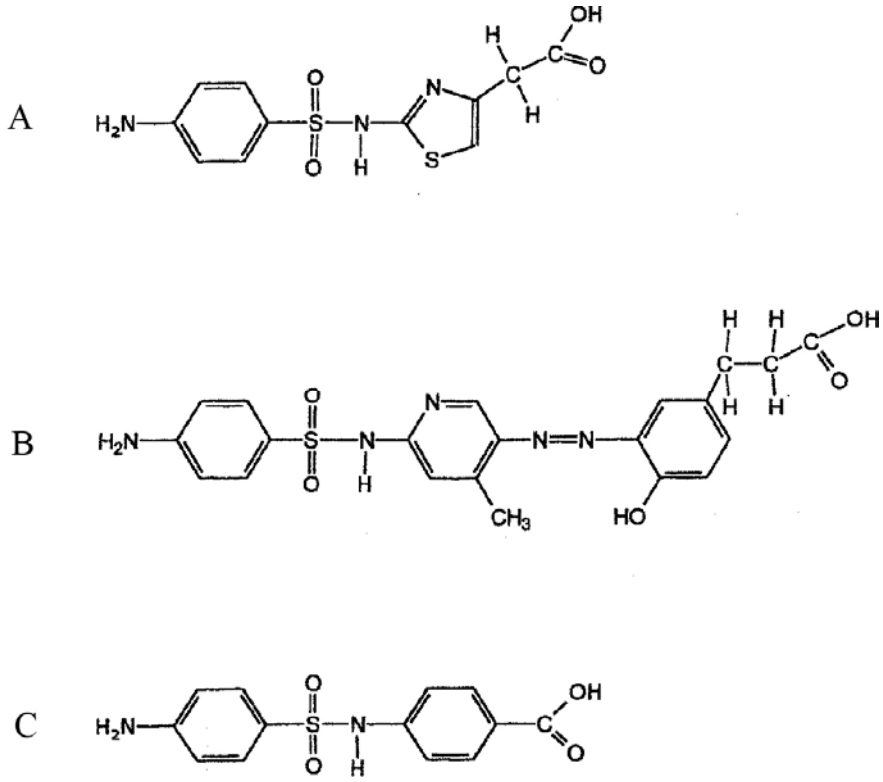

Figure 2. Molecular structures of the immunogens used for the development of generic Pabs and Mabs (A and B) and the structure of the hapten used by Muldoon et al. (1999; C). (A) = N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide (TS); (B) = N1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]sulfanilamide (PS); (C) = N-sulfanyl-4-aminobenzoic acid.

In Figure 3 the results are shown for two of these antisera. The Pabs from mouse 98008 (immunized with TS–BSA) showed good competition (> 50%) with five sulfonamides and the Pabs from mouse 98013 (immunized with PS-KLH) with nine sulfonamides. It is obvious that the blood obtained from the mouse immunized with TS reacted the best with those sulfonamides containing similar structures (sulfathiazole and sulfamethizole) but there is also a strong reaction with sulfonamides containing a pyridazinyl group (like sulfachloropyridazine and sulfamethoxypyridazine) or an isoxazolyl group (like sulfamethoxazole).
The antisera obtained from mouse 98013 showed a good reaction with sulfonamides containing a pyrimidinyl group (sulfadimidine, sulfamerazine, sulfadiazine, sulfamethoxydiazine and sulfadimethoxine), a pyridazinyl group (sulfamethoxy-pyridazine and sulfachloropyridazine), a quinoxalinyl group (sulfaquinoxaline) and a pyridinyl group (sulfapyridine). This serum was less reactive towards the sulfonamides containing a thiadiazolyl group (sulfamethizole) and an isoxazolyl group (sulfisoxazole).

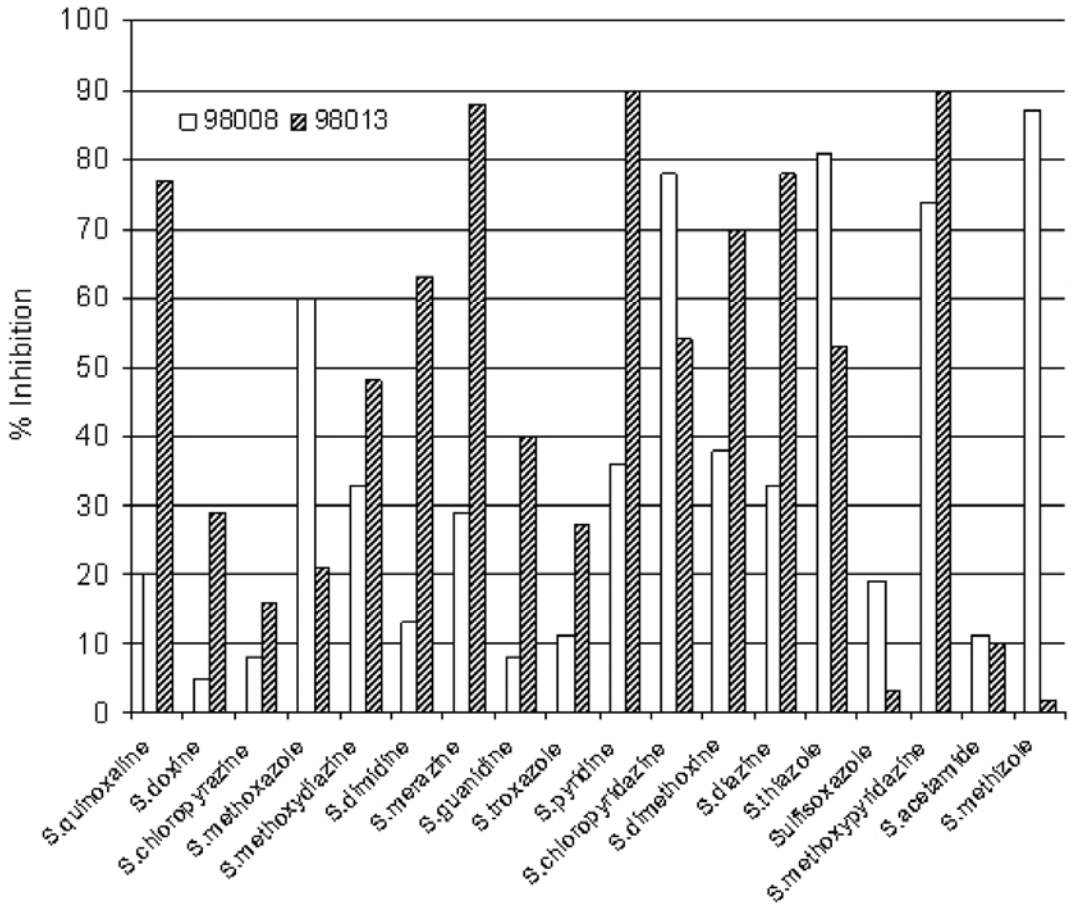

Figure 3. Inhibition (%) by 18 sulfonamides (at the 10 ppm level) in the ciELISA using bleeding sera of mouse 98008 (immunized with TS-BSA) and mouse 98013 (immunized with PS-KLH).

ELISAs based on generic Mabs

The spleen cells of mice 98008 and 98013 were used for fusion with myeloma cells. The hybridomas obtained with mouse 98013 did not produce antibodies specific for PS–OVA coated plates and this fusion was considered as failed. After the fusion with the spleen cells of mouse 98008, the culture supernatant of 21 wells showed reaction with TS–OVA coated plates. Competition with sulfonamides was found in five of these supernatants.

Table 2. Comparison of the inhibition concentrations at 50% (IC_{50}), expressed as ng/well, for the different sulfonamides used in ciELISA's with two of our Mabs (clone 27G3 and 4E10) with TS (see Figure 2) as the immunogen and the Mab (SULFA-1) of Muldoon et al. (1999) with N-sulfanyl-4-aminobenzoic acid (SUL; see Figure 2) as the immunogen.

Sulfonamide	IC_{50} (ng/well)		
	Clone 27G3	Clone 4E10	Clone SULFA-1
Sulfamethizole	0.03	0.1	7000
Sulfachloropyridazine	0.2	0.2	436
Sulfathiazole	0.5	6.5	32
Sulfamethoxypyridazine	0.7	0.6	-
Sulfapyridine	1.5	7.5	2.3
Sulfamethoxydiazine	1.5	1.5	-
Sulfadoxine	1.5	40	-
Sulfadiazine	4	4	976
Sulfamethoxazole	7.5	1.5	6000
Sulfadimethoxine	12.5	5	1572
Sulfisoxazole	12.5	2	20000
Sulfamerzine	25	12.5	374
Sulfaguanidine	25	>500	-
Sulfaquinoxaline	60	150	2500
Sulfachloropyrazine	90	400	-
Sulfacetamide	90	>500	1435
Sulfatroxazole	350	100	-
Sulfadimidine	400	350	7000
Sulfanitran	-	-	0.14
SUL	-	-	6.8
Sulfisomidine	-	-	314
Sulfasalazine	-	-	430
Sulfanilamide	-	-	6000
Sulfabenzamide	-	-	10000

–Not tested

These hybridoma cells were brought into limiting dilution (two or three times) and this procedure resulted in four stable clones (27G3; 14G11; 26E5 and 4E10) which all produced IgG1 kappa light chain antibodies. The preparation and evaluation of these Mabs were described before (Haasnoot *et al.*, 2000). By use of the best Mabs (27G3

and 4E10) and an optimized ciELISA protocol, eight structurally different sulfonamides showed 50% inhibition at concentrations less than 100 ng ml^{-1} or 5 ng/well (see Table 2). However, other relevant sulfonamides (such as sulfadimidine, sulfatroxazole and sulfachloropyrazine) were detected at a high level only.

DISCUSSIONS AND CONCLUSIONS

Most of the described immunoassays for sulfonamide detection are focused on one sulfonamide in which different coupling procedures for the preparation of the immunogens and enzyme conjugates are applied. In exception, Jackman *et al.* (1993) used succinylation for the coupling of 12 different sulfonamides to OVA and used these immunogens to raise Pabs in sheep. Although antisera titers were not described, the individual cdELISA (using sulfonamide–hemisuccinate–HRP) showed 50% inhibition in the range 0.2–5 ng ml^{-1}. In the present study, the succinylation worked well with sulfadimidine (high antibody titers and an high sensitivity ELISA) but with other sulfonamides the results were not so successful (low titers and low sensitivity). Trying several couplings, in our hands, the best general procedure for the coupling of the different sulfonamides seemed to be the use of epoxy-activated BSA.

A high variation of antibody titers in the sera obtained after the several booster injections was observed after all immunizations. Such variations in titers were reported also by Singh *et al.* (1989) who developed an enzyme immunoassay for screening sulfamethazine (= sulfadimidine). To obtain larger batches of Pabs with constant quality, some antisera obtained after different boosters were pooled.

Using the Pabs, the dose–response curves of the eight cdELISA's showed 50% inhibition between 0.2 and 8 ng ml^{-1}. These sensitivities are comparable to those reported by Jackman (1993) for his sulfonamide ELISA's.

Three of our cdELISA's (like the sulfadoxine-, sulfadimethoxine- and sulfachloropyrazine-ELISA) were very specific towards the sulfonamide used as the immunogen and cross-reactivities towards other sulfonamides were not observed (see Table 1). The sulfadimidine-ELISA cross-reacted with sulfamerazine (16%). Fodey *et al.* (1997), Fleeker and Lovett (1985) and Märtlbauer *et al.* (1992) described a cross-reactivity for sulfamerazine of 10, 30 and 56%, respectively, in their sulfadimidine-ELISA's. The sulfadiazine-ELISA showed cross-reactivity towards sulfathiazole (10%) and low cross-reactivity towards sulfamerazine (2.5%) and sulfapyridine (1%). Märtlbauer *et al.* (1992) also reported cross-reactivities to sulfathiazole (10.9%), sulfamerazine (11%) and sulfapyridine (1.1%) in the sulfadiazine-ELISA. The sulfatroxazole-ELISA showed low cross-reactivities towards three sulfonamides (see Table 1) and the sulfisoxazole-ELISA cross-reacted with sulfatroxazole (20%) and showed low cross-reactivity with four other sulfonamides (see Table 1). The sulfamethoxazole-ELISA showed 100% cross-reactivity with sulfamethizole due to the similarity in structure. In general, these ELISA's based on Pabs are very specific

and for the detection of several sulfonamides, different ELISA's have to be used simultaneously.

A better approach would be the development of generic antibodies by the immunization of animals with conjugates in which the common aromatic amino group is distal to the carrier protein. Such immunogens were described before. Sheth and Sporns (1991) described the use of TS and Assil *et al.* (1992) the use of PS.

For the synthesis of TS–protein conjugates, the method as described by Sheth and Sporns (1991) was followed although some modifications were applied during the syntheses (Haasnoot *et al.*, 2000). In the ciELISA, with TS–protein immobilized to the ELISA plate, Sheth and Sporns found no competition with high amounts (25 ppm) of free sulfonamides probably due to the greater affinity of antibodies for the protein-bound hapten. In our ciELISA, with the Pabs of the mice immunized with TS–proteins, all sulfonamides added at the 10 ppm level showed inhibition and five of them showed high inhibition (> 50%) (see Figure 3). Sheth and Sporns had to use another sulfonamide derivative (NS-conjugated OVA) bound to the microtiter plate to select a small subpopulation of the Pabs. In this ELISA, 11 sulfonamides showed 50 % inhibition below 25 ppm.

Using the Pabs of the mice immunized with PS–KLH, the ciELISA showed inhibition with all sulfonamides added at the 10 ppm level and nine of them showed high inhibition (> 50%; see Figure 3). Assil *et al.* (1992) used the same procedure and they found no competition with free sulfonamides added to the ELISA at the 10 ppm level. They had to select a subpopulation of the Pabs by affinity chromatography purification using OVA–TS bound to Sepharose. This fraction of the antiserum was used in a ciELISA with OVA-TS bound to the microtiter plate. In this ciELISA, seven sulfonamides showed inhibition when added at a level lower or equal to 10 ppm.

Instead of selecting subpopulations of the Pabs by using a deviating coating (Sheth & Sporns, 1991) or an affinity purified fraction (Assil *et al.*, 1992), we continued with the preparation of Mabs. To date we have succeeded in producing four Mabs producing hybridoma's after the fusion of myeloma cells with spleen cells of a mouse immunized with TS–BSA (Haasnoot *et al.*, 2000). As described in Table 2, we compared the generic performances of two of these Mabs with one recently described by Muldoon *et al.* (1999). Muldoon *et al.* used *N*-sulfanyl-4 aminobenzoic acid (see Figure 2) coupled to KLH as the immunogen to develop cross-reactive Mabs to sulfonamide antibiotics. The sensitivities of the Mab for sulfanitran, sulfapyridine and sulfathiazole (expressed as IC_{50} values) were 0.14, 2.3 and 32 ng/well, respectively. However, the sensitivity for other sulfonamides was much less (314–10000 ng/well).

In general, our Mabs (27G3 and 4E10) showed a higher sensitivity towards more sulfonamides (see Table 2). The binding of Mab 27G3 to OVA–TS coated microtiter plates was inhibited (50%) by all sulfonamides added at a level below 500 ng/well. Although these Mabs are unique, the difference in sensitivity towards the different sulfonamides is too high to call them true generic antibodies. Future experiments will

be focused on the development of Mabs against the PS-conjugate. As shown in Figure 3, this immunogen resulted in Pabs with high sensitivity towards interesting sulfonamides (sulfadimidine, sulfamerazine, sulfadiazine, sulfaquinoxaline, sulfadoxine and sulfadimethoxine) which were less detectable with the Pabs and Mabs obtained from TS immunized mice.

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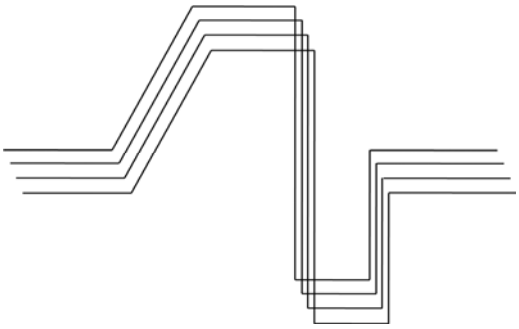
Part II

Biosensor immunoassay development for sulfonamides



Chapter 4

Biosensor immunoassay for the detection of eight sulfonamides in chicken serum

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ABSTRACT

A monoclonal antibody (Mab) raised against sulfamethazine (21C7) was applied in an optical biosensor (Biacore Q) to develop a rapid biosensor immunoassay (BIA) for the detection of several sulfonamides in chicken serum. The performance of this Mab was compared with two polyclonal antibodies (Pabs) raised against sulfamethazine (Qflex sulfamethazine binding protein (SBP) and RIKILT 464b). Using these Pabs, the limits of detection (LODs) in 10 times diluted chicken serum were approximately 30 ng ml^{-1} and the two BIAs were found to be specific for sulfamethazine. Using Mab 21C7, the LOD for sulfamethazine in 10 times diluted chicken serum was lower (10 ng ml^{-1}), high cross-reactivities were measured for sulfisoxazole (149%), sulfachloropyridazine (112%), sulfachloropyrazine (94%), sulfamerazine (87%), sulfadiazine (56%), sulfatroxazole (56%) and sulfathiazole (50%) and low cross-reactivities (11-25%) were measured with six other sulfonamides.
Compared with the Pabs, the Mab-based BIA resulted in a better sensitivity and was found suitable for the detection of 8 sulfonamides in 10 times diluted chicken serum with LODs between 7 and 20 ng ml^{-1}. The total run time for each cycle was 7 min.

INTRODUCTION

The sulfonamides are a group of antibacterial agents commonly given to food animals for prophylactic or therapeutic purposes. In the Netherlands, at least nine different sulfonamides are approved for veterinary application and for the medication of chicken, five sulfonamides (sulfamethazine, sulfadiazine, sulfachloropyridazine, sulfaquinoxaline and sulfamethoxazole) are approved. Intensive use of these drugs in animal breeding can lead to unwanted residues in food and to establish safe limits for human consumption, maximum residue limits (MRLs) for sulfonamides have been established in many countries. In the Netherlands and the European Union an MRL of 100 µg kg^{-1} for the total amount of sulfonamides is set for edible tissue [1].

For the detection of sulfonamides in edible tissue, many methods based on different technologies (microbial inhibition assays [2-3], immunoassays [4-6], liquid chromatography (LC) [7], LC-mass spectrometry (MS) [8], etc.) have been described. These methods are mainly used in laboratories and animal carcasses are processed before analytical results are available. For the "real-time" detection of sulfonamides in slaughterhouses, body fluids (bile, urine and blood serum) can be employed as markers for residue presence in tissue [4, 9-11]. These body fluids can normally be analyzed directly without extraction. Currently the most rapid technology for performing "real-time" analysis is based on a commercially available surface plasmon resonance (SPR) biosensor (Biacore^{TM}).

Sternesjö et al. [12] were the first who described an SPR-based biosensor immunoassay (BIA) for the determination of sulfamethazine residues in milk. Sulfamethazine was covalently immobilized to a carboxymethylated dextran-modified gold film. Polyclonal antibodies (Pab) raised against sulfamethazine were added to the sample and the immobilized surface was used to determine the amount of free antibodies. The limit of detection (LOD) of this inhibition assay in raw and defatted milk was <1 µg kg^{-1}, which was better than those obtained with microbiological, immunochemical and physical methods [13]. Other advantages of the biosensor assay they described were the lack of a sample preparation, its fully automated operation, the short time for analysis (20 min) and the specificity.

Elliott et al. [11] developed a more rapid immunobiosensor screening assay for the detection of sulfadiazine in pig bile. Pabs to sulfadiazine were added to 20 times diluted bile samples and the level of antibody binding to a sulfadiazine immobilized biosensor chip was determined after 20 s. The surface of the chip was then regenerated over a 1-min period prior to another sample injection. They found that an action level of 600 ng g^{-1} in pig bile could be used to control for the presence of sulfadiazine in edible tissue at the MRL level (100 ng g^{-1}). Crooks et al. [10] applied the same system and procedure to detect both sulfamethazine and sulfadiazine in pig bile on two separate biosensor chips and they found that the BIAs showed more

reliable results than conventional immunoassay methods. However, due to the specificity of the antibodies used, these BIAs were all specific for one of the sulfonamides and, to detect several sulfonamides, different BIAs had to be used. For the screening of more sulfonamides at the same time, several investigations were focused on the development of generic antibodies detecting the group of sulfonamides [14-16]. However, these approaches were not so successful and important sulfonamides (such as sulfamethazine) were not detected or at a high level only.
A Mab (21C7) raised against sulfamethazine [17] was identified as an antibody recognizing several important sulfonamides dissolved in buffer [18]. This Mab was used for the development of anti-idiotype antibodies and was applied in a BIA for the detection of sulfamethazine in urine [19] but was not used before for the detection of several sulfonamides in sample materials.

In the present study we aimed for a BIA suitable for the detection of several relevant sulfonamides in chicken blood. In a Dutch research project ("Development of monitoring and surveillance systems in the poultry meat chain", financed by the Dutch Ministry of Agriculture, Nature Management and Fisheries within research program no. 389) such a BIA should be combined with a BIA for the detection of *Salmonella* antibodies in chicken blood. The Biacore Q biosensor was used in combination with the Qflex Kit Sulfamethazine and the performance of the sulfamethazine binding protein (SBP), as supplied in the kit, was compared with an in-house developed polyclonal antiserum (464b) and with Mab 21C7.

EXPERIMENTAL

Materials

The *N*4-acetyl-sulfonamide kit (containing *N*4-acetyl-metabolites from sulfadiazine, sulfamethazine (sulfadimidine), sulfathiazole, sulfamethizole, sulfaquinoxaline, sulfanilamide and sulfamerazine), *N*4-acetyl-sulfamethoxazole, *N*4-acetyl-sulfadoxine, *N*4-acetyl-sulfadimethoxine, sulfadoxine, sulfachloropyrazine and sulfaquinoxaline were obtained from Serva (Heidelberg, Germany). Sulfamethazine, sulfamethoxazole, sulfamethoxypyridazine, sulfamerazine, sulfaguanidine, sulfadimethoxine, sulfadiazine, sulfachloropyridazine, sulfapyridine, sulfathiazole, sulfamethizole and sulfisoxazole were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). Sulfatroxazole was a gift from Leo Pharmaceutical Products (Weesp, the Netherlands).
The Qflex Kit Sulfamethazine (containing SBP, sulfamethazine derivative, HBS-EP buffer (composition: 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% Surfactant P20), a CM5 sensor chip and kit accessories), the Qflex Kit Sulfamethazine handbook and the amine coupling kit (containing 0.1 M *N*-hydroxysuccinimide (NHS),

0.4 M *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride-NaOH (pH 8.5)) were supplied by Biacore AB (Uppsala, Sweden). The CM-dextran sodium salt was obtained from Fluka Chemie (Zwijndrecht, the Netherlands) and dimethyl sulfoxide (DMSO) was from VWR International (Amsterdam, the Netherlands). The anti-sulfamethazine polyclonal antiserum (464b) was raised in a rabbit immunized with sulfamethazine-hemisuccinate-bovine serum albumine (BSA) and the performance of this antiserum in an enzyme-linked immunosorbent assay (ELISA) was described previously [4]. The raw serum was stored at -80 ºC until used. The preparation of the anti-sulfamethazine Mab (21C7) was also described previously [17]. Lyophilized ascites (0.5 ml) was dissolved in 0.5 ml of water and the solution was diluted 20 times in HBS-EP buffer and stored in 1 ml portions at -20 ºC until used.

Equipment

The Biacore Q biosensor was loaned from Biacore Benelux (Breda, the Netherlands). A Sigma Model 302K centrifuge was supplied by Salm en Kipp BV (Breukelen, the Netherlands) and the microtiter plate Varishaker-Incubator was obtained from Dynatech (Guernsey, UK).

Solutions

The sulfonamides and the *N*4-acetyl-sulfonamides were dissolved in methanol (1 mg ml^{-1}) and stored at -20 ºC until used. Of each standard, a first intermediate standard solution with a concentration of 27 µg ml^{-1} was prepared in water. From this intermediate standard solution, calibrant solutions (27, 9, 3 and 1 ng ml^{-1}), solutions used for the determination of the cross-reactivity (270, 27 or 2.7 ng ml^{-1}) and solutions used for the addition to chicken serum samples (20 and 50 ng ml^{-1}) were prepared in extraction buffer. The extraction buffer consisted of 20 mM sodium phosphate buffer and 0.15 M sodium chloride to which 0.3 g L^{-1} of CM-dextran sodium salt was added. This buffer was filtered though a 0.2 µm filter. The running buffer was ready-to-use HBS-EP buffer and the regeneration solution consisted of 20% acetonitrile in 0.2 M sodium hydroxide. The SBP stock solution provided in the kit was diluted in running buffer (one part stock solution to four parts HBS-EP).

Preparation of the biosensor chip

The sensor chip (CM5) consists of a gold surface coated with a carboxymethylated dextran matrix. The ready-to-use sulfamethazine derivative (supplied in the Qflex Kit) was immobilized on the sensor surface using the amine coupling kit and the Immobilization Wizard of the Biacore Q software. To eliminate the risk of carry-over of ligand from the immobilization procedure in the Integrated µ-Fluidic Cartridge (IFC) channels of the biosensor, immobilization was performed in the Surface Prep

unit, separate from the IFC. The Surface Prep unit is a sensor chip holder that fits directly onto the auto sampler rack base of the Biacore Q. Flow channels are formed on the sensor chip surface by docking a plastic block (the flow cell carrier) onto the chip. Solutions were injected directly into the flow channels with the auto sampler needle and effluents from the flow channels were collected in a small waste beaker.
At first, the sensor chip surface was activated by injecting a mixture of 0.4 M EDC and 0.1 M NHS (1:1; v/v) at a flow rate of 10 µl min^{-1} and with a contact time of 7 min. After washing with 0.1 M sodium hydroxide, the sulfamethazine derivative was injected over the surface during 7 min at a flow rate of 5 µl min^{-1}. To deactivate the remaining active sites, 1 M ethanolamine was injected during 3 min at a flow rate of 10 µl min^{-1}. An extra wash step with 50% DMSO was used to remove any adsorbed chemicals from the immobilization procedure.

Biosensor Immunoassays (BIAs)

The Qflex Kit Sulfamethazine was used in combination with the Biacore Q. The assay was designed as an inhibition (indirect) assay. The sensor chip, with immobilized sulfamethazine derivative, was used to bind antibodies against sulfamethazine (Qflex SBP). A known concentration of the SBP was mixed (1:1; v/v) with the sample (chicken serum which was ten times diluted in extraction buffer) in a microtiter plate automatically by the instrument. The mixture was injected over the sensor surface for 60 s at a flow rate of 40 µl min^{-1} and the response from free SBP was measured. The surface was regenerated by injecting a solution of 0.2 M sodium hydroxide containing 20% acetonitrile during 60 s at 40 µl min^{-1} and the sensor was ready for the next analysis cycle. The total run time under these conditions was 7 min. In the Concentration Analysis Wizard of the Biacore Q software, the responses for a set of calibration solutions (1, 3, 9 and 27 ng ml^{-1} sulfamethazine in dilution buffer) were used to generate a calibration graph (using the four-parameter equation as fitting function) and unknown samples were determined with reference to the calibration graph.
Applying Mab 21C7 (a 20 times dilution of the 20 times pre-diluted ascites (400 times diluted ascites)) instead of the SBP, the same procedure as described above could be applied.
When using Pab 464b instead of the SBP, a two-step regeneration (injecting 50 mM hydrochloric acid for 30 s, followed by 0.2 M sodium hydroxide containing 20% acetonitrile during 30 s) was necessary to clean the sensor surface from bound antibodies (run time 7 min).

Determination of cross-reactivities

For each of the three antibodies, calibration graphs of sulfamethazine in extraction buffer (27, 9, 3 and 1 ng ml^{-1}) were prepared.

With the rather specific polyclonal anti-sulfamethazine antibodies, standard solutions of the sulfonamides and the *N*4-acetyl-metabolites (27 and 270 ng ml^{-1}) in extraction buffer were injected and the concentrations were calculated by means of the calibration results in extraction buffer. For *N*4-acetyl-sulfamethazine, a lower concentration (2.7 ng ml^{-1}) was also injected. For each of the sulfonamides, the percentage of cross-reactivity was calculated (the calculated concentration (sulfamethazine equivalents) divided by the injected concentration times 100 %).
For the less specific Mab 21C7, standard solutions of the sulfonamides with concentrations of 2.7 and 27 ng ml^{-1} in extraction buffer were injected and cross-reactivities were calculated as described above.
For the calculation of the cross-reactivity in diluted chicken serum (10 or 4 times diluted), a chicken serum was spiked at the 100 ng ml^{-1} level with each of the sulfonamides. Of the sulfonamides, standard solutions of 20 and 50 ng ml^{-1} were prepared in extraction buffer. In the case of 10 times diluted chicken serum, 20 µl of chicken serum (mixture of 10 different blank chicken sera) was mixed in a microtiter plate with 100 µl of the sulfonamide standard solutions (20 ng ml^{-1}) and 80 µl of extraction buffer. The sulfamethazine equivalents were calculated with a calibration graph for sulfamethazine in chicken serum (10, 30, 90 and 270 ng ml^{-1}) which was also 10 times diluted. In the case of the 4 times diluted chicken sera, 50 µl of the chicken serum was mixed in a microtiter plate with 100 µl of the sulfonamide standard solutions (50 ng ml^{-1}) and 50 µl of extraction buffer. The sulfamethazine equivalents were calculated with a calibration graph for sulfamethazine in chicken serum (4, 12, 36 and 108 ng ml^{-1}) which was also 4 times diluted.

Samples

Blank chicken sera samples (n=66) were obtained from the Institute for Animal Science and Health (ID-Lelystad, Lelystad, the Netherlands). Of these samples, 42 were taken from 21-day-old chickens and 24 were taken from 44-day-old broilers.

RESULTS AND DISCUSSION

Calibration graphs obtained with the three antibodies.

In the Biacore Q, the performance of the antibody (SBP) supplied in the Qflex Kit Sulfamethazine was compared with a Pab developed in RIKILT (464b) and with the Mab developed in the Weizmann Institute of Science (Mab 21C7). All three antibodies were developed against sulfamethazine. During this comparison, the conditions prescribed in the Qflex Kit Sulfamethazine handbook (flow rate, injection volume, regeneration, etc.) were used (see Experimental section). These conditions worked well for the SBP and also for Mab 21C7. However, applying Pab 464b, the

regeneration conditions had to be adjusted to a two step regeneration with 50 mM HCL (20 µl), followed by 0.2 M NaOH containing 20% acetonitrile (20 µl).
Under the conditions prescribed in the kit handbook, the SBP was diluted in running buffer and, prior to the injection, mixed in the Biacore with extraction buffer (1:1; v/v), which resulted in an average maximum response of 807 ± 31 RU. A comparable average maximum response in buffer (783 ± 34 RU) was obtained with a 385 times dilution of Pab 464b. In an ELISA [4], this antiserum could be used in a 2500 times dilution (25 µl). This means that the BIA consumes approximately 10 times more antibodies than the ELISA. The 20 times pre-diluted Mab 21C7 containing ascites could be diluted another 20 times (400 times diluted ascites) to obtain an average maximum response in buffer of 726 ± 12 RU. Using these antibody dilutions mixed (1:1; v/v) with standard solutions of sulfamethazine (1, 3, 9 and 27 ng ml^{-1}), the calibration graphs showed 50% inhibition at 11.5, 9.5 and 8 ng ml^{-1} for the Qflex SBP, Pab 464b and Mab 21C7, respectively (see Figure 1).

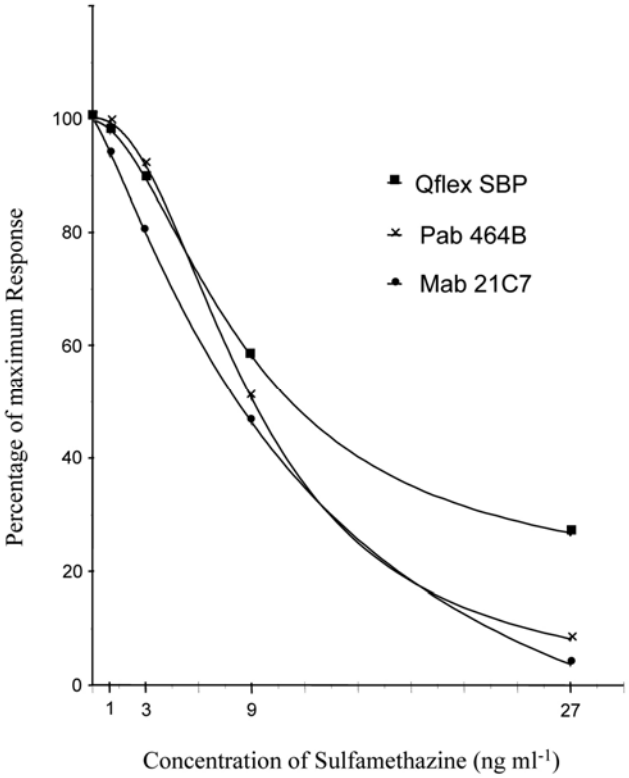

Figure 1. Normalized calibration graphs of sulfamethazine in buffer using the three different antibodies in the BIA.

The limits of detection (LOD = the concentration of sulfamethazine corresponding to the average maximum response minus three times standard deviation (S.D.)), obtained with the three calibrations for sulfamethazine in buffer were 3.0, 3.5 and 1.5 ng ml^{-1} for the SBP, 464b and Mab 21C7, respectively. The inhibition of the response at the highest calibration standard (27 ng ml^{-1}) was also less for the calibration using the

SBP. This means that the calibration graph for the BIA could be improved if the SBP was replaced by Pab 464b or Mab 21C7.

Performance of the BIAs in chicken sera.

Table 1: Performances of the three BIAs (using the Qflex SBP, Pab 464b and Mab 21C7) in the Biacore Q for the determinations of sulfamethazine in 10 times diluted blank chicken sera samples (n=66) and these samples spiked with sulfamethazine (100 ng ml^{-1}).

	ANTIBODY		
	Qflex SBP	**Pab 464B**	**Mab 21C7**
Maximum response with buffer (RU)	807±31	783±34	726±12
Maximum response with blank chicken sera (RU)	833±27	828±31	773±15
LOD in 10 x diluted chicken sera (ng ml^{-1})	30	28	10
LDM in 10 x diluted chicken sera (ng ml^{-1})	45	43	18
Response with sulfamethazine spiked (100 ng ml^{-1}) chicken sera (RU)	465±47	341±45	323±9
Inhibition of maximum response with sulfamethazine spiked chicken sera (%)	44.2	58.8	58.2
Recovery of sulfamethazine (%)	89±5	103±9	93±3

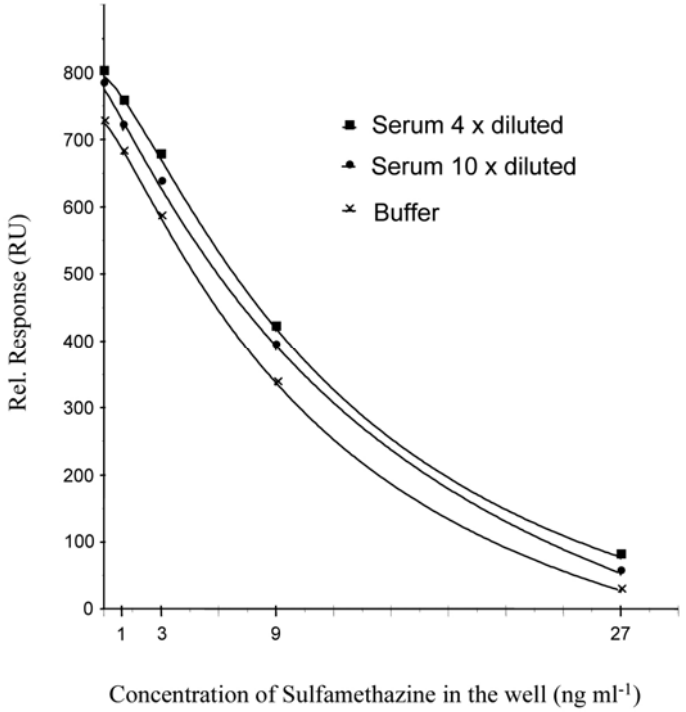

Figure 2. Comparison of sulfamethazine calibration graphs in buffer and chicken serum (10 and 4 times diluted) obtained with Mab 21C7 in the BIA.

Of each blank chicken serum (n=66), 20 µl were pipetted into a microtiter plate well and 180 µl of extraction buffer was added. After 2 min of mixing on a microtiter plate shaker, the chicken sera were analyzed without the addition of antibody (1:1 dilution

with running buffer; v/v) to investigate the average background response (nonspecific binding) which was low (21 ± 10 RU).

Thereafter, the 10 times diluted chicken sera (n=66) were analyzed after mixing (1:1; v/v) with the three different antibodies (diluted in running buffer). The average maximum responses obtained with the three antibodies in the diluted chicken sera were somewhat higher than those obtained in buffer (see Table 1), which could only be partly explained by the small background obtained from the chicken sera without the addition of antibodies. After the addition of sulfamethazine to the chicken sera (100 ng ml^{-1}), the average responses obtained with the 10 times diluted chicken sera in the three BIAs were strongly decreased compared to the maximum responses. The average inhibitions of the maximum responses were between 44 - 59 % (see Table 1).

Table 2: Cross-reactivities of the antibodies towards several sulfonamides and N^4-acetyl-metabolites in buffer and chicken serum (4 and 10 x diluted).

SULFONAMIDE	**CROSS-REACTIVITY (%)**				
	Pab Qflex SBP buffer	**Pab 464B buffer**	**Mab 21C7**		
			buffer	**SERUM (10x)**	**SERUM (4x)**
Sulfamethazine (sulfadimidine)	100	100	100	100	100
*N*4-Acetyl-sulfamethazine	81	79	70	68	73
Sulfadiazine	1	2	63	56	52
*N*4-Acetyl-sulfadiazine	1	2	50	52	43
Sulfamerazine	**7**	8	86	87	85
*N*4-Acetyl-sulfamerazine	10	10	83	70	73
Sulfachloropyrazine	2	2	127	94	86
Sulfisoxazole	<0.4	<0.4	130	149	>108
Sulfachloropyridazine	<0.4	<0.4	100	112	99
Sulfatroxazole	<0.4	<0.4	47	56	51
Sulfathiazole	<0.4	1	48	50	37
*N*4-Acetyl-sulfathiazole	0.4	1	26	30	19
Sulfamethizole	<0.4	<0.4	30	15	12
*N*4-Acetyl-sulfamethizole	<0.4	<0.4	11	<10	<4
Sulfadimethoxine	1	3	22	20	20
*N*4-Acetyl-sulfadimethoxine	1	3	7	<10	5
Sulfamethoxypyridazine	<0.4	<0.4	22	25	26
Sulfamethoxydiazine	<0.4	<0.4	21	22	23
Sulfapyridine	1	1	15	13	17
Sulfamethoxazole	<0.4	<0.4	8	11	14
*N*4-Acetyl-sulfamethoxazole	<0.4	1	2	<10	<4
Sulfaquinoxaline	<0.4	<0.4	0.9	<10	<4
*N*4-Acetyl-sulfaquinoxaline	0.4	<0.4	<0.4	<10	<4
Sulfadoxine	<0.4	<0.4	<0.4	<10	<4
*N*4-Acetyl-sulfadoxine	<0.4	<0.4	<0.4	<10	<4
Sulfaguanidine	<0.4	<0.4	<0.4	<10	<4

Calculating the concentration in the spiked sera by means of the calibration graph in buffer resulted in average recoveries between 89 and 103 % (see Table 1).

Calibration graphs in chicken serum were prepared for the three BIAs and compared with the calibrations in buffer. The responses obtained with those in 10 times diluted chicken sera were somewhat higher than those obtained in buffer. With the Mab-based BIA, a calibration graph in four times diluted chicken serum was also prepared. As shown in Figure 2, the responses obtained were again slightly higher, which proved the influence of the chicken serum, and, for quantitative results, calibration graphs in chicken serum should be used to calculate the concentrations in the sample.
Using the calibration plots in 10 times diluted chicken serum, the limits of detection (LOD = concentration of sulfamethazine corresponding with the average response of blank chicken sera minus three times the S.D.) of the two Pab-based BIAs in 10 times diluted chicken sera were calculated as 28 and 30 ng ml^{-1} (see Table 1). The limits of determination (LDM = concentration of sulfamethazine corresponding with the average response of blank chicken sera minus six times S.D.) were 43 and 45 ng ml^{-1} (see Table 1). With the Mab-based BIA, the LOD and LDM in 10 times diluted chicken sera were lower (10 and 18 ng ml^{-1}, respectively). These lower values were obtained due to a smaller variation in the responses obtained with the blank chicken sera and due to a more sensitive calibration graph. With this Mab, the LOD and LDM in four times diluted chicken sera were 5 and 9 ng ml^{-1}, respectively.
Therefore, of the three antibodies used in this study, the Mab showed the best results in the BIA for the determination of sulfamethazine in chicken sera.

Cross-reactivity.
The cross-reactivities of the three antibodies in the BIAs were compared using standard solutions of different sulfonamides and *N*4-acetyl-metabolites of some of the sulfonamides dissolved in buffer. As shown in Table 2, the SBP and the Pab 464b were very specific towards sulfamethazine and its *N*4-acetyl metabolite. With both antibodies, low cross-reactivities were observed for sulfamerazine and its *N*4-acetyl-metabolite.
Mab 21C7 showed high cross-reactivity (47 - 130%) with eight sulfonamides and with the *N*4-acetyl-metabolites of three of these sulfonamides, medium cross-reactivity (15-30%) with five sulfonamides and low cross-reactivity (8%) with one of the sulfonamides. These cross-reactivities obtained with the Mab were also determined in chicken sera (10 and 4 times diluted) and the results were almost the same with those obtained in buffer (see Table 2). The different sulfonamides were added at the 100 ng ml^{-1} level (MRL in tissue) to a mixture of the blank chicken sera and the samples were 10 and 4 times diluted in extraction buffer. As shown in Figure 3, the responses obtained with the spiked serum samples were compared with the average responses obtained with the blank chicken sera (n=66) which were calculated as 773 ± 15 RU for 10 times diluted sera and 798 ± 15 RU for 4 times diluted sera. From these average responses obtained with blank sera, threshold levels (average

response minus six times the standard deviation) were calculated as 683 RU (10 times diluted sera) and 708 RU (4 times diluted sera). As shown in Figure 3A, the addition of 8 of the sulfonamides at the 100 ng ml^{-1} level resulted in strongly reduced responses using 10 times diluted samples. With 4 times diluted samples the responses obtained with 14 of the sulfonamides were below the threshold (Figure 3B).

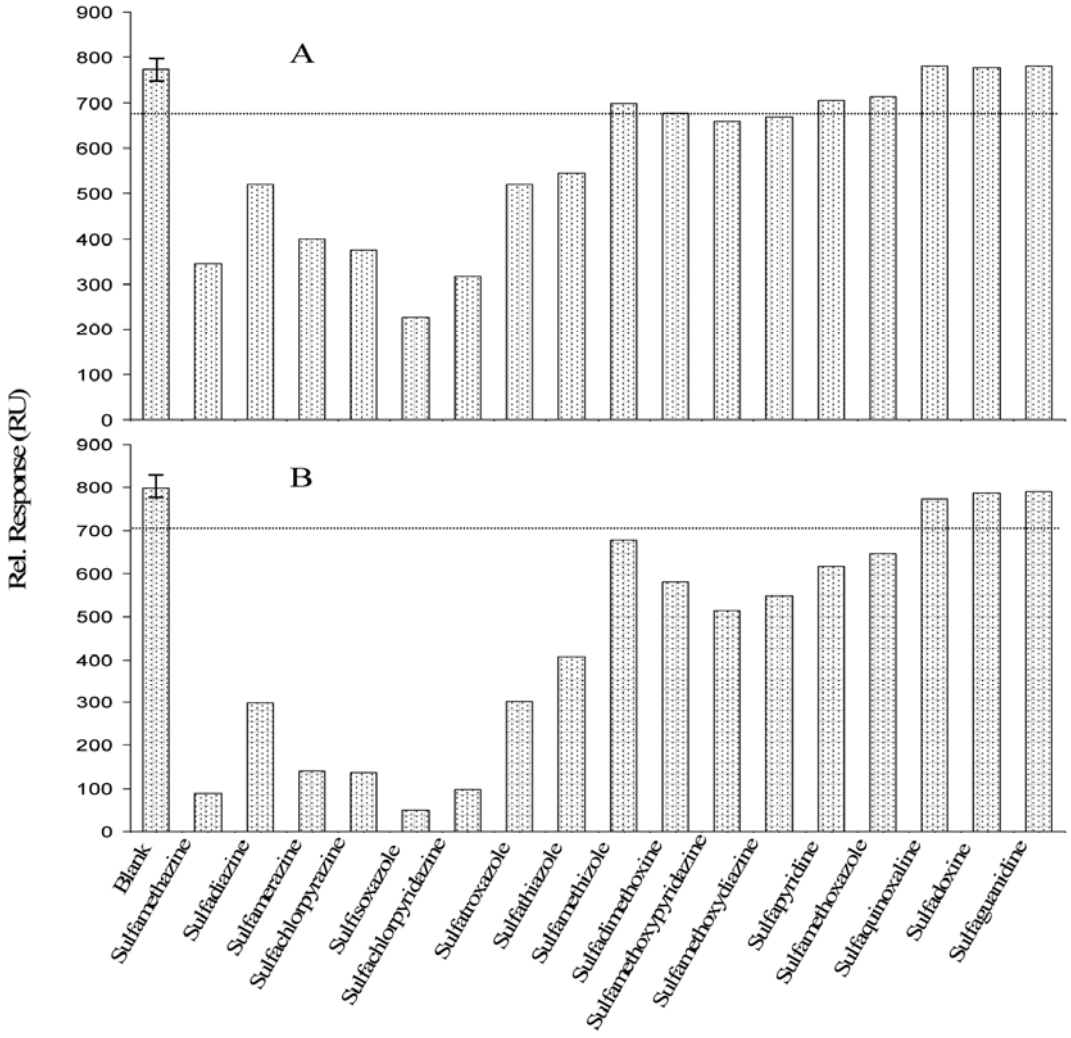

Figure 3. Responses obtained in the Mab-based BIA with a blank chicken serum towards different sulfonamides were added at the 100 ng ml^{-1}level, after a 10 times dilution (A) and after a 4 times dilution in extraction buffer (B). The thresholds (dotted lines) were calculated from the average responses (minus six times the standard deviation) obtained with the blank chicken sera (n=66).

Of the five sulfonamides allowed to be used in chicken in the Netherlands, this Mab recognized three of them (sulfamethazine, sulfadiazine and sulfachloropyridazine) with high cross-reactivity (see Table 2) which makes it a unique antibody. However, the cross-reactivities with the other two sulfonamides allowed for the treatment of chicken were low (11% for sulfamethoxazole and <10% for sulfaquinoxaline in 10

times diluted serum) and additional research is needed to include these sulfonamides into the assay. An option might be the use of a multi-channel approach in which one or two antibodies are added to the assay in combination with immobilized sulfonamides in additional flow channels. Such a multi-channel approach on a high-throughput biosensor was recently described for the simultaneous determination of sulfamethazine and sulfadiazine in porcine bile [20].

CONCLUSIONS

The Qflex Kit Sulfamethazine in combination with the Biacore Q resulted in a fast BIA (total run time of 7 min) that could be used for the detection of sulfamethazine in 10 times diluted chicken sera with an LOD of 30 ng ml^{-1}. This BIA and the BIA based on Pab 464b were very specific for sulfamethazine and its *N*4-acetyl metabolite. The BIA could be improved by the application of Mab 21C7. The Mab-based BIA resulted in a lower LOD for sulfamethazine in 10 times diluted chicken sera (10 ng ml^{-1}) and could detect 8 sulfonamides at comparable sensitivities (LODs between 7 and 20 ng ml^{-1}). During the experiments described in this study, the biosensor chip with the immobilized sulfamethazine derivative was used for approximately 1100 cycles and no significant change in performance was observed using the same antibody, which makes this BIA very robust.
Future experiments with medicated chicken are necessary to investigate the relationship between concentrations of the sulfonamides in blood serum and edible tissue to establish an action level in blood serum of chicken.

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Chapter 5

Comparison of multi-sulfonamide biosensor immunoassays

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ABSTRACT

Three different group-specific anti-sulfonamide antibodies were compared in inhibition assay formats in an optical biosensor (Biacore 3000) using CM5 sensor chips coated with three different sulfonamide derivatives. The antibodies used were an anti-sulfamethazine monoclonal antibody (Mab) 21C7, the sulfonamide binding protein (SBP) in the Qflex Kit Sulfonamides and a recently developed mutant antibody (M.3.4). Each of these antibodies showed interactions with all 17 sulfonamides tested and one (Mab 21C7) was sensitive for the *N*4-acetyl metabolites also. The limits of detection of the different sulfonamides in chicken serum varied between 7 and >1000 ng ml^{-1} (Mab 21C7), 15 and 340 ng ml^{-1} (Qflex) and 4 and 82 ng ml^{-1} (mutant M.3.4). The mutant M.3.4-based assay was found to be the most sensitive towards most of the sulfonamides whereas the Qflex Kit Sulfonamides detected the five sulfonamides registered for application in poultry in the Netherlands within the narrowest measurement range.

INTRODUCTION

One of the aims of the Dutch research project "Development of monitoring and surveillance systems in the poultry meat chain" is to evaluate the on-line detection of drug residues in slaughterhouses and the detection of sulfonamides was chosen as a first model. In the Netherlands, at least nine different sulfonamides are approved for veterinary application. For the medication of poultry, five sulfonamides (sulfamethoxazole, sulfadiazine, sulfamethazine, sulfachloropyridazine and sulfaquinoxaline) are approved of which the first two are most frequently applied. Intensive use of these drugs in animal breeding can lead to unwanted residues in food and to establish safe limits for human consumption, the European Union established a maximum residue limit (MRL) of 100 μg kg^{-1} for the total amount of sulfonamides in edible tissue [1].

For the detection of sulfonamides in edible tissue, many methods based on different technologies (microbial inhibition assays [2,3], immunoassays [4–6], LC [7], LC–MS [8], etc.) have been described. These methods are mainly used in laboratories and animal carcasses are processed before analytical results are available. For the detection of sulfonamides in slaughterhouses, body fluids (bile, urine and blood serum) can be employed as markers for residue presence in tissue [4,9–11]. These body fluids can normally be analyzed directly without extraction. Currently the most rapid technology for performing analysis is based on a commercially available surface plasmon resonance (SPR) biosensor (Biacore^{TM}). Applications were described for the determination of sulfamethazine residues in milk [12,13] and the detection of sulfamethazine and sulfadiazine in pig bile [10,11]. However, due to the specificity of the antibodies used, these biosensor immunoassays (BIAs) were all specific for one of the sulfonamides and, to detect several sulfonamides, different BIAs had to be used. As an example, a two-channel approach on a high-throughput biosensor was described for the simultaneous determination of sulfamethazine and sulfadiazine in porcine bile [14].

For the screening of more sulfonamides at the same time, several investigations were focused on the development of multi-sulfonamide antibodies detecting the group of sulfonamides [15–18]. However, these approaches were partly successful because important sulfonamides (such as sulfamethazine) were not detected or at a high level only.

A monoclonal antibody (Mab) 21C7 raised against sulfamethazine [19] was identified as an antibody recognizing several important sulfonamides dissolved in buffer [20]. This Mab was applied in BIAs for the detection of sulfamethazine in urine [21] and for the detection of eight sulfonamides in chicken serum with limit of detections (LODs) between 7 and 20 ng ml^{-1} [22]. However, the sensitivity of this assay for the detection of important sulfonamides, such as sulfamethoxazole and sulfaquinoxaline

was less. A mutant antibody (A.3.5) was selected after protein engineering of a broad specificity sulfonamide binding monoclonal antibody (Mab 27G3 [17]). In a competitive time-resolved fluoroimmunoassay this mutant showed an improved sensitivity towards several sulfonamides compared with the wild-type Mab [23]. However, the sensitivity towards important sulfonamides such as sulfamethazine, sulfadimethoxine and sulfaquinoxaline was low. In order to improve the properties of the antibodies, mutants from the previous study [23] were recombined and more mutations introduced [24]. In the time-resolved fluoroimmunassay, the selected mutant from that study (M.3.4) showed an improved sensitivity for all 13 sulfonamides tested and even sulfamethazine, sulfadimethoxine and sulfaquinoxaline could be measured below the 100 ng ml^{-1} level.

In the present study, the performance of the new mutant M.3.4 was compared with that of Mab 21C7 and with a recently launched multi-sulfonamide kit (Qflex® Kit Sulfonamides) of Biacore (Uppsala, Sweden) in the Biacore 3000 using chicken serum as sample material. The performances of these antibodies in the biosensor are compared for (i) sensitivities towards 26 sulfonamides and *N*4-acetyl metabolites added to chicken serum and (ii) the influence of chicken serum on the assays.

MATERIALS AND METHODS

Materials and instrument

Sulfatroxazole was a gift from Leo Pharmaceutical Products (Weesp, the Netherlands). All other sulfonamides, including an *N*4-acetyl-sulfonamide kit (not available anymore) were obtained from Serva (Heidelberg, Germany) or Sigma–Aldrich (Zwijndrecht, the Netherlands). Biacore AB (Uppsala, Sweden) delivered the Biacore 3000, the Qflex Kit Sulfamethazine, the Qflex Kit Sulfonamides, HBSEP buffer, CM5 biosensor chips and an amine coupling kit (containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine-hydrochloride (pH 8.5)). All other chemicals were obtained from Sigma–Aldrich. The preparation of *N*1-[4-(carboxymethyl)-2-thiazolyl] sulfanilamide (TS) [17], the development of Mab 21C7 [19] and the application in the biosensor [22] and the development of mutant M.3.4 were described previously [24].

Biosensor immunoassays (BIAs)

Preparation of the biosensor chips.

For each of the three assays, a different sulfonamide derivative was bench top immobilized onto the surface of a CM5 sensor chip. In the Mab 21C7-based assay, a sulfamethazine derivative as supplied in the Qflex Kit Sulfamethazine was immobilized [22]. Using the Qflex Kit Sulfonamides, another sulfonamide derivative

(supplied in the kit) was immobilized. During both immobilizations, the procedures as prescribed by the manufacturer were followed.
For the application of mutant M.3.4, the chip was coated with TS using the following procedure. The chip surface was activated with 50 µl of a mixture of 0.4 M EDC and 0.1 M NHS (1:1; v/v) during 15 min at RT. The chip surface was washed with water and dried under a stream of nitrogen gas. To the chip, 50 µl of 0.1 M ethylene diamine (pH of 8.5) was added and after 15 min incubation at RT, the chip was washed with water and dried under a stream of nitrogen gas.
The sulfonamide derivative (TS) was immobilized on the activated sensor surface using the following procedure. TS (2 mg) was dissolved in 0.2 ml dimethylsulfoxide (DMSO) and 0.8 ml of sodium carbonate buffer (1.59 g Na_2CO_3 + 2.93 g $NaHCO_3$ in 1 l water, adjusted to pH 9.6) was added. Of this TS solution, 50 µl was mixed with 50 µl 0.4 M EDC and 50 µl 0.1 M NHS and after an incubation at RT for 1 h, this mixture (50 µl) was added to the activated chip. After an incubation of 1 h at RT, the chip was washed with water, dried under a stream of nitrogen and docked into the Biacore 3000.

Assays for serum samples

In the Mab 21C7-based and mutant M.3.4-based assays, chicken serum (20 µl) was pipetted into a 96-well microtiter plate and 80 µl of extraction buffer (20 mM sodium phosphate and 0.15 M sodium chloride; pH 7.2) was added. Using the Qflex Kit Sulfonamides, 60 µl of extraction buffer and 20 µl of a concentrated Hepes buffer (0.1 M Hepes, 34 mM EDTA, 1 M NaCl and 0.01% P20) were added to chicken serum (20 µl) in the well of the microtiter plate. Prior to injections, the diluted samples were mixed with diluted antibody (1:1; v/v) in the Biacore 3000. The antibody solutions were diluted to result in maximum responses between 700 and 1200 response units (RU) applying injection volumes of 50 µl at a flow rate of 20 µl min^{-1} (Mab 21C7 or mutant M.3.4) or 27 µl at a flow rate of 80 µl min^{-1} (according to the Qflex Kit Sulfonamide manual) with HBS-EP as the running buffer. For the regenerations, 10 µl of 0.1 M HCl followed by 5 µl of 0.1 M NaOH (mutant M.3.4) or 20 µl of 0.1 M NaOH (Qflex) or 20 µl of 0.2 M NaOH plus 20% acetonitrile (21C7) were injected at a flow rate of 20 µl min^{-1}.

Sample materials

Blank chicken serum samples (n = 66) were obtained from the Animal Sciences Group (ASG, Lelystad, the Netherlands). Of these samples, 42 were taken from 21-days-old chicken and 24 were taken from 44-days-old broilers. These samples were mixed to create a large batch (approximately 100 ml) of blank chicken serum.
An animal experiment with 4-weeks-old broilers was performed at ASG and started on 18-02-2003. One group of broilers (10 male and 10 female) was not treated with

sulfonamides and served as blanks. These broilers were slaughtered on the first day of the experiment and serum samples were stored at −20 °C until analyzed.

To obtain incurred samples, another group of chickens (15 male and 15 female) was given an oral treatment with Trimethosulfmix 50% (80 mg trimethoprim and 420 mg sulfadiazine sodium per g; Eurovet Animal Health BV, Bladel, the Netherlands) using a dose of 0.45 g l^{-1} of drinking water. The average water consumption was determined as 200 ml (approximately 38 mg of sulfadiazine) per kg body weight. During the 4 days treatment, two groups of six chickens each were slaughtered at days 2 and 4 and after the treatment three groups of six chickens each were slaughtered at days 7, 10 and 14 (3, 6 and 10 days after treatment) and serum samples were stored at −20 °C until analyzed.

RESULTS AND DISCUSSION

Assay conditions

The performances of the three inhibition assays were, as much as possible, tested under comparable conditions. Chicken serum samples were five times diluted in extraction buffer and, prior to the injection, the sample solutions were mixed with the antibody solutions (1:1; v/v) in the Biacore. The final dilutions of the antibodies in the three assays were adjusted to result in maximum responses between 700–1200 RU using an injection volume of 50 µl at a flow rate of 20 µl min^{-1} with HBS-EP as the running buffer. However, the performance of the Qflex Kit Sulfonamides increased (better sensitivity) using the protocol described in the kit handbook (for pork muscle only) in which a concentrated Hepes buffer was added to the sample solution and short injections (20 s) at a high flow rate (80 µl min^{-1}) were applied.

During the comparison, these conditions were applied with the Qflex kit only. In all assays, the relative responses obtained 30 s after the sample injections were used for the calculations as described below. Due to the different sulfonamide derivatives immobilized onto the chip surface and the different antibodies used, the regeneration conditions of the three assays varied also (see materials and methods section). These differences in assay conditions are visualized in the sensorgrams obtained with the three different assays (Fig. 1). The total time of analysis using the Qflex kit was 8 and 10 min for the other two assays. Of this time, about 3 min were used for mixing the sample with the antibodies, 1–2.5 min for sample injection and 0.5–1 min for the regeneration. The rest of the analysis time was used by the Biacore during washing steps. Due to the addition of concentrated Hepes buffer, resulting in a higher bulk response (BR), and the short injection at a high flow rate, the sensorgram obtained with the Qflex kit was deviating from the sensorgrams obtained with the other two assays (Fig. 1).

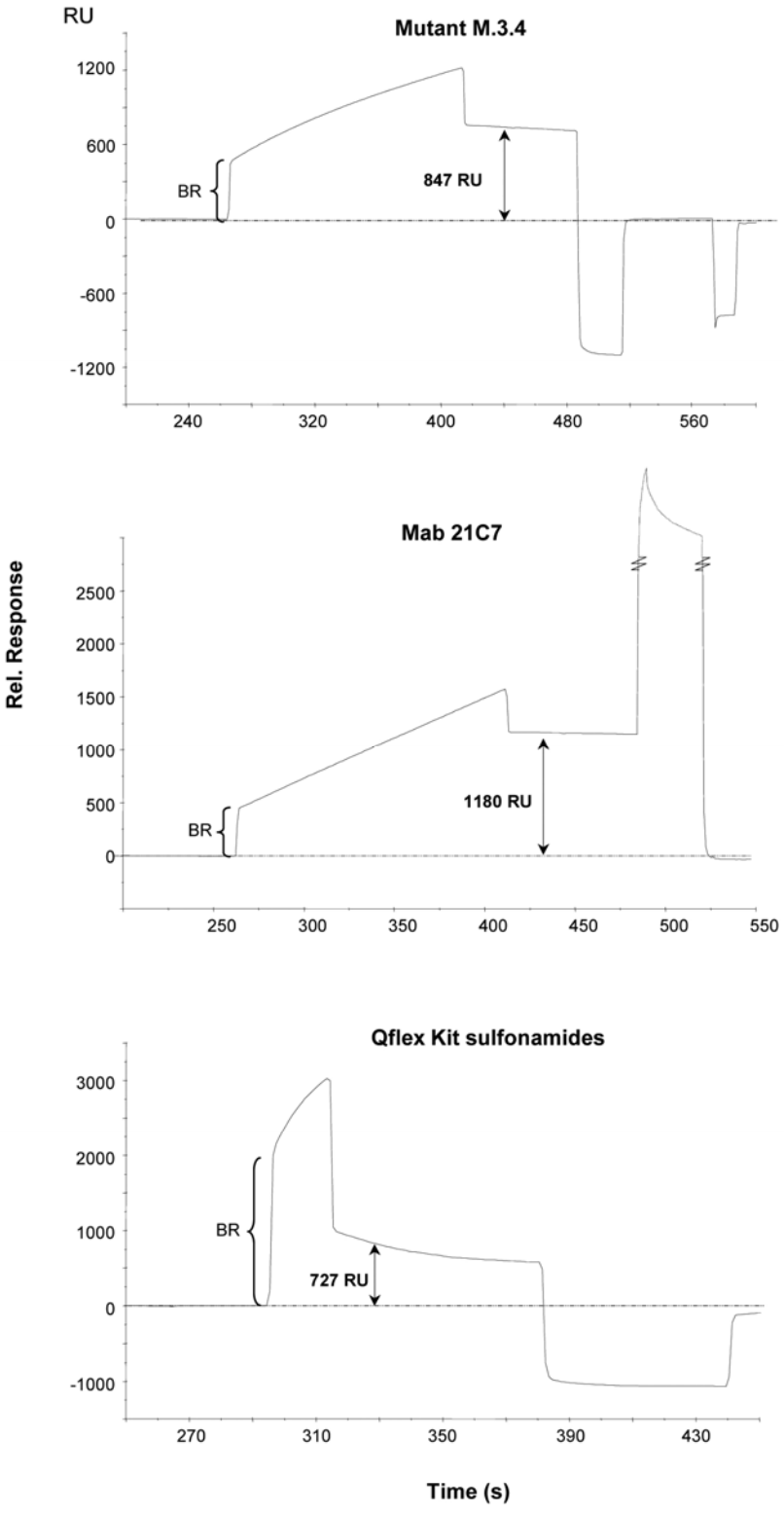

Figure 1. Sensorgrams obtained in the three different assays with a blank chicken serum. In the Mab 21C7-based and mutant M.3.4-based assay, 50 µl was injected at a flow rate of 20 µl min^{-1} and for the Qflex Kit Sulfonamides, 27 µl was injected at a flow rate of 80 µl min^{-1} (according to the kit handbook). For the regeneration conditions, see Materials and methods. BR = bulk response.

Sensitivity of the three assays towards different sulfonamides

The batch of blank chicken serum samples was spiked with 26 different sulfonamides and metabolites (100 ng ml^{-1}) and analyzed in the three different assays. The responses were compared with the average responses obtained after analyzing the blank chicken serum and percentages of inhibition were calculated. As shown in Fig. 2, the assay based on Mab 21C7 was sensitive towards the *N*4-acetyl-metabolites of some sulfonamides and the other two assays showed minor reactions with these metabolites (showing a cross-reactivity of <10% compared to the parent compounds).

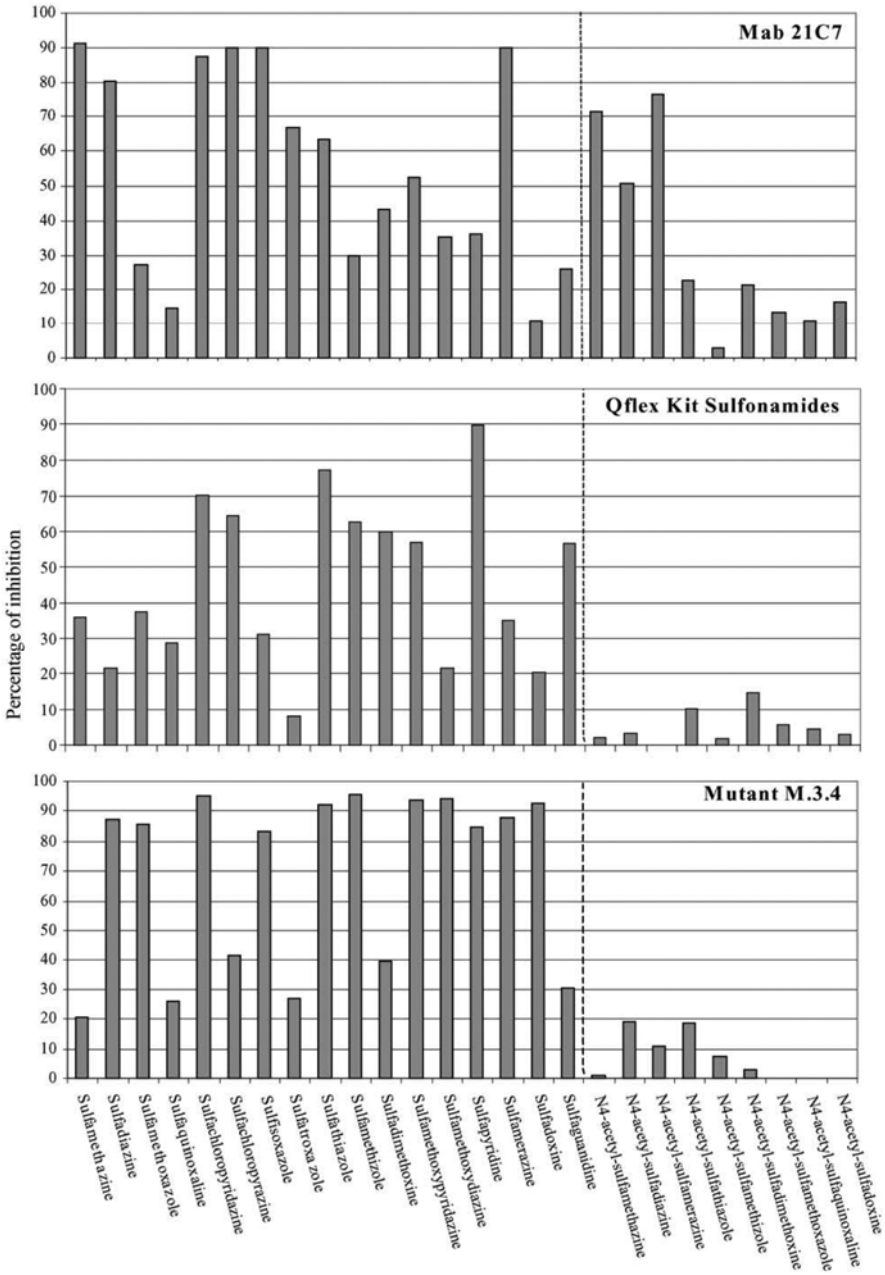

Figure 2. Inhibition of responses (%) obtained in three assays with a blank chicken serum spiked at the 100 ng ml^{-1} level with different sulfonamides and N4-acetyl-metabolites.

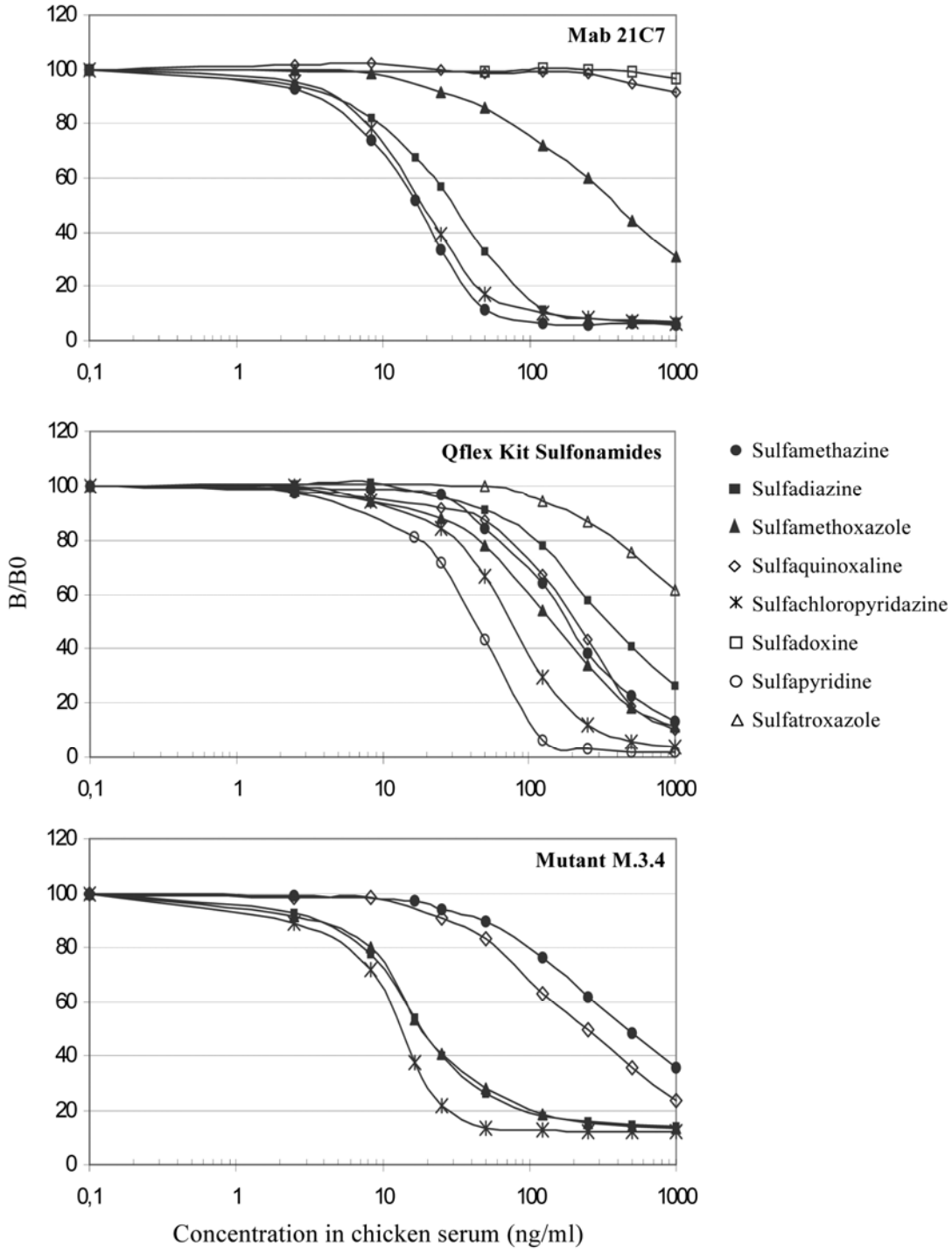

Figure 3. Calibration curves in chicken serum obtained in the three assays with the five different sulfonamides registered for application in broilers in the Netherlands (sulfamethazine, sulfadiazine, sulfamethoxazole, sulfaquinoxaline and sulfachloropyridazine) and with some sulfonamides showing the highest and lowest sensitivity in the assays.

In the three assays, all sulfonamides were detected however, with different sensitivities. The number of sulfonamides showing more than 50% inhibition (inh.) was 8 (Qflex), 9 (Mab 21C7) and 11 (mutant M.3.4).

Calibration curves were prepared in blank chicken serum for the five sulfonamides registered in the Netherlands for application in poultry (sulfamethazine (SMZ), sulfadiazine (SDZ), sulfamethoxazole (SMX), sulfaquinoxaline (SQX) and sulfachloropyridazine (SCP)) and for the sulfonamides showing the highest and lowest sensitivities in the assays (Fig. 3).

The assay based on Mab 21C7 was most and least sensitive towards sulfamethazine and sulfadoxine (50% inh. at 14 and >1000 ng ml^{-1}, respectively). The Qflex kit was most and least sensitive towards sulfapyridine and sulfatroxazole (50% inh. at 40 and >1000 ng ml^{-1}, respectively). This is in agreement with the data presented in Application note 44 (Biacore AB, Uppsala, Sweden), describing the detection of sulfonamides in porcine muscle. In general, for the sulfonamides applied in this study, the results obtained are comparable with the results described in this application note except for sulfadiazine. For this sulfonamide the calculated cross-reactivity (compared to sulfamethazine) was 60% whilst 124% was described in the note. The cross-reactivity was expressed as a percentage of the concentration at 50% inhibition (IC_{50}) of sulfadiazine compared to sulfamethazine. Another difference was found in the LOD for sulfadiazine (16.9 ng g^{-1} for pork tissue) whilst in chicken serum this LOD was calculated as 62 ng ml^{-1}. The difference in sample material (serum versus muscle) might cause such differences.

Table 1. Average maximum responses obtained in the three assays with blank chicken serum samples (n=20) and the calculated limits of detection (LODs) using five different calibration curves in chicken serum (sulfamethazine (SMZ), sulfadiazine (SDZ), sulfamethoxazole (SMX), sulfaquinoxaline (SQX) and sulfachloropyridazine (SCP)).

ASSAY	BLANK SERUM AVERAGE MAX. RESPONSE (RU) (n=20)	LOD IN CHICKEN SERUM (ng ml^{-1})				
		SMZ	SDZ	SMX	SQX	SCP
21C7	1180 ± 100	7	11	79	>1000	10
Qflex kit	727 ± 26	44	62	25	45	19
M.3.4	847 ± 40	82	5	5	44	4

The mutant-based assay was most and least sensitive towards sulfachloropyridazine and sulfamethazine (50% inh. at 12 and 450 ng ml^{-1}, respectively).

The 20 blank chicken sera, obtained from the untreated broilers in the animal experiment, were analyzed with the three assays and the average responses obtained (minus three times the standard deviation) were used to calculate the LODs using the five calibration curves of the registered sulfonamides (Table 1). The lowest LOD for sulfamethazine was found in the Mab 21C7-based assay, which had a good sensitivity for sulfadiazine and sulfachoropyridazine as well. However, the LOD for

sulfamethoxazole and sulfaquinoxaline was much higher. The mutant M.3.4-based assay was found to be the most sensitive towards most of the sulfonamides and of the three assays tested, this assay showed the lowest LOD for the two sulfonamides (sulfamethoxazole and sulfadiazine) most frequently applied for poultry in the Netherlands. The highest LOD was found for sulfamethazine (82 ng ml^{-1}) and this was also observed in a lanthanide fluoroimmunoassay (LFIA) using the same mutant [25] in which an LOD in chicken serum of 25 ng ml^{-1} was obtained. In this LFIA a sample preparation, to remove the proteins from chicken serum, had to be applied whilst the Biacore sample preparation consisted of a dilution step only.

The Qflex Kit Sulfonamides detected the five sulfonamides within the narrowest measurements range and this is an advantage for the screening of total sulfonamide residues in chicken serum in the Netherlands.

Samples from the batch of blank serum were spiked with sulfamethoxazole or sulfadiazine at the 50 ng ml^{-1} level and these samples were measured on 2 days with 1 week in between and six times each day. The concentrations were calculated using calibration standards of the two sulfonamides added to the blank chicken serum. The LODs of the Qflex kit for sulfadiazine and of the Mab 21C7-based assay for sulfamethoxazole were higher than this level and were not analyzed. With the sulfadiazine-spiked samples the average concentration found with the Mab 21C7-based assay was 52.0 ± 2.4 ng ml^{-1} and in the mutant-based assay 55.0 ± 2.6 ng ml^{-1}. With the sulfamethoxazole-spiked samples, the average concentration found with the Qflex kit was 50.2 ± 6.9 ng ml^{-1} and with the mutant-based assay 55.7 ± 2.8 ng ml^{-1}.

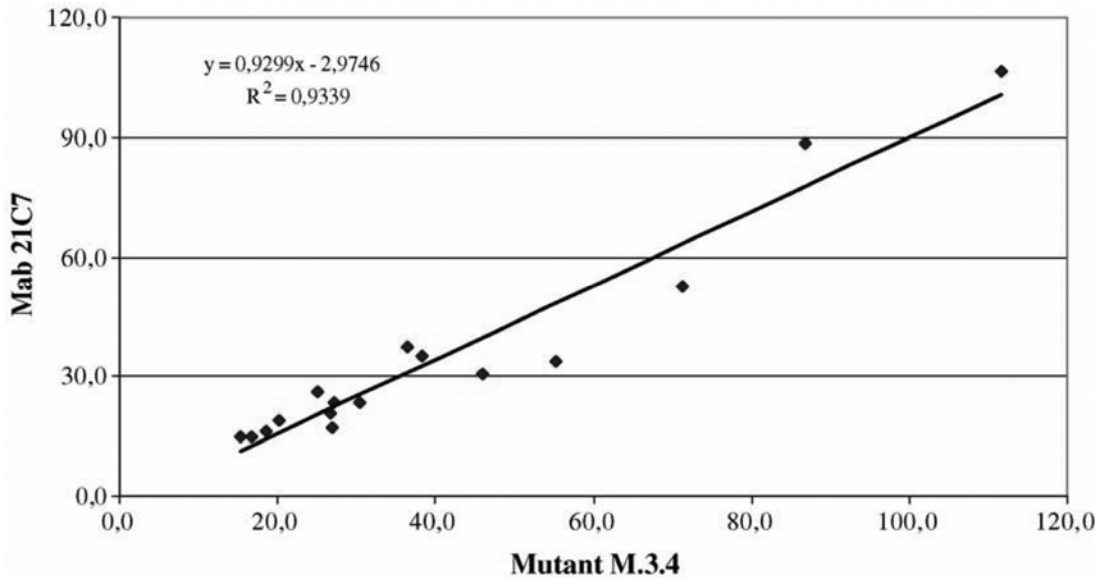

Figure 4. Concentrations of sulfadiazine (ng ml^{-1}) found with the mutant- and the Mab 21C7-based assays after analyzing the incurred serum samples.

Incurred serum samples

Incurred serum samples were obtained from an animal experiment in which broilers were treated with sulfadiazine. As found with the mutant-based assay, the sera taken during the treatment contained high concentrations of sulfadiazine (>2700 ng ml^{-1}). The sera taken 3, 6 and 10 days after the treatment contained concentrations between 15 and 110 ng ml^{-1} and these results were compared with those obtained with the Mab 21C7-based assay (Fig. 4). Although the Mab-21C7 based assay detects metabolites of sulfadiazine and the mutant M.3.4-based assay does not (Fig. 2), comparable concentrations were found with the two assays. This suggests a low (not detectable) concentration of sulfadiazine metabolites present in chicken serum. The depletion of another sulfonamide (sulfamethazine) in hens was studied [26] and the concentration of *N*4-acetyl-sulfamethazine in plasma was 6–8% only of the parent drug concentration.

CONCLUSIONS

For the application of the three multi-sulfonamide assays, three different small molecules were directly immobilized onto the sensor surface of a CM5 chip and this resulted in stable surfaces suitable for hundreds of cycles. The three assays were all found to be suitable for the multi-sulfonamide detection in chicken serum however, with different sensitivities within each assay for different sulfonamides. The mutant M.3.4 is a single chain variable fragment (scFv) consisting of the variable domains of the heavy and light chains of an antibody bound by a linker domain. The scFv has a molecular weight of 26-27 kDa and it is more than five times smaller than the IgG molecule (*M*w =150 kDa). The mutant performed very well (high association and low dissociation) in the inhibition biosensor assay and was found to be the most sensitive towards most of the sulfonamides. The Qflex Kit Sulfonamides detected the five sulfonamides registered for application in poultry within the narrowest measurements range which is preferential for the multi-sulfonamide screening. Based on the comparison of results obtained with the incurred serum samples using the assay recognizing *N*4-acetyl metabolites (Mab 21C7) and the mutant M.3.4 assay (not sensitive towards these metabolites) it was concluded that only low concentrations of this metabolite for sulfadiazine occurred in chicken serum. Future experiments will be focused on the relation between concentrations of sulfonamides in chicken serum and tissue to establish predictive values for the application in control systems.

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Chapter 6

Application of a multi-sulfonamide biosensor immunoassay for the detection of sulfadiazine and sulfamethoxazole residues in broiler serum and its use as a predictor of the levels in edible tissue

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ABSTRACT

A multi-sulfonamide biosensor immunoassay (BIA), based on a previously developed mutant antibody (A.3.5) in an optical biosensor (Biacore 3000), was applied to analyze serum and plasma samples obtained from the broilers treated with sulfamethoxazole and sulfadiazine. The assay was fast (5 min per sample), the sample preparation was easy (dilution in antibody-containing buffer only) and an equal sensitivity for the two sulfonamides was obtained with limits of detection in serum and plasma below 10 ng ml^{-1}. The concentrations found with the BIA in serum and plasma of the treated broilers were comparable and higher than the concentrations found in tissue by LC-MS/MS. The average serum/tissue ratio's for sulfamethoxazole were 6.2 (leg meat), 2.5 (liver) and 1.3 (skin+fat) and for sulfadiazine 8.7 (leg meat), 3.1 (liver) and 2.2 (skin+fat). To predict the concentrations of the two sulfonamides below the maximum residue limit (MRL) of 100 ng g^{-1} in the tissue with the highest level (skin+fat), the proposed action level of the multi-sulfonamide BIA in serum is 130 ng ml^{-1}.

A later developed mutant antibody (M.3.4), with a better sensitivity towards more sulfonamides, was applied during a survey. Serum samples (n=300) of broilers from 30 different flocks were found negative. Concentrations between <5 and 152 ng ml^{-1} (sulfamethoxazole equivalents) were found in serum samples of one flock (n=160) with an average of 25 ± 21 ng ml^{-1}. The sulfonamide identified by LC-MS/MS in these samples was sulfamethoxazole.

INTRODUCTION

One of the aims of the Dutch research project "Development of monitoring and surveillance systems in the poultry meat chain" is to evaluate the on-line detection of sulfonamide residues in slaughterhouses. In the Netherlands, at least nine different sulfonamides are approved for veterinary application. For the medication of poultry, five sulfonamides (sulfamethoxazole, sulfadiazine, sulfamethazine, sulfachloro-pyridazine and sulfaquinoxaline) are approved of which the first two are most frequently applied. Unauthorized use of these drugs in animal breeding can lead to unwanted residues in food. To ascertain safe limits for human consumption, the European Union established a maximum residue limit (MRL) of 100 μg kg^{-1} for the total amount of sulfonamides in edible tissue [1].

For the detection of sulfonamides in slaughterhouses, body fluids (bile, urine and blood serum) can be employed as markers for residue presence in tissue [2-5]. These body fluids can normally be analyzed directly without extraction. Currently, the most rapid technology for performing an analysis is based on a commercially available surface plasmon resonance (SPR) biosensor (Biacore^{TM}). Applications were described for the determination of sulfamethazine residues in milk [6,7], the detection of sulfamethazine and sulfadiazine in pig bile [4,5] and for the multi-sulfonamide detection in chicken serum [8-10]. A monoclonal antibody 21C7 (Mab 21C7) raised against sulfamethazine [11] was identified as an antibody suitable for the biosensor detection of eight sulfonamides in chicken serum with LODs between 7 and 20 ng ml^{-1} [8]. However, the sensitivity of this assay for the detection of important sulfonamides, such as sulfamethoxazole and sulfaquinoxaline was not good enough. A mutant antibody (A.3.5) was selected after protein engineering [13] of a broad specificity sulfonamide-binding Mab (27G3) [12]. In a competitive time-resolved fluoroimmunoassay this mutant showed an improved sensitivity towards several sulfonamides compared with the wild-type Mab [13]. However, the sensitivity towards important sulfonamides such as sulfamethazine, sulfadimethoxine and sulfaquinoxaline was low. In order to improve the properties of the antibodies, mutants from the previous study were recombined and more mutations introduced [14]. In the time-resolved fluoroimmunoassay, the selected mutant from this study (M.3.4) showed an improved sensitivity for all 13 sulfonamides tested and even sulfamethazine, sulfadimethoxine and sulfaquinoxaline could be measured below the 100 ng ml^{-1} level. This fluoroimmunoassay was successfully applied for the detection of sulfonamides in meat, milk and serum samples [15].

In an optical biosensor, the performances of the mutants (A.3.5 and M.3.4) were compared with the wild-type Mab with chicken serum as sample material [10]; improved sensitivities for the mutants-based assays were observed for all

sulfonamides, and mutant M.3.4 was found to be the most sensitive towards the 17 sulfonamides tested.

In another study [9], the performance of the best mutant M.3.4 was compared with the multi-sulfonamide Mab 21C7 and with a recently launched multi-sulfonamide kit (Qflex® Kit Sulfonamides) of Biacore (Uppsala, Sweden) [16]. The mutant M.3.4-based BIA was also selected as most sensitive towards most of the sulfonamides added to the chicken serum, and minor reactions with the *N*4-acetyl-metabolites were observed.

The aims of the present study were (1) to evaluate the performance of the multi-sulfonamide mutant A.3.5-based BIA for the detection of sulfadiazine and sulfamethoxazole (most applied sulfonamides in the Netherlands) in the incurred samples (serum and plasma samples obtained from broilers treated with these sulfonamides (animal experiment performed in February 2003)), and to determine the concentrations of these sulfonamides in serum and plasma samples after different withdrawal times; (2) to determine the predictive levels of the biosensor assay in the serum for the levels of the two sulfonamides in edible tissue, by comparing the concentrations found in the serum with the concentrations detected by LC-MS/MS in edible tissue (leg meat, liver and skin plus fat); and (3) to evaluate the performance of a later developed and more sensitive mutant M.3.4-based BIA in a multi-channel combined assay for the detection of sulfonamides (one flow-channel) and three anti-salmonella serotypes (three flow-channels), in which the sulfonamide assay conditions were adapted to the serological assay conditions, and to apply this assay during a survey (performed in 2004) in which serum samples from 31 different Dutch broiler farms were analyzed for the possible presence of sulfonamides.

EXPERIMENTAL

Materials

The preparation of the sulfonamide derivative (*N*1[4-(carboxymethyl)-2-thiazolyl] sulfanilamide (TS)) was described previously [12]. Sulfamethoxazole, sulfadiazine and guanidine hydrochloride were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). HBS-EP buffer (composition: 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% Surfactant P20), CM5 sensor chips, the amine coupling kit (containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride-NaOH (pH 8.5)) and 10 mM glycine-HCl buffer (pH 1.5) were supplied by Biacore AB (Uppsala, Sweden). The CM-dextran sodium salt was obtained from Fluka Chemie (Zwijndrecht, the Netherlands). Tween 20, Tween 80, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and Triton X-

100 were obtained from VWR International (Amsterdam, the Netherlands). Stomacher bags (BA6041) were obtained from Salm en Kipp b.v. (Breukelen, the Netherlands). The developments of the mutant antibodies A.3.5 and M.3.4 were described before [13,14].

Equipment

The Biacore 3000 biosensor was purchased from Biacore Benelux (Breda, the Netherlands) and the microtiter plate Varishaker-Incubator was obtained from Dynatech (Guernsey, UK). The Sigma-302k centrifuge and the Stomacher®400 were supplied by Salm en Kipp bv (Breukelen, the Netherlands).

Biosensor immunoassays (BIAs)

The coupling of the sulfonamide derivative TS to the CM5 sensor chip surface was described before [9].

Solutions applied in the BIAs

Sample buffer I (used in the mutant A.3.5-based BIA) consisted of 20 mM sodium phosphate buffer containing 0.15 M sodium chloride and 0.3 g l^{-1} of CM-dextran sodium salt. Sample buffer II (used in the mutant M.3.4-based BIA) consisted of HBS-EP to which 5 g l^{-1} CM-dextran sodium salt, 0.3 M sodium chloride and 0.05% Tween 80 were added. Regeneration solution I (used in the mutant A.3.5-based BIA) was 0.1 M HCl. Regeneration solution II (used in the mutant M.3.4-based BIA) was prepared by mixing 10 ml of 18 M guanidine hydrochloride in detergent solution (0.3% of Tween 20, Tween 80, Triton X-100 and CHAPS) with 30 ml 10 mM glycine-HCl buffer (pH 1.5).

Mutant A.3.5-based BIA for the analyses of serum and plasma samples.

Prior to the analysis of the thawed chicken serum and plasma samples from the animal experiment, a 4 min centrifugation at 500 x g was applied. Of the supernatant, 20 µl was pipetted into a well of a microtiter plate. Mutant A.3.5 (approximately 0.6 mg ml^{-1}) was diluted in sample buffer I (2000 times) and 100 µl of it was added to the well. After mixing for 2 min, 50 µl was injected at a flow rate of 20 µl min^{-1} with HBS-EP as the running buffer, and the response obtained 10 s prior to the injection of regeneration solution I (10 µl) was used for the calculations.

For the calculation of concentrations of sulfamethoxazole and sulfadiazine in the serum and plasma, calibration graphs of the two sulfonamides were prepared in blank chicken serum and plasma using the following procedure. The sulfonamides were dissolved in methanol (1 mg ml^{-1}) and stored at -20 ºC until used. Of each sulfonamide solution, a first intermediate standard solution with a concentration of 270 µg ml^{-1} was prepared in HBS-EP buffer. The first intermediate standard solutions

(27 µg ml^{-1} sample) in mixed chicken serum or plasma samples (mixtures of materials coming from 20 blank broilers) were prepared by mixing 450 µl of the sample with 50 µl of the standard solution with a concentration of 270 µg ml^{-1}. Second intermediate standard solutions (2.7 µg ml^{-1}) were prepared by a 10 times dilution in blank serum or plasma. From these intermediate standard solutions, calibrant solutions in the serum or plasma (270, 90, 30 and 10 ng ml^{-1} in serum or plasma) were prepared by dilution with the blank serum or plasma. The concentrations of the sulfonamides in the samples were directly calculated from the calibration curves (dilution factor =1).

Mutant M.3.4-based BIA for the analyses of serum samples (surveillance).

During the surveillance, a combined assay for the detection of sulfonamides (one flow-channel) and three anti-salmonella serotypes (three flow-channels) was applied in the four flow-channel biosensor and the biosensor conditions for the detection of sulfonamides (sample dilution, sample buffer, running buffer and regeneration conditions) were adapted to the serological assay conditions.

Of the serum samples, 40 µl portions were pipetted into the wells of a microtiter plate and 60 µl of sample buffer II were added. The mutant (0.82 mg ml^{-1}) was diluted in sample buffer II (450 times) and, in the Biacore, 50 µl of the diluted sample was added to 50 µl of diluted mutant. After mixing in the Biacore, 40 µl was injected at a flow rate of 20 µl min^{-1} with HBS-EP + 0.05% Tween 80 as running buffer, which was followed by the injection (10 s at 100 µl/min) of regeneration solution II. The response obtained 10 s prior to the injection of regeneration solution was used for the calculations. For the calculation of the concentrations of sulfamethoxazole and sulfadiazine in serum samples, calibration graphs of the two sulfonamides were prepared. Hereto, 40 µl portions of blank serum were pipetted into microtiter plate wells and 40 µl of standard solutions in sample buffer II (250; 125; 62.5; 25; 12.5; 4.3 and 1.25 ng ml^{-1}) and 20 µl of sample buffer II were added. In the Biacore, 50 µl of the diluted sample was mixed with 50 µl of the diluted mutant in sample buffer II, and the standards were analyzed as described above. The concentrations of the sulfonamides in the samples were directly calculated from the calibration curves (dilution factor =1).

Sample materials

Animal experiments

The animal experiment with 4-weeks-old broilers was performed at the facilities of the Animal Sciences Group of Wageningen UR (ASG, Lelystad, the Netherlands) and started on 18-02-2003.

One group of broilers (10 males and 10 females) was not treated with sulfonamides and served as controls. These broilers were slaughtered before administration of the sulfonamides to the other groups.
Another group of broilers (15 males and 15 females) received an oral treatment for 4 days with Methoxasol-T (20 mg trimethoprim and 100 mg sulfamethoxazole per ml; Eurovet Animal Health BV, Bladel, the Netherlands). The dose was 2 ml L^{-1} of drinking water. The average daily water consumption of each broiler was determined to be 200 ml, resulting in a dose of approximately 40 mg of sulfamethoxazole per kg body weight. During the treatment, groups of six broilers (3 males and 3 females) were slaughtered at day 2 and 4, respectively, and after the treatment groups of six broilers each were slaughtered at day 6, 8 and 10 (2, 4 and 6 days after treatment). The prescribed withdrawal time for Methoxasol-T in broilers is 6 days.
The third group of broilers (15 males and 15 females) received for 4 days an oral treatment with Trimethosulfmix 50% (80 mg trimethoprim and 420 mg sulfadiazine sodium per g; Eurovet Animal Health BV, Bladel, the Netherlands). The dose was 0.45 g L^{-1} of drinking water. The average water consumption was determined to be 200 ml per broiler resulting in a dose of approximately 38 mg of sulfadiazine per kg body weight. During the treatment, groups of six broilers (3 males and 3 females) were slaughtered at day 2 and 4, respectively, and after the treatment, groups of six broilers each were slaughtered at day 7, 10 and 14 (3, 6 and 10 days after treatment). The prescribed withdrawal time for Trimethosulfmix 50% in broilers is 12 days.
At slaughter, blood samples (approximately 10 ml) were taken from each individual broiler. Half of the blood was used to obtain serum samples after a 24 h clotting. Plasma samples were prepared from the other half by transfer into heparinized tubes followed by a 10 min centrifugation at approximately 1500 x g. Furthermore, from each broiler, liver, 40-50 g portions of breast meat and leg meat and about 10 g of skin+fat were sampled. The samples were ground and stored at -20°C.

Survey

Serum samples from 31 different flocks (10 samples per flock) originating from 31 different Dutch broiler farms were taken at a slaughterhouse and were supplied by ASG in 100-200 µl quantities. From one flock, in which the presence of sulfonamides was detected during the survey, serum samples from another 150 broilers, slaughtered at the same day as the previous 10 broilers, were obtained.

LC-MS/MS analyses.

Tissue samples

The ground tissue samples (10 g each) were extracted with dichloromethane (25 ml) in a Stomacher® bag for 2 min. After filtration (folded filter of Schleicher & Schuell (Grade 595½, 150 mm diameter)) of the dichloromethane, the extraction was repeated twice, the filtrates were combined in an erlenmeyer flask and 25 ml of petroleum ether was added. This mixture was passed through a Sep-Pak® silica cartridge (Waters no. 51900) and the erlenmeyer flask was washed with 5 ml of dichloromethane which was passed through the cartridge as well. The cartridge was dried for 15 min under a mild stream of nitrogen gas, and the sulfonamides were eluted with phosphate buffer (pH 10) of which the first 4 ml was collected. After mixing (Vortex), the pH was measured and, if necessary, adjusted to a value between 6.5 and 7 by means of phosphoric acid (0.5 M) or sodium hydroxide (0.1 M) solutions. After the addition of ethyl acetate (2 ml), mixing (Vortex) for 20 s and centrifugation (1 min at 2000 rpm), the upper layer was transferred into a test tube. The ethyl acetate extraction was repeated twice and the combined ethyl acetate fraction was evaporated at 30 °C under a stream of nitrogen gas. The residue was dissolved in 0.4 ml of 10 mM ammonium acetate (pH 3.5) and after the injection of 100 µl, an acetonitrile-10 mM ammonium acetate eluent was used with a 15 min linear gradient from 0-90 vol.% acetonitrile, starting 5 min after injection, with a flow-rate of 400 µl/min. The applied LC-MS/MS conditions were described previously [17]. The limit of detection for sulfadiazine and sulfamethoxazole in tissue samples was approximately 1 ng g^{-1}.

Serum samples

Two different mixed serum samples obtained from one positive flock of the surveillance study were analyzed by LC-MS/MS to identify the sulfonamide applied by the farmer. A comparable sample preparation (ultrafiltration) as described for the detection of sulfonamides in milk [17] was applied. In short, the serum sample (50 µl) was mixed with water (950 µl), and 20 µl of an internal standard solution (d_7-sulfadimidine [10 ng ml^{-1}]) was added. Blank serum samples were fortified with the internal standard and, for quantification, with the sulfonamides of interest in the range of 0 - 1000 ng ml^{-1}. After mixing, the sample was allowed to stand for 30 min at room temperature to allow equilibration. The mixture was centrifuged for 15 min at room temperature at 3500 x *g*, 0.5 ml of the supernatant was transferred to a Microcon 0.5 ml ultrafilter (molecular weight cut-off (MWCO) of 30 kDa (Millipore, Bedford, MA, USA)), centrifuged at 5000 x *g* for 30 min at room temperature, and 100 µl of the ultrafiltrate was injected into the LC-MS/MS system. The applied LC-MS/MS procedure was described previously [17]. The limits of detection for most sulfonamides in serum were 5 ng ml^{-1}.

RESULTS AND DISCUSSION

BIA for sulfadiazine and sulfamethoxazole in serum and plasma.

A previously developed Mab 27G3 raised against the generic structure of sulfonamides [12] was modified by protein engineering [13], and in the BIA, the selected mutant A.3.5 showed an improved sensitivity towards several sulfonamides [10]. The sensitivities for sulfamethoxazole and sulfadiazine were improved 10 and 50 times, respectively, and a similar sensitivity was obtained for both sulfonamides in the mutant-based assay. Compared with these two sulfonamides, this multi-sulfonamide BIA showed a comparable or better sensitivity with at least eight other sulfonamides [10]. The increased and similar sensitivity for sulfadiazine and sulfamethoxazole (the most frequently applied sulfonamides in broilers in the Netherlands) was interesting for the analyses of the serum and plasma samples obtained from the animal experiment. The sample preparation consisted of the dilution of the serum or plasma (20 µl) with 100 µl of mutant A.3.5 containing sample buffer only. After mixing, 50 µl was injected at a flow rate of 20 µl min^{-1} followed by a regeneration step with 0.1 M HCl (0.5 min). The total time of analysis, including washing steps in the Biacore, was 5 min. A typical sensorgram (responses versus time) of one cycle of analysis obtained with a blank broiler serum is shown in Figure 1.

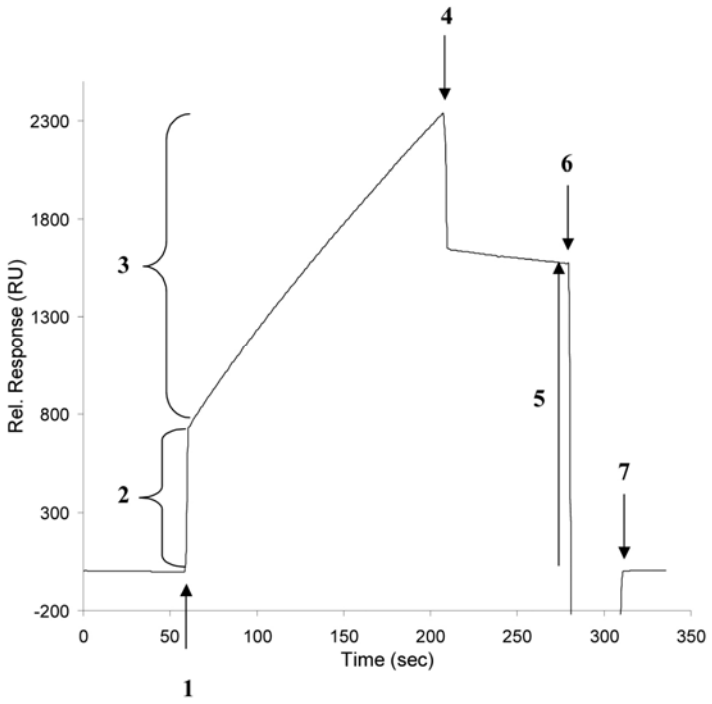

Figure 1. Sensorgram obtained in the Biacore 3000 after the injection of 50 µl of diluted broiler serum (20 µl serum diluted with 100 µl of mutant A.3.5.) followed by a 20 µl injection of 0.1 M hydrochloric acid for the regeneration: (1) start sample injection (at this moment the relative response is set to 0); (2) bulk response; (3) binding of the mutant; (4) stop sample injection; (5) response used for calculation; (6) start injection regeneration solution; and (7) stop injection regeneration solution.

The response obtained 10 sec prior to the injection of regeneration solution was used during the calculations. For quantitative analyses, calibration graphs of both sulfonamides (see Figure 2) were prepared in blank broiler serum and plasma (mixtures of the 20 blank samples obtained from the animal experiment) using four calibrants with concentrations of 10, 30, 90 and 270 ng ml^{-1} (expressed as ng of the sulfonamide per ml of broiler serum).

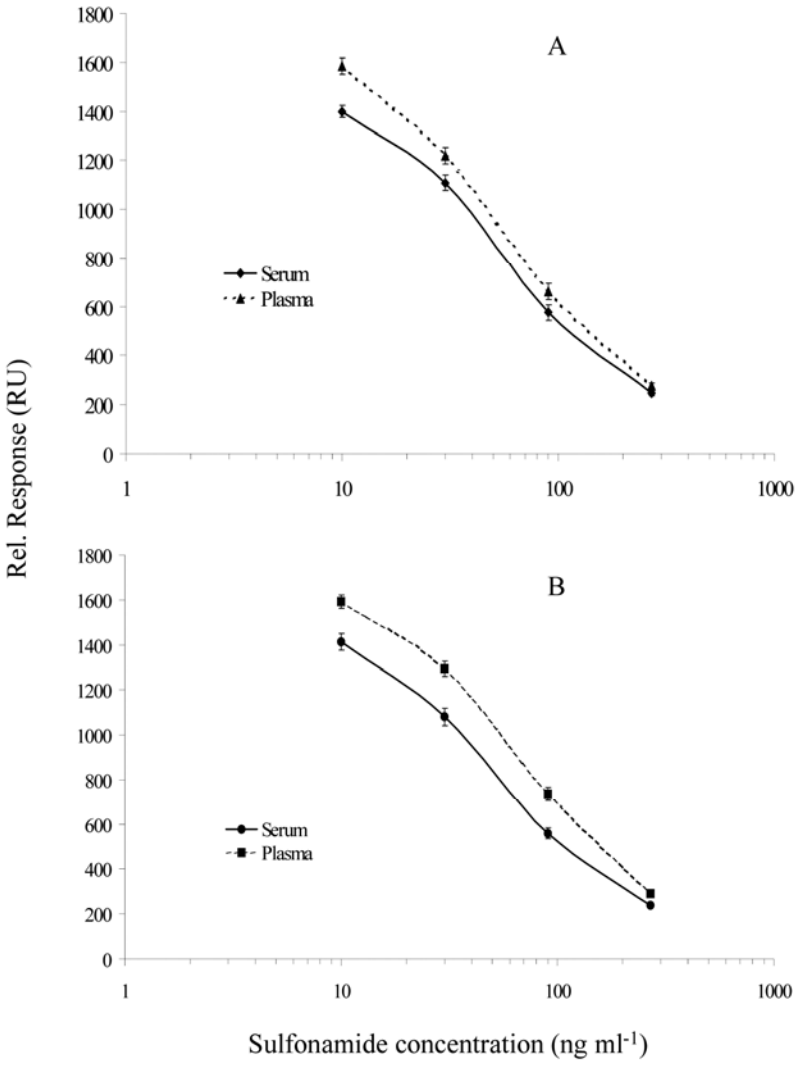

Figure 2. Calibration graphs of sulfadiazine (A) and sulfamethoxazole (B) in broiler serum and plasma in the mutant A.3.5-based BIA. Rel. responses were obtained after the injection of 50 µl of a mixture of sample and antibody-containing buffer (20:100; v/v).

In this assay, the mutant solution (approximately 0.6 mg ml^{-1}) was diluted 2000 times to obtain high average maximum relative responses of 1640 ± 30 RU in blank broiler serum (n=20) and 1750 ± 30 RU in blank plasma (n=20). Using these values and the calibration graphs as presented in Figure 2, the LOD of both sulfonamides in serum and plasma (determined as the concentration corresponding to the average maximum

responses minus three times the standard deviation) were less than the lowest calibration standards in serum and plasma (<10 ng ml^{-1}).
As shown in Figure 2, the calibration graphs for both sulfonamides in serum showed a similar sensitivity (50 % inhibition at 50 ng ml^{-1}). The sensitivities for sulfadiazine and sulfamethoxazole in plasma were somewhat less (50% inhibitions at 60 and 70 ng ml^{-1} for sulfadiazine and sulfamethoxazole, respectively).

Concentrations in the sera and plasma samples from the animal experiment.
The relative responses and calculated concentrations (using the calibration graphs of both sulfonamides in serum and plasma) obtained in the BIA with the sera and plasma samples from the animal experiments are presented in Table 1 (sulfamethoxazole) and Table 2 (sulfadiazine). Compared with the average relative responses obtained with the blank sera and plasma samples (20 each; day 0 of the experiment), all samples taken during treatment (days 2 and 4 of the animal experiment) and after treatment (days 6 to 14 of the experiments) showed lower responses due to the presence of the sulfonamides (inhibition of antibody binding).

Table 1: Average relative response units (RU) and calculated concentrations (ng ml^{-1}) obtained with the mutant A.3.5-based BIA in serum and plasma samples obtained from broilers treated with Methoxasol-T (sulfamethoxazole).

Day of exp.	Day after treatment	Number of broilers	Average Rel. Response (RU)		Average concentration (ng ml^{-1})	
			Serum	Plasma	Serum	Plasma
0		20	1640±30	1750±30	<10	<10
2		6	100±80	80±70	>2700 [a]	>2700 [a]
4		6	80±40	70±60	>2700 [a]	>2700 [a]
6	2	6	620±300	710±340	97±54	115±62
8	4	6	940±340	1120±370	48±39	56±47
10	6	6	1050±320	1200±330	36±27	44±33

[a]Analysed after an extra 10 times sample dilution

During the treatment at daily doses of 40 mg kg^{-1} bw (days 2 and 4), low responses were obtained due to the presence of high concentrations of the sulfonamides. Even after an extra 10 times dilution of these samples, the concentrations in serum and plasma were out of range in the calibration graphs (>2.7 µg ml^{-1}). This level is in accordance with Furusawa and Kishida [18] who fed laying hens for a longer period (7 days) with sulfadiazine and sulfamethoxazole at lower daily doses of 5.2 mg per kg bw and at the seventh day of the treatment, the average concentrations found in the plasma were 1.72 and 1.86 µg ml^{-1}, respectively.

With the samples obtained from the broilers slaughtered after the treatments (2-10 days after treatments) responses were obtained which fitted in the calibration graphs. Among the six broilers slaughtered at each day of the experiments, a high variation in concentration was observed which was due to the varying concentrations in the individual broilers and not due to the variation of the method of analysis (CVs < 5%). Good correlations between the concentrations in serum and plasma were found for both sulfonamides (R^2= 0.996 and 0.993 for sulfamethoxazole and sulfadiazine, respectively), which means that both sample materials can be applied for screening. The prescribed Dutch withdrawal time for Methoxasol-T in broilers is 6 days, however, even after a 2 days withdrawal, the average concentration of sulfamethoxazole in serum and plasma (Table 1) decreased from >2700 ng ml^{-1} (during treatment) to a level of approximately 100 ng ml^{-1}.

Table 2: Average relative response units (RU) and calculated concentrations (ng ml^{-1}) obtained with the mutant A.3.5-based BIA in serum and plasma samples obtained from broilers treated with Trimethosulfmix (sulfadiazine).

Day of exp.	Day after treatment	Number of broilers	Average Rel. Response (RU)		Average concentration (ng ml^{-1})	
			Serum	Plasma	Serum	Plasma
0		20	1640±30	1750±30	<10	<10
2		6	70±10	50±20	>2700[a]	>2700[a]
4		6	50±10	40±10	>2700[a]	>2700[a]
7	3	6	690±170	890±250	75±29	75±32
10	6	6	1020±360	1230±380	43±45	41±46
14	10	6	850±250	1030±260	56±33	53±31

[a]Analysed after an extra 10 times sample dilution

After a withdrawal time of 6 days, none of the serum and plasma samples contained concentrations higher than 100 ng ml^{-1}. The prescribed Dutch withdrawal time for Trimethosulfmix in broilers is 12 days but after 3 days withdrawal period, the average concentration of sulfadiazine in serum and plasma (Table 2) had decreased from >2700 ng ml^{-1} (during treatment) to average levels of 75 ng ml^{-1}.

After a 10 days withdrawal period, the highest concentrations in serum and plasma (119 and 110 ng ml^{-1}, respectively) were found in one of the broilers while the others contained concentrations < 100 ng ml^{-1}. The rapid elimination of sulfadiazine from plasma of orally treated broiler chickens (a mean half-life of 3.7 h) was previously described [19]. The elimination of sulfamethoxazole in plasma of pigs (after an oral single dose administration) was even more rapid (half-life of 2.5 h [20]).

Neither the concentrations of sulfamethoxazole nor those of sulfadiazine in the plasma and serum showed a significant decline during the (later stage of the) withdrawal period (from days 4 and 6 after the treatment, respectively (see Tables 1 and 2)).

Concentrations in tissue as determined by LC-MS/MS.

The MRL for the total amount of sulfonamides (parent compounds only) is 100 µg kg^{-1} in edible tissue. To establish a predictive level in broiler serum, the correlation with the parent compounds in the edible tissue had to be determined.

Table 3: Concentrations of sulfamethoxazole in serum samples (ng ml^{-1}, as determined with the BIA) and in tissue samples (ng g^{-1}, as determined with LC-MS/MS) obtained 2, 4 and 6 days after the treatment of broilers (n=18) with Methoxasol-T (sulfamethoxazole).

Chicken no.	**Day after treatment**	**BIA Serum ng ml^{-1}**	**LC-MS/MS**					
			Leg meat		**Liver**		**Skin+fat**	
			ng g^{-1}	**S/T[a]**	**ng g^{-1}**	**S/T[a]**	**ng g^{-1}**	**S/T[a]**
6428	2	147	30	4.9	75	2.0	121	1.2
6429	2	142	42	3.4	54	2.6	91	1.6
6430	2	49	8	6.1	24	2.0	45	1.1
6453	2	146	23	6.3	54	2.7	90	1.6
6454	2	29	5	5.8	20	1.5	26	1.1
6455	2	68	12	5.7	41	1.7	29	2.3
Average		*97±54*	*20±14*	*5.4±1.1*	*45±21*	*2.1±0.5*	*67±39*	*1.5±0.5*
6431	4	17	3	5.7	8	2.1	37	0.5
6432	4	116	19	6.1	45	2.6	56	2.1
6433	4	70	8	8.8	28	2.5	43	1.6
6456	4	18	2	9.0	8	2.2	12	1.5
6457	4	28	6	4.7	14	2.0	42	0.7
6458	4	41	6	6.8	15	2.7	37	1.1
Average		*48±39*	*7.3±6.1*	*6.8±1.7*	*20±14*	*2.4±0.3*	*38±14*	*1.2±0.6*
6434	6	32	6	5.3	14	2.3	29	1.1
6435	6	21	4	5.2	8	2.6	48	0.4
6436	6	86	12	7.2	28	3.1	41	2.1
6459	6	5	1	5.0	3	1.7	7	0.7
6460	6	40	5	8.0	13	3.1	25	1.6
6461	6	33	4	8.2	7	4.7	38	0.9
Average		*36±27*	*5.3±3.7*	*6.5±1.5*	*12±9*	*2.9±1.0*	*31±15*	*1.1±0.6*
Overall average		**60±47**	**11±11**	**6.2±1.5**	**25±20**	**2.5±0.7**	**45±29**	**1.3±0.6**

[a] Serum/Tissue ratio

Therefore, tissue samples (leg meat, liver and skin+fat) from the broilers with quantified concentrations of the sulfonamides in serum (originating from the 36 broilers slaughtered 2 to 10 days after the treatment with the two sulfonamides) were analyzed by LC-MS/MS.

In the samples from the sulfamethoxazole experiment (18 broilers, see Table 3), the overall average concentration found by the BIA in serum during the withdrawal was 60 ± 47 ng ml^{-1}. The overall average concentrations of sulfamethoxazole found by LC-MS/MS in leg meat, liver and skin+fat were 11 ± 11, 25 ± 20 and 45 ± 29 ng g^{-1}, respectively. All the tissue samples, except for one sample of skin+fat (2 days after the treatment), had concentrations less than the MRL of 100 ng g^{-1}.

Table 4: Concentrations of sulfadiazine in serum samples (ng ml^{-1}, as determined with the BIA) and in tissue samples (ng g^{-1}, as determined with LC-MS/MS) obtained 3, 6 and 10 days after the treatment of broilers (n=18) with Trimethosulfmix (sulfadiazine).

Chicken no.	**Day after treatment**	**BIA Serum ng ml^{-1}**	**LC-MS/MS**					
			Leg meat		**Liver**		**Skin+fat**	
			ng g^{-1}	**S/T[a]**	**ng g^{-1}**	**S/T[a]**	**ng g^{-1}**	**S/T[a]**
6478	3	57	7	8.1	21	2.7	36	1.6
6479	3	114	13	8.8	31	3.7	37	3.1
6480	3	105	8	13.1	26	4.0	32	3.3
6503	3	47	4	11.8	17	2.8	52	0.9
6504	3	74	9	8.2	23	3.2	43	1.7
6505	3	50	4	12.5	16	3.1	39	1.3
Average		*75±29*	*7.5±3.4*	*10±2.3*	*22±6*	*3.3±0.5*	*40±7*	*2.0±1.0*
6481	6	18	2	9.0	8	2.2	15	1.2
6482	6	39	2	19.5	9	4.3	15	2.6
6483	6	133	23	5.8	46	2.9	50	2.7
6506	6	28	5	5.6	10	2.8	21	1.3
6507	6	33	4	8.2	14	2.4	16	2.1
6508	6	8	2	4.0	6	1.3	17	0.5
Average		*43±45*	*6.3±8.3*	*8.7±5.6*	*16±15*	*2.7±1.0*	*22±14*	*1.7±0.9*
6484	10	44	4	11.0	11	4.0	14	3.1
6485	10	63	12	5.2	18	3.5	16	3.9
6486	10	119	18	6.6	34	3.5	41	2.9
6509	10	41	7	5.9	13	3.2	12	3.4
6510	10	40	6	6.7	13	3.1	20	2.0
6511	10	29	5	5.8	13	2.2	15	1.9
Average		*56±33*	*8.7±5.4*	*6.9±2.1*	*17±9*	*3.2±0.6*	*20±11*	*2.9±0.8*
Overall average		**58±37**	**7.4±5.8**	**8.7±3.8**	**18±10**	**3.1±0.7**	**27±14**	**2.2±1.0**

[a] Serum/Tissue ratio

For leg meat and liver, all corresponding serum samples had higher concentrations of sulfamethoxazole and in the case of skin+fat, five samples had higher concentrations of sulfamethoxazole compared with the serum samples. For the sulfamethoxazole-containing samples, the differences between the daily average serum/tissue ratios were small with overall averages of 6.2 (leg meat), 2.5 (liver) and 1.3 (skin+fat). In the samples from the sulfadiazine experiment (18 broilers, see Table 4), the overall

average concentration found by the BIA in serum during the withdrawal was 58 ± 37 ng ml^{-1}. The overall average concentrations of sulfadiazine found by LC-MS/MS in leg meat, liver and skin+fat were 7 ± 6, 18 ± 10 and 27 ± 14 ng g^{-1}, respectively. All tissue samples had concentrations below the MRL of 100 ng g^{-1} and, except for two skin+fat samples, all concentrations of sulfadiazine in serum were higher than the concentrations in the corresponding tissue samples.

For the sulfadiazine containing samples, the differences between the daily average serum/tissue ratios were small with overall averages of 8.7 (leg meat), 3.1 (liver) and 2.2 (skin+fat).

Plotting the individual concentrations of sulfamethoxazole and sulfadiazine in serum found with the BIA (X) versus the tissue concentrations found with LC-MS/MS (Y) resulted in correlation data as presented in Table 5. The highest correlations were obtained between the serum and the liver samples and the lowest between the serum and skin+fat samples.

Table 5: Correlation data (Y = aX +b) obtained by plotting the concentrations found in broiler serum (n = 108) with the BIA (X) against the concentrations found in tissue with LC-MS/MS (Y) using the samples obtained from the broilers after the treatment with sulfamethoxazole and sulfadiazine (n = 18 for each tissue and sulfonamide).

Sulfonamide	**Tissue**	**a**	**b**	R^2
Sulfamethoxazole	Leg meat	0.2140	-1.98	0.8538
Sulfamethoxazole	Liver	0.4099	+0.69	0.9113
Sulfamethoxazole	Skin+Fat	0.5278	+13.52	0.7594
Sulfadiazine	Leg meat	0.1400	-0.68	0.7887
Sulfadiazine	Liver	0.2714	+2.57	0.9152
Sulfadiazine	Skin+Fat	0.2523	+12.72	0.4560

We were surprised that during the total withdrawal periods, the concentrations of both sulfonamides found in serum samples were higher than the concentrations in tissue. However, Furusawa and Kishida [18] also found higher concentrations of sulfonamides in plasma compared to the levels in tissue. During the treatment of laying hens with sulfamethoxazole and sulfadiazine, the ratio (%) of the sulfonamides in the tissue to that in the plasma were reported as 40 % (liver) and 28 % (muscle) for sulfamethoxazole and 20% (liver) and 15% (muscle) for sulfadiazine. In an experiment with calves treated with sulfadiazine [21], the concentrations in kidney, liver and muscle, determined 1, 3 and 7 days after the last dose, respectively, were also generally lower than the corresponding plasma concentrations, although the tissue-to-plasma concentration ratio increased for each tissue over the 7 days period. This increase was not observed in our study.

Survey in chicken sera.

Broiler sera (n=310) were obtained from 31 different flocks of 31 different Dutch farms (10 sera from each farm). These samples were analyzed with the mutant M.3.4-based BIA which proved to be more sensitive towards different sulfonamides compared with mutant A.3.5 [10]. The 50% inhibition values for sulfamethoxazole and sulfadiazine in serum were 20 ng ml^{-1} and this is 2.5 times more sensitive compared with the mutant A.3.5-based BIA. Although a two times higher amount of mutant was injected, the average maximum response obtained with blank sera (approx. 750 RU) was lower compared with the A.3.5-based BIA (1640 RU). This is caused by the relative high amounts of NaCl (0.3 M versus 0.15 M in the A.3.5-based BIA) and CM-dextran (0.5% versus 0.03% in the A.3.5-based BIA) in the sample buffer of the mutant M.3.4-based BIA (adapted to the serological assay conditions).

The samples (*n*=300) from 30 farms were found negative (<5 ng ml^{-1} (sulfamethoxazole equivalents)) and the 10 serum samples obtained from one farm contained levels varying from 10 to 72 ng ml^{-1} with an average of 26 ± 20 ng ml^{-1} (sulfamethoxazole equivalents). Another 150 serum samples, taken from that flock at the same time, were analysed and measured concentrations ranged from <5 to 152 ng ml^{-1} with an average of 25 ± 21 ng ml^{-1} (sulfamethoxazole equivalents). Of these samples, 4 were found negative (<5 ng ml^{-1}) and in 3 samples the concentrations were >100 ng ml^{-1}.

Two different combined serum samples from that flock were prepared (each by mixing approximately 50 µl portions of 13 different serum samples with a relative high concentration (sulfamethoxazole equivalents)). In the BIA, the concentrations found in these combined samples were 26 ± 0.7 and 42 ± 4 ng ml^{-1} (sulfamethoxazole equivalents). The samples were also analysed by LC-MS/MS according to a previously described and fully validated procedure for the analysis of milk samples [17] and sulfamethoxazole was identified and quantified (33 and 49 ng ml^{-1}).

CONCLUSIONS

The mutant antibody-based multi-sulfonamide BIAs proved to be robust and fast (5 min per sample) for the detection of sulfamethoxazole and sulfadiazine in serum and plasma of untreated and treated broilers. The sample preparation procedure was easy and consisted of a dilution of the sera and plasma samples with antibody containing buffer only. The assays showed equal sensitivities for the two sulfonamides in serum or plasma with LODs of <10 ng ml^{-1} (mutant A.3.5-based BIA) and <5 ng ml^{-1} (mutant M.3.4-based BIA). The concentrations found with the BIA in serum and plasma of the treated broilers were comparable and generally higher than the concentrations found in tissue by LC-MS/MS, which made these body fluids suitable

for screening. The decline in sulfonamide residues at the beginning of the withdrawal was very rapid and variability in the residue levels between animals was quite large.
The overall average serum/tissue ratios for sulfamethoxazole were 6.2 (leg meat), 2.5 (liver) and 1.3 (skin+fat) and for sulfadiazine 8.7 (leg meat), 3.1 (liver) and 2.2 (skin+fat). The proposed action level of the multi-sulfonamide BIA in serum, to predict concentrations of the two sulfonamides below the MRL of 100 ng g^{-1} in the tissue containing the highest level (skin+fat), is 130 ng ml^{-1}. In the serum samples from the animal experiments, taken 2 to 10 days after the treatment, only 4 of the 36 samples (11 %) contained concentrations higher than the proposed action level and of the corresponding edible tissues only one sample contained a level higher than the MRL (121 ng g^{-1} in skin+fat) while the concentrations in the others ranged between 50 and 91 ng g^{-1}.
The mutant antibody (M.3.4), with the best sensitivity towards most sulfonamides, was applied in the BIA during a survey and in the 10 serum samples from one flock (out of 31 different flocks), concentrations between 10 and 72 ng ml^{-1} (sulfamethoxazole equivalents and later identified by LC-MS/MS as sulfamethoxazole) were found. A high variation in concentrations (<5 to 152 ng ml^{-1}, with only one sample above the proposed action level) was observed after the analyses of another 150 serum samples of that flock sampled at the same time. High variations were also observed in the serum samples obtained on the same days in the animal experiments (Tables 3 and 4). Such a high variation in concentration is probably due to differences in water consumption and intestinal absorption of the sulfonamide by the broilers. This implies that control of broiler flocks on the possible presence of sulfonamides should always be based on sampling of at least 10 animals.
Another point of concern with the multi-sulfonamide assays is the differences in sensitivity towards different sulfonamides. Although the assays showed an equal sensitivity towards sulfamethoxazole and sulfadiazine, the mutant M.3.4-based assay showed a higher sensitivity towards at least six other sulfonamides (including the for poultry approved sulfachloropyridazine) and a lower sensitivity towards two other approved sulfonamides (sulfaquinoxaline and sulfamethazine) [9]. Based on one action level in the serum, the application of such a multi-sulfonamide screening assay will result in a higher number of false-negative and -positive results and confirmation by LC-MS/MS is mandatory.

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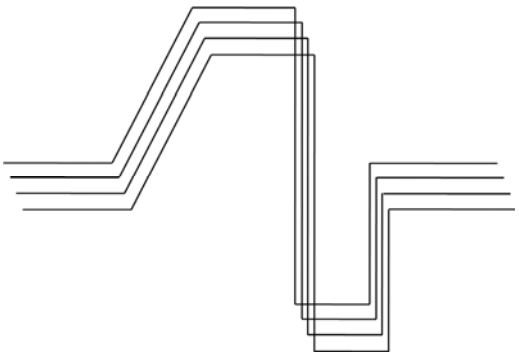
Part III

Biosensor immunoassay development for aminoglycosides



Chapter 7

Direct versus competitive biosensor immunoassays for the detection of aminoglycoside residues in milk

Composed from the following two publications:

- A direct (non-competitive) immunoassay for gentamicin residues with an optical biosensor.
  Willem Haasnoot[1] and Ron Verheijen[1], published as short communication in *Food and Agricultural Immunology* (2001) 13, 131-134.

- Direct versus competitive biosensor immunoassays for the detection of (dihydro)-streptomycin residues in milk.
  Willem Haasnoot[1], Elma E.M.G. Loomans[2], Geert Cazemier[1], Richard Dietrich[3], Ron Verheijen[1], Aldert A. Bergwerff[4], Rainer W. Stephany[4] in *Food and Agricultural Immunology* (2002) 14, 15-27.

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ABSTRACT

Using sensor chip (CM5) immobilized anti-gentamicin monoclonal antibodies (Mabs) in an optical biosensor (Biacore 3000) resulted in the first single antibody-based and label free non-competitive immunoassay for the direct detection of residues of a low molecular weight compound in a food product. Calibration curves for gentamicin (Mw. 466 Da) in buffer and milk showed 50% binding at 20 and 35 ng ml^{-1}, respectively, which is well below the maximum residue limit of 100 ng ml^{-1}.

For the detection of streptomycin (STREP; mw. 581 Da) and dihydrostreptomycin (DHS; Mw. 583 Da), a Mab against DHS (4G8) was developed and its performance compared with a previously developed Mab against STREP (4E2). Direct biosensor immunoassays (BIAs) for the detection of DHS and STREP were developed and compared with competitive inhibition BIAs, using a STREP–protein conjugate immobilized on the chip. The sensitivities of the direct and competitive BIAs for both drugs in buffer were comparable (10–20 ng ml^{-1} at 50% binding or inhibition). With milk, interferences, probably due to the nonspecific binding of proteins to the sensor chips, were observed in both BIAs. These interferences could be largely reduced using ultra filtration (UF) as sample pre-treatment. Another option was the use of a reference flow channel to correct for nonspecific binding. Using this option with five times diluted milk, Mab 4G8 was found to be suited for the direct BIA of both drugs with a limit of detection (LOD) of 20 ng ml^{-1} and both Mabs could be applied in the competitive BIA format with similar LODs.

INTRODUCTION

Gentamicin and the closely related compounds streptomycin (STREP) and dihydrostreptomycin (DHS) are antibiotics belonging to the group of aminoglycosides. They are used in veterinary medicine for treatment of Gram-negative infections. The use in lactating cows may lead to the presence of residues of these antibiotics in milk. With regard to consumer protection, the European Union (EU) has established maximum residue limits (MRL) of 100 μg kg^{-1} for gentamicin and 200 μg kg^{-1} for STREP and DHS in milk (EU regulation no. 1140/96).

For the detection of aminoglycosides, three techniques are mainly applied: the microbial inhibition screening tests (IDF Bulletin, 1991; Nouws *et al.*, 1991; Nouws *et al.*, 1995), immunoassays or receptor assays (IDF Bulletin, 1991; Dietrich *et al.*, 1993; Schnappinger *et al.*, 1993; Haasnoot *et al.*, 1999; Verheijen *et al.*, 2000) and HPLC (Essers, 1984; Gambardella *et al.*, 1985; Albracht & de Wit, 1987; Gerhardt *et al.*, 1994; Guggisberg & Koch, 1995a; Guggisberg & Koch, 1995b). HPLC might be the most suitable technique for the confirmation of positive screening results. For a large scale first screening, microbiological methods are preferred because of their convenience, low costs and broad-spectrum characteristics. However, with such methods it takes at least 6 h before the results are known (Nouws *et al.*, 1995). More rapid methods for the detection of these antibiotics are microtiter plate ELISAs with results being available within 2 h (Haasnoot *et al.*, 1999). However, such ELISAs are specific and normally performed in a laboratory. The fastest and easiest assay described so far is the one-step strip test in which the results can be obtained within 10–15 min (Verheijen *et al.*, 2000). This test was developed for on-site screening of raw milk samples for the presence of DHS and STREP on a farm. However, this test is qualitative only and not suitable for large scale screening.

For a few years, rapid and automated surface plasmon resonance (SPR)-based biosensor immunoassays (BIAs) are described for the detection of sulfamethazine (Sternesjö *et al.*, 1995), enrofloxacin and ciprofloxacin (Mellgren & Sternesjö, 1998) in bovine milk and for sulfamethazine and sulfadiazine residues in pig bile (Elliott *et al.*, 1999; Crooks *et al.*, 1998). The small size of these low molecular weight compounds (LMWC), with molecular weights (Mw) of 466 (gentamicin), 581 (DHS) and 583 Da (STREP), prevents a simultaneous exposure of more than one antibody binding site (epitope) or receptor binding site. Therefore, for the detection of these compounds, the competitive inhibition immunoassay format is mandatory and the LMWC were covalently immobilized to a carboxy-methylated dextran modified gold film. Polyclonal antibodies (Pabs) against the LMWC were added to the sample and the immobilized surface was used to determine the amount of free antibodies. The major advantage of SPR-based biosensor assays is the label free detection. Other described advantages are the lack of a sample preparation, their fully automated

operation, the short time of analysis (5–20 min) and the high specificity. The newest biosensor model, Biacore 3000 (Biacore AB, Uppsala, Sweden), was designed to measure LMWC directly. This was confirmed during an investigation of interactions between immobilized thrombin and its inhibitors in solution (Karlsson *et al.*, 2000).
The objective of the present study was to demonstrate that a direct immunoassay for LMWC could work at a level below the maximum residue limit (MRL) in a food product. The aminoglycosides gentamicin, DHS and STREP with Mw between 466 and 583 Da) were chosen as the target analytes and milk as the model matrix. For the direct detection of gentamicin, a commercially available monoclonal antibody (Mab) was used. For the detection of DHS and STREP, a Mab against DHS was developed and its performance in the Biacore 3000 was compared with a Mab previously developed against STREP. With these Mabs, direct and competitive BIAs were developed and their performances with regard to the detection of DHS and STREP in buffer and milk were compared. The influence of several parameters (origin of antibodies, flow rate, coating concentration, sample volume and especially matrix effects from milk) on the performance of the assays are described.

MATERIALS AND METHODS

Instruments and Reagents

The Biacore 3000 biosensor system, sensor chips (CM5), HBS-EP buffer and the Amine Coupling Kit (containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride-NaOH (pH 8.5)) were supplied by Biacore AB (Uppsala, Sweden). A Sigma model 2K15 centrifuge was obtained from Salm and Kipp BV (Breukelen, the Netherlands). The FPLC system and HiTrap Protein G columns (1 ml) were supplied by Amersham Pharmacia Biotech AB (Uppsala, Sweden). Specol was obtained from the Institute for Animal Science and Health (ID-Lelystad, Lelystad, the Netherlands). Dulbecco modified Eagle's medium (DMEM) was obtained from Bio Wittaker (Verviers, Belgium). Hybridocult and Hybridokine were purchased from Immuno Quality Products (Groningen, the Netherlands) and Fetal Clone I (FCI) came from HyClone (Logan, UT, USA). Hypoxanthine–thymidine (HT), aminopterine (A) and chlortetracycline were obtained from Life Technologies (Breda, the Netherlands). Gentamicin sulfate, neomycin sulfate, spectinomycin, tobramycin, kanamycin, amikacin, streptomycin sulfate (STREP) and dihydrostreptomycin sesquisulfate (DHS), succinic anhydride, *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), ethanolamine hydrochloride, triethylamine, sodium sulfate, *N*,*N*-dimethylformamide (DMF), dichloromethane, ethyl acetate, 2-(*N*-morpholino)ethanesulfonic acid (MES),

tris(hydroxymethyl)aminomethane (Tris), glycin hydrochloride, L-glutamine, L-glucose, sodium carbonate, glutaraldehyde, sodium borohydride, goat anti-mouse immunoglobulins-alkaline phosphatase (GAM-AP), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin (OVA) and polyethyleneglycol (PEG; Hybri-Max) were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). Mouse anti-gentamicin Mab (Clone 905.1; 0.5 ml with a concentration of 1.9 mg ml^{-1}) was obtained from HybriDomus (Rhoon, the Netherlands) and affinity purified rabbit IgG (10 mg ml^{-1}) from Lampire (Piersville, PA, USA). The BCA protein assay was supplied by Pierce (Rockford, IL, USA). Flat bottom microtiter ELISA (EIA/RIA high binding plates, 96 wells), tissue culture plates and culture flasks came from Costar (Schiphol-Rijk, the Netherlands). Ultrafree-0.5 centrifugal filter devices (Biomax-10, –30 and –100) were obtained from Millipore (Bedford, USA).

Preparation of Immunogen and Coating Conjugates

Activated carbodiimid coupling method (acc method)

DHS was coupled to pre-activated KLH or BSA through the activated carbodiimid coupling method (acc method). Preactivation of the proteins was performed with succinic anhydride in order to introduce additional coupling sites and to reduce the chance of creating inter-protein cross-links. Subsequently, the conjugation reaction between the protein carboxyl groups and the aminogroups of DHS was performed using the water soluble carbodiimide reagent EDC.

In order to produce NHS–succinate, necessary for the pre-activation of the proteins, NHS (5 g) and succinic anhydride (4.35 g) were dissolved in dichloromethane (30 ml). Triethylamine (7.2 ml) was added and the reaction mixture was stirred for 3 h at room temperature. Ethyl acetate (50 ml) was added and the mixture was transferred to a separator funnel. The organic phase was washed twice with 25 ml ice-cold 1 M HCl, dried over sodium sulfate and concentrated in a rotary evaporator. An amount of 1 g off-white solid was obtained that was used without further purification.

The protein (2 mg in 1 ml PBS at pH 7) was transferred to an eppendorf vial. NHS–succinate was dissolved in DMF (10 mg ml^{-1}), and 60 µl was added to the protein solution. The reaction mixture was vortexed, left at room temperature for 45 min and another 60 µl of the NHS–succinate solution was added. The reaction mixture was vortexed, left for 45 min at room temperature, transferred to a Biomax concentrator (cut-off 30000 Da) and concentrated by centrifugation for 30 min at 3000 x *g* and at 4°C. A volume of 1 ml 5 mM MES buffer at pH 5 was added to the retentate and the solution was centrifuged for 30 min at 3000 x *g* at 4°C. This step was repeated twice to ensure complete buffer exchange. The protein solution was taken up in 1 ml 5 mM MES buffer. A portion of 200 µl of 10 mg ml^{-1} DHS in 5 mM MES buffer was added

to the protein solution followed by 200 µl of a 10 mg ml^{-1} EDC solution in 5 mM MES buffer, which was prepared just before use. The reaction mixture was vortexed, kept at room temperature for 2 h, transferred to a Biomax concentrator and concentrated by centrifugation for 30 min at 3000 x *g* at 4°C. A volume of 1 ml of PBS at pH 7 was added and the solution was centrifuged again. This step was repeated twice to ensure complete buffer exchange. The protein solution was diluted with PBS to give a 1 mg ml^{-1} solution.

Glutaraldehyde coupling method (GDA)

The glutaraldehyde (GDA) coupling method was performed at two pH values (7.3 and 8.5) because of observed differences in solubility at these pH values. KLH or BSA (2 mg ml^{-1}) was dissolved in a mixture of 0.1 M sodium carbonate and 0.15 M NaCl (pH 7.3 or 8.5) and subsequently DHS was added to the protein carrier solution to obtain a concentration of 2 mg ml^{-1}. After adding 1% (m/v; final concentration) freshly prepared glutaraldehyde, the reaction mixture was vortexed and kept for 3 h at 4 °C under gentle agitation. Sodium borohydride was then added to a final concentration of 10 mg ml^{-1} and the solution was incubated for 1 h at 4°C. Finally, the conjugate was purified by gelfiltration or dialysis to remove excess reagents.

STREP–OVAL conjugate

The STREP–OVAL conjugate was prepared according to a similar procedure as previously described for the preparation of STREP–BSA (Verheijen *et al.*, 2000).

Immunization of Mice

Two female Balb/cByHlco mice (99049/99050) of 9 weeks old were immunized (subcutaneously) with 75 µg KLH–DHS in 200 µl PBS/Specol (1:1; v/v). The immunogen consisted of a cocktail of three KLH–DHS conjugates prepared by three different coupling methods; glutaraldehyde pH 7.3 (GDA7.3), glutaraldehyde pH 8.5 (GDA8.5) and the activated carbodiimid coupling (acc). One month before and 14 days after immunization, blood was taken by orbital punction to determine the specific antibody titer in ELISA. Mice were boostered (subcutaneously) twice, at various intervals, with 25 µg conjugate in 200 µl PBS/Specol (1:1; v/v). One week after each booster, the serum was analyzed with regard to the presence of specific antibodies by indirect ELISA, using microtiter plates coated with BSA–DHS conjugates. Competitive ELISAs were used to assess the ability to recognize free DHS.

Production of Mabs

Four days after the final booster (intraveneously), the spleen of one mouse (99049) was used to produce Mabs by the hybridoma technology (Köhler & Milstein, 1975).

Spleen cells were fused with myeloma cells (NS-20, American Type Culture Collection, Rockville, USA) at a ratio 2:1, using PEG 1500 precipitation. Cells were suspended in HAT selection medium [culture medium (DMEM supplemented with 8% FCI + 1% hybridocult + 1% tetracycline) + 1% HT (hypoxanthine/ thymidine) + 1% A (aminopterin)] and plated in 96-well tissue culture plates at 150000 cells per well. After one week, the medium was changed to HT medium (culture medium + 1% HT) and seven days thereafter to normal culture medium. In this period, hybridoma culture supernatants were screened by ELISA for antibody activity on specific binding to the homologous coating conjugate (1–5 μg ml^{-1}) and displacement capacity in the presence of the free hapten (1 μg per well). Positive cultures were further cloned by limiting dilutions (5, 1 and 0.2 cells per well) in 'limiting dilution' medium (DMEM + 8% FCI + 2.5% Hybridokine). When positive again, larger quantities of Mabs were obtained by growth from larger-well plates to tissue culture flasks (up to 20 ml).

Isotyping was performed using a multi-analyte one-step lateral flow assay in combination with anti-mouse isotype antibodies conjugated to carbon particles (developed at ATO).

To culture supernatant (50 ml of Mab 4G8, see Results and Discussion), 50 ml of saturated ammonium sulfate were added slowly. After centrifugation, the supernatant was removed and the residue was dissolved in 1 ml of PBS and dialyzed against PBS. The dialyzed product was transferred to a protein G column and, after washing with PBS, the bound IgG was eluted with 2.5 ml of 0.1 M glycine. HCl (pH 2.5). The pH of the eluted fraction was immediately neutralized by the addition of 0.5 ml of 1 M Tris-HCl buffer (pH 9.5). The final volume was 3 ml with a protein concentration of 0.53 mg ml^{-1} (as determined by the Pierce protein assay, according to the instructions of the manufacturer, with BSA as standard protein).

Anti-STREP Mab

The development of the Mab against STREP (4E2) was described before (Dietrich *et al.*, 1993; Schnappinger *et al.*, 1993). The antibody (IgG1) was affinity-purified from hybridoma culture supernatant on protein A Sepharose to a concentration of 0.52 mg ml^{-1} in PBS. The cross-reactivity with DHS was 86% (Dietrich *et al.*, 1993).

ELISAs

Microtiter plates were coated (overnight at 4 °C or 1 h at 37 °C) with 100 μl of a BSA–DHS conjugate at different coating concentrations (maximum concentration of 1 μg per well) in carbonate/ bicarbonate coating buffer (0.05 M; pH 9.6). The plates were washed three times with PBS-Tween (0.05% Tween 20; pH 7.2) and blocked (incubation of 2 h at 37 °C with 200 μl of BSA or casein (2% in PBS, pH 7.2). After washing, 100 μl serum or culture supernatants (different dilutions in dilution buffer (PBS + 0.1% BSA or casein)) were added to the wells and the plate was incubated for

1 h at 37°C. After washing, 100 µl GAM-AP (1:1000 in dilution buffer) was added to the wells and the plate was incubated for 1 h at 37°C. After washing, color was developed by adding 200 µl of the substrate solution (1 mg 4-nitrophenylphosphate disodium salt per ml of 0.05 M carbonate/bicarbonate buffer containing 100 ng 1^{-1} $MgCl_2$·H2O, pH 9.6). The optical density of each well was measured with a plate reader at 405 nm after various time intervals.

For the competition with free DHS, 50 µl of the diluted antibodies were mixed with 50 µl of a 20 µg ml^{-1} DHS solution in PBS. The plates were further treated as described above.

BIAs

Proteins (Mabs or STREP–OVAL) were immobilized on the sensor surface of a CM5 sensor chip by using the amine coupling kit and the Surface Preparation Wizard, as present in the Biacore 3000 control software. The biosensor surface was activated by injection of 35 µl of a mixture of EDC and NHS (1:1; v/v) in one of the four flow channels (Fcs) at a flow rate of 5 µl min^{-1}. Then the proteins, diluted in coupling buffer, were injected and attached via primary amine groups to the carboxy-methylated dextran surface. After coupling, active groups were blocked with 1 M ethanolamine.

Direct BIAs.

The Mab-containing solutions were diluted to an end concentration of 0.1 mg ml^{-1} in coupling buffer (10 mM-sodium acetate, pH 4.5). In order to obtain measurable responses from binding gentamicin, DHS and or STREP, the amount of antibodies on the sensor surface should be as high as possible and a level of 15000 RU was aimed at in the Surface Preparation Wizard. Immobilized total rabbit IgG was used in the reference Fc (Fcl). Standard solutions of gentamicin, STREP or DHS were injected (20, 50 or 100 µl) at different flow rates (20 or 50 µl min^{-1}) and antibody-bound gentamicin was regenerated by the injection of 5 µl of a 20 mM sodium hydroxide solution and bound STREP or DHS were regenerated by the injection of 5 µl 5 mM hydrochloric acid solution. The response measured 10 s prior to the start of the regeneration was used for calculations.

Defatted milk samples were five-fold diluted in HBS-EP buffer and portions of 20 µl were injected at a flow rate of 20 µl min^{-1} (direct method A) or 0.5 ml diluted milk was pipetted into the centrifugal filter device (Biomax-10). After centrifugation for 30 min at 12000 x *g*, volumes of 20 µl or 50 µl of the filtrate were injected at a flow rate of 20 µl min^{-1} (direct method B). The total run times between samples for the 20 and 50 µl injections were 3 and 5 min, respectively.

Competitive BIAs.

The STREP–OVAL conjugate-containing solution was diluted in coupling buffer (10 mM sodium acetate, pH 5.0) to a final concentration of 25 μg ml^{-1}. Different immobilization levels and reference Fcs were applied (see Results and Discussions). In the final method, STREP–OVAL was immobilized in Fc2 (1000 RU) and OVAL was immobilized in Fc1 (950 RU), which served as a reference Fc. A volume of 80 μl in HBS–EP buffer diluted antibodies was added to 20 μl defatted milk sample, mixed and 50 μl was injected at a flow rate of 20 μl min^{-1}. The regeneration was performed with 10 μl 50 mM hydrochloric acid. The response measured 10 s prior to the start of the regeneration was used for calculations. The total run time was 5 min.

Milk Samples

A pasteurized skimmed milk powder was supplied by NIZO Food Research (Ede, The Netherlands). To reconstitute milk, 9 ml water was added to 1 g of powder. Blank milk samples (n = 17) from five different cows and taken at different milking moments were obtained from the Research Institute for Animal Husbandry (Lelystad, the Netherlands). The samples were previously found negative (< 1 ng ml^{-1}) with regard to the presence of DHS and STREP by LC-MS and were stored at –20 °C before analysis. Prior to the use of thawed milk, samples were centrifuged for 5 min at 1000 x g and the defatted milk sample was pipetted from below the layer of fat.

RESULTS AND DISCUSSIONS

Mab Production

DHS was coupled to the carrier protein (KLH) through three different coupling methods; glutaraldehyde pH 7.3 (GDA7.3), glutaraldehyde pH 8.5 (GDA8.5) and the activated carbodiimid coupling (acc). A cocktail of these conjugates was used to immunize two BALB/c mice. After the second booster, both mice (99049 and 99050) showed good titers and, in the competitive indirect ELISA, sera of day 35 showed 70% and 40% inhibition, respectively, caused by the addition of 1 μg DHS to the BSA–DHS (acc) coated wells. Spleen cells from mouse 99049 were used for PEG-fusion and the resulting hybridomas were cultured in a total of 1152 wells of 96-well microtiter plates. An indirect ELISA, using microtiter plates coated with a cocktail of BSA–DHS conjugates, was used to screen the supernatants from each well showing cell-growth for antibodies against DHS. A total of 15 wells contained specific antibody producing hybridomas. These positive hybridoma cells were transferred into 1 ml bottles and tested once more in a competitive ELISA for their ability to recognize unconjugated (free) DHS. Only one cell line (62–4G8) showed a high titer and competition with DHS with all DHS conjugates. This cell line was brought into

limiting dilution (LD). Four stable cell lines (62–4G8-C10/D10/E8/G3) produced anti-DHS antibodies, of which the binding to DHS coated plates was inhibited by 1 μg ml^{-1} DHS. The supernatants of the first LDs of 62–4G8-G3 and 62–4G8-E8 contained Mabs (IgG1 with a kappa light chain). A second LD for 62–4G8-C10 and -D10 was performed to guarantee their monoclonal origin.

In a competitive ELISA with BSA–DHS (acc or GDA8.5) coated plates, the culture supernatant of 62–4G8-E8 (further abbreviated as 4G8) showed the highest sensitivity with IC_{50} values of approximately 100 and 50 ng ml^{-1} for DHS in raw milk and buffer, respectively. This Mab (4G8) and the previously developed Mab against STREP (4E2), after concentration from culture supernatant by affinity chromatography, were used in the BIAs.

Direct BIAs

Gentamicin.

Using the immobilization wizard of the Biacore 3000 control software, the coupling of antibodies in Fc1 and Fc2 resulted in final responses of 9940 and 10 000 RU, respectively. During the measurements, Fc1 served as the reference channel and the obtained response was subtracted from that in Fc2. Standard solutions of gentamicin were injected (20 μl) at a flow rate of 20 μl min^{-1}. This resulted in sensorgrams as shown in Figure 1.

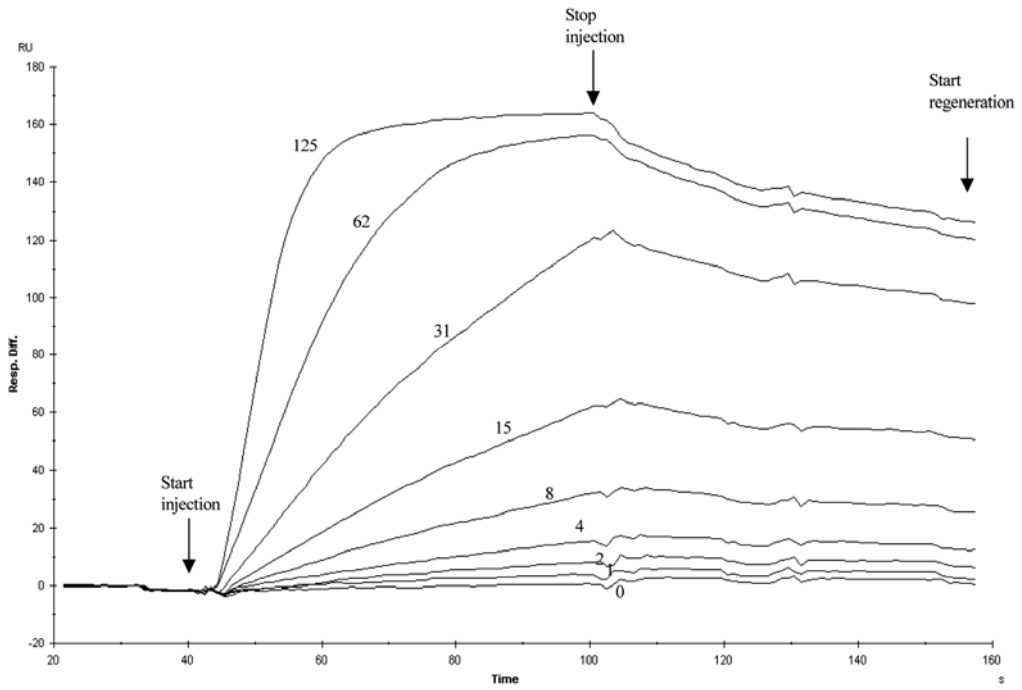

Figure 1. Sensorgrams obtained with standard solutions of gentamicin (0-125 ng ml^{-1}).

Gentamicin-bound antibodies were regenerated by the injection of 5 µl of a 20 mM sodium hydroxide solution. The total run time (sample injection and regeneration) per sample was 5 min. As shown in Figure 1, the immobilized antibodies were saturated with gentamicin at a concentration of 125 ng ml^{-1} (2.5 ng per injection) and lower concentrations resulted in reduced responses during and after injection. The detection limit in buffer (three times background response) was 2 ng ml^{-1} (40 pg per injection). The response differences, measured 20 s before the regeneration started (100 s after injection, see Figure 1), obtained from the different standard solutions in buffer and in milk were plotted against the concentration of gentamicin (see Figure 2). This resulted in a calibration curve in buffer with 50% binding at 20 ng ml^{-1}.

The calibration curve in milk (reconstructed from skimmed milk powder) showed a 50% binding at 35 ng ml^{-1} (see Figure 2) and a detection limit of 10 ng ml^{-1} which is well below the MRL of 100 ng ml^{-1}.

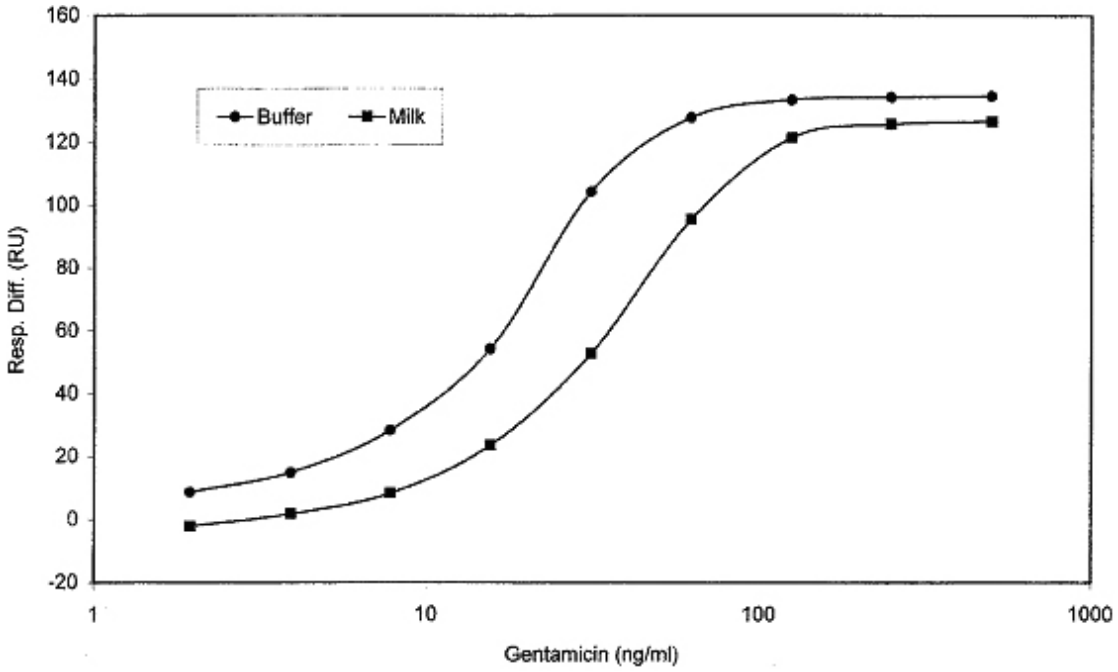

Figure 2. Calibration curves of the direct gentamicin immunoassay in buffer and milk.

STREP and DHS.

In the direct assays for DHS and STREP we were aiming for an immobilization level of 15000 response units (RU) in the Surface Preparation Wizard and final responses of 14000 RU (Mab 4E2 in flow channel 2 (Fc2)) and 12800 RU (Mab 4G8 in Fc4) were obtained (biosensor chip A). Total rabbit IgG was immobilized in Fc1 and Fc3 (9000 RU) which served as reference Fcs. After 20 µl injections of standard solutions (0–1000 ng ml^{-1}) of STREP (581 Da) in the four serially connected Fcs, maximum responses of 105 RU in Fc2 and 90 RU in Fc4 were found (see Figure 3).

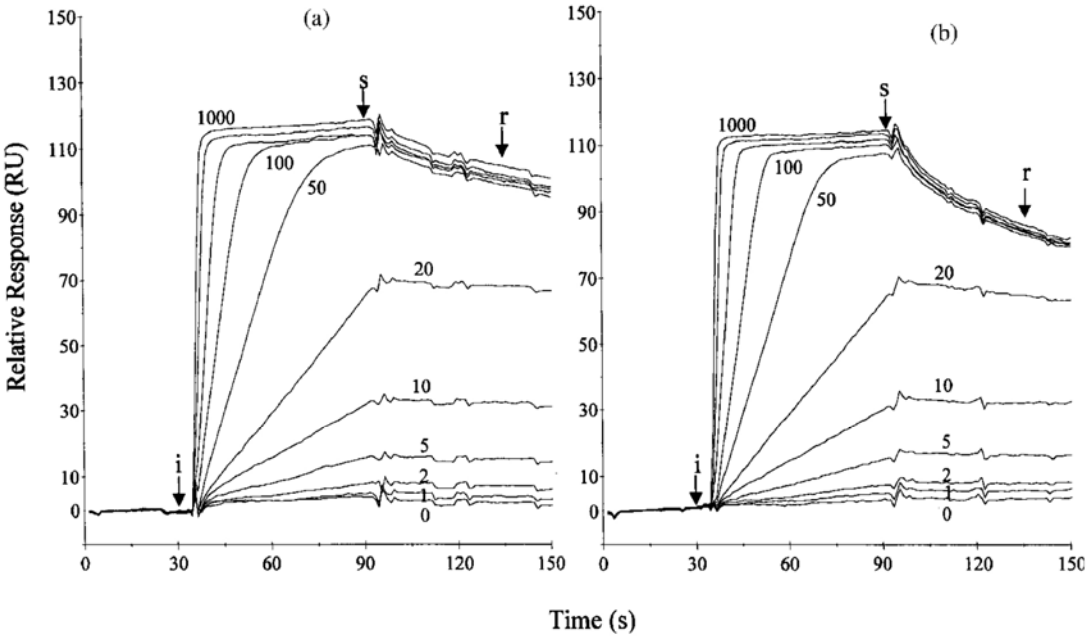

Figure 3. Sensorgrams showing the binding of STREP to the immobilized Mabs in the direct BIAs: (a) Mab 4E2 in Fc2 and (b) Mab 4G8 in Fc4. Several standard solutions of STREP in HBS-EP buffer (0–1000 ng ml^{-1}) were injected (20 µl) using a flow rate of 20 µl min^{-1}. (i) injection start; (s) stop injection; (r) response (RU) used for calibration curves.

The immobilized antibodies were saturated with STREP after 20 µl injections of standard solutions with a concentration of 50 ng ml^{-1} (1 ng absolute) or higher at a flow rate of 20 µl min^{-1}. Lower concentrations of STREP resulted in reduced signals (see Figure 3). These results were comparable with the results obtained with the direct BIA for gentamicin. The antibodies were regenerated by the injection of 5 µl 5 mM HCl at a flow rate of 20 µl min^{-1}. The responses, measured 10 s before the start of the regeneration (40 s after the injection stop, see Figure 3), obtained for the different concentrations of STREP and DHS in buffer were plotted against their corresponding concentrations (see Figure 4). With both Mabs, STREP injections resulted in higher responses than DHS injections. Due to the differences in dissociation (see Figure 3), the responses obtained with STREP and DHS binding to Mab 4E2 (Fc2) were higher than those obtained with Mab 4G8 (Fc4).

The concentration corresponding with 50% binding of STREP and DHS to the two Mabs was 11 ± 1 ng ml^{-1}. The measurement ranges for both aminoglycosides were narrow, from 5 (10% binding) to 30 ng ml^{-1} (90% binding). Increasing the injection volumes from 20 to 50 and 100 µl resulted in increased sensitivities (from 11 to 6 and 3 ng ml^{-1} at 50% binding, respectively). Increasing the flow rate from 20 to 50 µl min^{-1} resulted in reduced maximum signals, due to a less efficient binding. Solutions of 1000 ng ml^{-1} of other aminoglycosides (spectinomycin, tobramycin, kanamycin, amikacin, gentamicin and neomycin) were injected and neither BIA responsed, demonstrating the specificity of both Mabs with regard to DHS and STREP.

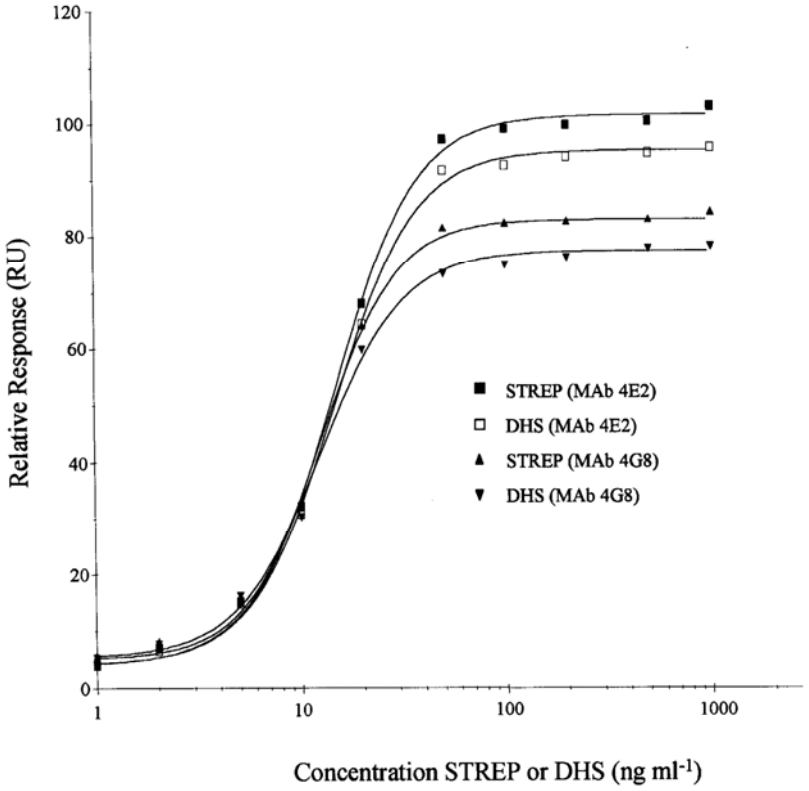

Figure 4. Calibration curves of STREP and DHS obtained in the direct BIAs with both Mabs (4E2 and 4G8). Injection volumes were 20 µl at a flow rate of 20 µl min^{-1}.

DHS and STREP in milk.

The calibration curves of DHS (binding to Mab 4G8 in Fc4) in buffer and reconstituted milk from skimmed milk powder were compared (see Figure 5). Due to matrix effects, the calibration curve for DHS in milk showed less sensitivity (22 ng ml^{-1} at 50% binding) compared with the curve in buffer (12 ng ml^{-1} at 50% binding). However, with an LOD (determined as three times the average response of the blank milk) of 10 ng ml^{-1}, the assay was still useful for the detection far below the MRL of 200 ng ml^{-1}. These results were similar to the results obtained with the direct BIA for gentamicin using the same milk sample. By diluting the milk sample with buffer (five times), the difference between the curves in buffer and milk was reduced, supporting the suggestion of matrix interference.

The 17 different blank milk samples were diluted five times in HBS–EP buffer and 20 µl of each sample was injected (20 µl min^{-1}) in the four serially connected Fcs. Average background responses of 42 ± 16, 118 ± 64, 49 ± 16 and 41 ± 9 RU were obtained in Fc1, Fc2, Fc3 and Fc4, respectively. The average background response in Fc2 (Mab 4E2) was very high compared with those obtained in the other Fcs and also compared with the maximum responses obtained after binding of STREP or DHS (approximately 100 RU) and Mab 4E2 was considered to be not suitable for a direct

BIA in milk without a sample preparation. The best results were obtained with Mab 4G8 (Fc4) and when corrected for the responses obtained in the reference Fc (Fc3), an acceptable average background was obtained (–8 ± 8 RU).

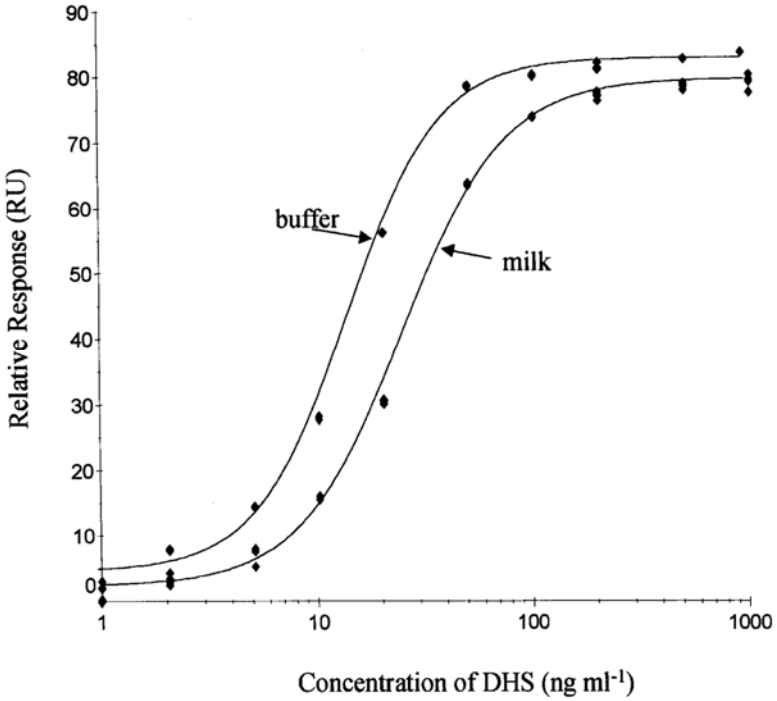

Figure 5. Comparison of calibration curves of DHS (20 µl injections at 20 µl min^{-1}) in the direct BIA (Mab 4G8) in buffer and undiluted milk (reconstituted milk powder).

Using this Mab, the detection limit in milk (using five times diluted samples) was 20 ng ml^{-1}.

The observed background responses were caused by matrix effects (probably protein–protein interactions), which could be reduced effectively by means of an ultra filtration (UF) device with a cut-off of 10 kDa. Unlike the reconstituted skimmed milk powder, the 17 different milk samples could not be used undiluted in the UF device because of blocking of the membrane. Using such devices with five times diluted milk, the filtrates were clear and, after 20 µl injections of the filtrates of the 17 blank milk samples, average background responses of 0.4 ± 0.6, 3.6 ± 2.0, 0.0 ± 2.0 and 3.5 ± 1.4 were obtained in Fc1, Fc2, Fc3 and Fc4, respectively. A calibration curve of STREP in milk (0–1000 ng ml^{-1}), injected after UF, was almost comparable to the curve in buffer (50% binding at 40 and 50 ng ml^{-1} in buffer and milk, respectively, in five times diluted standards and samples). Using UF as sample preparation, both Mabs could be applied for the detection of DHS and STREP at the 20 ng ml^{-1} level. However, a direct BIA without sample preparation is easier to perform and this could be achieved with Mab 4G8 only.

Competitive BIAs

Most BIAs for the detection of LMWC use the competitive inhibition assay format in which the LMWC is directly immobilized onto the sensor surface. In these immobilization procedures, a high concentration of the LMWC (2 mg ml^{-1}) was used (Sternesjö *et al.*, 1995). In order to avoid contamination of the biosensor system with the LMWC, causing interferences with following sample analysis, these immobilizations were performed outside the biosensor system in which the whole sensor surface was coated with the same LMWC. Having a biosensor system in which four different Fcs could be used for different BIAs, immobilizations in the individual Fcs were preferred. Another disadvantage of the direct coupling of the LMWC to the sensor surface was that for each type of LMWC a new immobilization procedure on the chip had to be developed. For these reasons, a drug–protein conjugate (STREP–OVAL) was chosen for the immobilization. The immobilization of proteins on the CM5 sensor chip by amine coupling is a standard procedure in which low concentrations of protein are used (25–100 μg ml^{-1}). This enables the direct coupling in the different Fcs and gives the possibility to vary and control the coating concentration.

To examine the influence of coating concentration on the BIA performance, two different amounts of STREP–OVAL were bound to the sensor surface of a new biosensor chip (chip B). In Fc4, we aimed for a high amount (10000 RU) bound to the sensor surface and obtained, after the injection of 63 μl of a 25 μg ml^{-1} solution, a final response of 5900 RU. In Fc2, a 10 times lower concentration of STREP–OVAL was aimed at (600 RU) and, after injection of 13 μl of the 25 μg ml^{-1} solution, a final response of 620 RU was obtained. Bound antibodies could be released from the surface by injection of 5 μl of 50 mM HCl. For the preparation of calibration curves of STREP and DHS with the two Mabs in the two Fcs, 50 μl of standard solutions were mixed (just prior to the injection, no incubation) with 50 μl of diluted Mabs. The final dilution of Mab 4E2 used in combination with Fc2 was 800 times (4.3 nM) and 2000 times (1.7 nM) in combination with Fc4, which resulted in maximum responses of 240 RU in both Fcs. The calibration curves for STREP showed 50% inhibitions of 10 and 9 ng ml^{-1} in Fc2 and Fc4, respectively, which means that the effect of the Fc capacity on the sensitivity of the calibration curves is minimal. Final dilutions of Mab 4E2 of 400 times (8.6 nM) and 4000 times (0.86 nM) in Fc4 resulted in 50% inhibition at 30 and 8 ng ml^{-1}, respectively, which means that the sensitivity of the assay decreased with higher concentrations of the Mab.

The calibration curves for STREP and DHS obtained with both Mabs (each fd 2000 times; 1.7 nM) in Fc4 are shown in Figure 6.

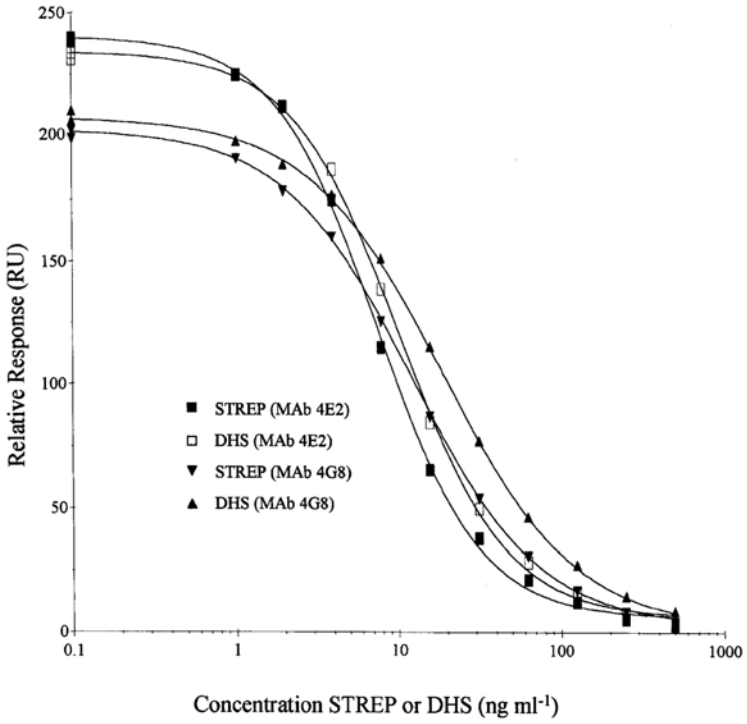

Figure 6. Calibration curves of STREP and DHS obtained in the competitive BIAs in Fc4 of chip B using Mabs 4E2 and 4G8 at final dilution of 2000 times (1.7 nM). Standard solutions (50 μl) were mixed with diluted antibodies (50 μl) and 50 μl was injected at a flow rate of 20 μl min^{-1}.

With Mab 4E2, the 50% inhibitions for STREP and DHS were 9 and 12 ng ml^{-1}, respectively, which means a cross-reactivity for DHS of 75%, which is comparable with the cross-reactivity (86%) obtained in the ELISA (Dietrich *et al.*, 1993). The 50% inhibition values obtained with Mab 4G8 were 12 and 20 ng ml^{-1} for STREP and DHS, respectively, which means that this Mab, although raised against DHS, had a better sensitivity towards STREP.

Competitive BIAs in milk

The blank milk samples (n = 17), with and without the addition of STREP, were analyzed. After injection of milk, increased responses due to matrix effects were observed. This nonspecific binding could easily be detected by injecting milk without the addition of antibodies. The nonspecific binding was highest in Fc4 (STREP–OVAL 5900 RU) where, with undiluted milk, responses were recorded up to 500 RU. Dilution of the milk samples reduced the nonspecific binding only slightly. To study this nonspecific binding in more detail, a new biosensor chip (chip C) was prepared with immobilized OVAL (950 RU) in Fc1 and STREP–OVAL (1000 RU) in Fc2. Fc3 was activated with NHS/EDC and directly blocked with ethanolamine (EA; blank reference Fc) and Fc4 was not treated at all. Chip C was first used to measure the nonspecific binding from the 17 blank milk samples (five times diluted) in the four Fcs.

Table 1. Nonspecific binding in the four Fcs (without Mab) and specific binding in Fc2 (with Mab 4E2) of biosensor chip C using the competitive BIA with 17 blank milk samples with and without the addition of STREP (0, 10, 20, 50, 100, 200 and 400 ng ml^{-1}). The milk samples were diluted five times prior to injection.

Addition of STREP (ng ml^{-1})	**Addition of Antibo-dies**	***n***	**RESPONSE (RU)**				
			Fc1 OVAL	**Fc2 STREP –OVAL**	**Fc3 Blocked with EA**	**Fc4 No coating**	**Fc2–Fc1**
0	No	17	67 ± 33[a]	53 ± 25[a]	60 ± 34[a]	75 ± 26[a]	–15 ± 7
0	Yes	17	66 ± 32[a]	286 ± 30[b]	59 ± 34[a]	70 ± 25[a]	220 ± 10[c]
10	Yes	17	62 ± 33[a]	249 ± 37[b]	56 ± 33[a]	63 ± 25[a]	187 ± 12[c]
20	Yes	17	62 ± 31[a]	233 ± 35[b]	56 ± 32[a]	63 ± 24[a]	171 ± 9[c]
50	Yes	17	63 ± 33[a]	139 ± 29[b]	58 ± 36[a]	67 ± 26[a]	76 ± 7[c]
100	Yes	17	62 ± 33[a]	106 ± 28[b]	56 ± 34[a]	66 ± 26[a]	44 ± 7[c]
200	Yes	17	69 ± 38[a]	84 ± 30[b]	64 ± 40[a]	74 ± 31[a]	15 ± 9[c]
400	Yes	17	72 ± 37[a]	74 ± 31[b]	68 ± 39[a]	78 ± 30[a]	1 ± 8[c]

[a] Nonspecific binding.
[b] Total response (nonspecific binding + specific binding of Mab 4E2 to OVAL—STREP).
[c] Corrected response (specific binding of Mab 4E2).

As shown in Table 1 (no addition of antibodies), nonspecific binding was observed in all Fcs. Compared with Fc2 (STREP–OVAL), in general, the nonspecific binding in the other Fcs was higher. This proved that the low level of immobilized STREP–OVAL did not contribute to this nonspecific binding. With most samples, the nonspecific binding was highest in Fc4. This should be due to the binding of proteins to the negative charged carboxymethylated dextran surface. Activating this surface with EDC/NHS and blocking with ethanolamine, as in Fc3, resulted in less nonspecific binding. Compared with Fc2, the nonspecific binding in Fc1 was slightly higher with all samples. This might be due to the serial connection of the four Fcs through which samples were first introduced in Fc1. Fc1 may therefore have filtered some interfering compounds.

Mixing the milk samples with Mab 4E2 (in a final dilution of 500) gave a total response in Fc2 of 286 ± 30 RU (see Table 1). By subtracting the responses obtained in Fc1 (reference Fc) from Fc2, the corrected responses varied less (220 ± 10 RU). The addition of antibodies did not result in an increase of nonspecific binding in Fc1, Fc3 and Fc4. The 17 blank milk samples were also analyzed after the addition of increasing concentrations of STREP, which resulted in decreasing responses in Fc2 (see Table 1). Especially at low concentrations of added STREP, the variation in the corrected responses (Fc2-Fc1) was less than the variation in the responses obtained in Fc2, which reduced the detection limit to 20 ng ml^{-1}, which is 10 times below the MRL.

CONCLUSIONS

In our opinion, this study presents the first non-competitive single antibody-based and label free immunoassay for the direct detection of low molecular weight compounds in a food matrix at relevant levels (well below the MRL). In these direct BIAs, antibodies are the only reagents needed. However, in order to obtain sufficient high responses (approximately 100 RU) for a binding LMWC, highly pure antibodies and high immobilization levels are required. These high immobilization levels result in background responses probably due to nonspecific binding of milk components to the immobilized antibodies. This nonspecific binding can be reduced considerably by removing the high molecular weight components from milk by means of UF. One of the Mabs (4G8) showed less background responses in the direct BIA and a limit of detection (LOD) of 20 ng ml^{-1} was obtained without the sample pretreatment by UF.
In general, the competitive BIA format is more flexible. Here, different amounts of antibodies can be used and high responses can be obtained. Drug–protein conjugates can be used as ligands immobilized on the sensor chip surface. If the ligand concentration is low (<1000 RU), the background response, due to nonspecific binding of milk components, is comparable with the nonspecific binding to the dextran coating on the CM5 chip. The use of drug–protein conjugates as ligands, instead of a direct immobilization of the drug, has the advantages that immobilizations can be performed directly into the biosensor, without a risk of contamination of the system, and that the immobilization level can be adjusted. This provides the possibility to create multi-analyte BIAs by means of immobilizing different drug–protein conjugates in the different Fcs. The use of a reference Fc allows to subtract the background responses, obtained as a result of matrix interferences, from the measurements in both the direct and competitive BIAs, which results in lower LODs.

ACKNOWLEDGEMENTS

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Chapter 8

Single biosensor immunoassay for the detection of five aminoglycosides in reconstituted skimmed milk

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ABSTRACT

The application of an optical biosensor (Biacore 3000), with four flow channels (Fcs), in combination with a mixture of four specific antibodies resulted in a competitive inhibition biosensor immunoassay (BIA) for the simultaneous detection of the five relevant aminoglycosides in reconstituted skimmed milk. Four aminoglycosides (gentamicin, neomycin, kanamycin and a streptomycin derivative) were immobilized onto the sensor surface of a biosensor chip (CM5) in the four Fcs of the biosensor system by amine coupling. In the Biacore, milk (reconstituted from skimmed milk powder) was 10 times diluted with a mixture of the four specific antibodies and injected through the four serially connected Fcs (1 min at a flow rate of 20 μl min^{-1}). The responses measured just prior to the injection (20 μl at a flow rate of 20 μl min^{-1}) of the regeneration solution (0.2 M NaOH + 20% acetonitrile) were indicative for the presence or absence of the aminoglycosides in reconstituted milk. The limits of detection were between 15 and 60 ng ml^{-1}, which was far below the maximum residue limits (MRLs) (varying from 100 to 500 ng ml^{-1}) and the total run time between samples was 7 min.

INTRODUCTION

Aminoglycosides are broad-spectrum antibiotics most commonly used in veterinary drug medicine in the treatment of infections caused by aerobic Gram-negative bacteria, such as mastitis [1]. The presence of residues of these drugs in food is considered a high risk to the consumer and maximum residue limits (MRLs) have been established. In the European Union (EU), the MRLs for gentamicin (Genta), kanamycin (Kana), streptomycin (Strep), dihydrostreptomycin (DHS) and neomycin (Neo) in milk are 100, 150, 200, 200 and 500 ng ml^{-1}, respectively.

For the detection of aminoglycosides, three techniques are mainly applied: microbial inhibition screening tests [2–4], immunoassays or receptor assays [2,5–8] and liquid chromatography (LC) [9–14]. LC might be the most suitable technique for the confirmation of positive screening results. For a large-scale screening, first microbiological methods are preferred because of their convenience, low cost and broad-spectrum characteristics. However, with such methods it takes at least 6 h before the results are known [4]. More rapid methods for the detection of these antibiotics are microtiter plate enzyme-linked immunosorbent assays (ELISAs) with results being available within 2 h [7]. However, such ELISAs are specific only for one or two of the aminoglycosides and are normally performed in a laboratory. The fastest and easiest assay described so far is the one-step strip test in which the results can be obtained within 10–15 min [8]. This test was developed for on-site screening of raw milk samples for the presence of DHS and Strep on a farm. However, this test is specific for DHS and Strep only, and is not suitable for large-scale screening.

Rapid and automated surface plasmon resonance (SPR)-based biosensor immunoassays (BIAs) have been proposed for the detection of sulfamethazine [15], enrofloxacin and ciprofloxacin [16] and Strep [17] in bovine milk. For the detection of these low molecular weight compounds, the competitive inhibition immunoassay format was used in which the drugs were covalently immobilized on to carboxymethylated dextran-modified gold film on the biosensor chip. Applications for the direct detection of Genta [18] and Strep and DHS [19] were also described. In these unique direct assays, monoclonal antibodies were immobilized onto the biosensor chip and the binding of the aminoglycosides was measured directly. Although these direct assays were applied in milk, the competitive inhibition format was found to be more flexible and robust [19].

The assays described above are specific for one or two of the aminoglycosides. To detect the five relevant aminoglycosides in milk simultaneously, the four flow channel biosensor (Biacore 3000) was applied in the present study. Genta, Neo, Kana and a Strep derivative were immobilized onto the surface of a biosensor chip (CM5) in the four Fcs of the biosensor system and a mixture of selected specific antibodies was used in the competitive inhibition format in which the four Fcs were serially

connected. The combined assay was constructed, with regard to sensitivity, to allow the determination of the five aminoglycosides below and at the regulatory MRL levels. To avoid the instability of milk during the several experiments performed with the different assays over a longer period, reconstituted milk from skimmed milk powder was used as a model matrix in this study.

EXPERIMENTAL

Materials

Gentamicin sulphate, neomycin trisulphate, kanamycin disulphate, streptomycin sulphate and dihydrostreptomycin sesquisulphate were purchased from Sigma–Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). The Qflex Kit Streptomycin (containing streptomycin binding protein (SBP), streptomycin derivative, streptomycin calibration stock solution, HBS-EP buffer (composition: 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% surfactant P20), a CM5 sensor chip and kit accessories), the Qflex Kit Streptomycin handbook, 10 mM sodium acetate (pH 4.5) and the amine coupling kit (containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M *N*-ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride-NaOH (pH 8.5)) were supplied by Biacore AB (Uppsala, Sweden). The anti-gentamicin polyclonal antiserum (422 batch 8.2) was raised in a rabbit; the performance of this antiserum in an ELISA has been described before [7]. The development of the anti-dihydrostreptomycin monoclonal antibody (4G8) was also described before [19]. An affinity purified culture supernatant with a final IgG1 concentration of 0.53 mg ml^{-1} was used as the stock solution. The anti-neomycin (CR2389R) and anti-kanamycin (CR2383S) polyclonal antisera were supplied by Cortex Biochem Inc. (San Leandro, CA). The purified monoclonal antibody and the polyclonal antisera were stored at −20 °C.

Equipment

The Biacore Q biosensor system was loaned from NIZO Food Research (Ede, the Netherlands). The Biacore 3000 biosensor system was supplied by Biacore AB (Uppsala, Sweden).

Preparation of the biosensor chip

The immobilization of the aminoglycosides onto the biosensor chip surface and into the four flow channels (Fcs) was performed in the Surface Prep unit of the Biacore Q and was guided by the immobilization wizard. The Surface Prep unit is a sensor chip holder that fits directly onto the auto sampler rack base of the Biacore Q. Fcs are formed on the sensor chip by docking a plastic block (the flow cell carrier) onto the

chip. Solutions were injected directly into the Fcs with the autosampler needle and effluents from the flow channels were collected in a small waste beaker.
The immobilization of the Strep derivative in Fc4 of the sensor chip (CM5) was performed according to the instructions prescribed in the Qflex Kit Streptomycin handbook. Prior to immobilization, the sensor chip surface in Fc4 was activated by injecting a mixture of 0.4 M EDC and 0.1 M NHS (1:1; v/v) at a flow rate of 10 µl min^{-1} and with a contact time of 7 min. After washing with 0.1 M sodium hydroxide, the Step derivative (10 times diluted in 10 mM sodium acetate (pH 4.5)) was injected through Fc4 for 7 min at a flow rate of 5 µl min^{-1}. To deactivate the remaining active sites, 1 M ethanolamine was injected for 3 min at a flow rate of 10 µl min^{-1}. An extra wash step with water was used to remove any adsorbed chemicals at the end of the immobilization procedure.
Genta, Neo and Kana were immobilized into Fc1, Fc2 and Fc3, respectively. Here, the sensor chip surfaces in the Fcs were activated with the mixture of EDC and NHS for 40 min at a flow rate of 2 µl min^{-1}. After washing with water, solutions of the aminoglycosides (3 mg ml^{-1} in borate buffer (pH 8.5)) were injected over the surface for 60 min at a flow rate of 2 µl min^{-1}. The sensor surfaces were deactivated and washed as described above for the immobilization of the Strep derivative.

Biosensor immunoassays (BIAs)

The Biacore Q was used for the immobilization of the four aminoglycosides onto the surface of the biosensor chip into the four Fcs. A major disadvantage of this biosensor system is that the four different Fcs can only be used separately. For that reason, the chip with the immobilized aminoglycosides was docked into the Biacore 3000 system in which the four Fcs can be used individually or serially connected. The running buffer was HBS-EP at a flow rate of 20 µl min^{-1}. For all the assays, the standard solutions in buffer or milk and the milk samples (reconstituted from skimmed milk powders) were pipetted (100 µl) into the wells of a microtiter plate. Using the Biacore 3000 control software, 10 µl of the standards or samples was mixed with 90 µl of the antibody solution in the microtiter plate. Of this mixture, 20 µl was injected at a flow rate of 20 µl min^{-1}. After this injection, the surface was regenerated by injection of 0.2 M sodium hydroxide containing 20% acetonitrile (20 µl at 20 µl min^{-1}). The responses measured 10 s prior to the injection of the regeneration solution were used for the calculations in the Biacore Evaluation software. The total run time between samples was 7 min.

Milk powder samples

Ten skimmed milk powders obtained from the General Inspection Service (Kerkrade, the Netherlands) in the year 2001 and taken from 10 different suppliers were used during the experiments. The average fat, protein and lactose content of these milk

powders were 0.8 ± 0.4, 36.0 ± 0.9 and 47 ± 2 %, respectively. To reconstitute milk, 90 ml of water were added to 10 g of the milk powders and after stirring for 30 min at room temperature on a magnetic stirrer, the milk samples were ready to use. The reconstituted milk samples were stored at 4–6 °C for a maximum of 5 days.

RESULTS AND DISCUSSION

Biosensor chip

The primary amine-containing aminoglycosides were immobilized onto the NHS-activated biosensor chip surface by amine coupling. Of the aminoglycosides used is this study, Strep and DHS have only two primary amine groups, Genta has three or four, Kana has four or five (dependent of the component) and Neo has six amine groups. For the immobilization of Strep, the derivative as supplied in the Qflex Streptomycin Kit and the procedure described in the handbook (see Experimental section) were used. For the immobilization of the other three aminoglycosides in the other Fcs, a coupling buffer of pH 8.5 was used because the attachment of primary amine-containing ligands to the NHS-activated surface required an uncharged primary amine function [20]. Long contact times during the NHS activation (40 min) and during the immobilization (60 min) of the aminoglycosides were applied to ensure that enough of the aminoglycosides were bound to the surface. Directly after the immobilizations, the base responses, measured in the Biacore 3000 with HBS-EP as running buffer, were 22150 RU in Fc1 (Genta), 22600 RU in Fc2 (Neo), 23125 RU in Fc3 (Kana) and 21340 in Fc4 (Strep derivative). After 250 cycles, the base responses in the first three Fcs were at the same level as directly after the immobilization, while the base response in Fc4 decreased to 20600 RU and stayed at that level during the next 250 cycles.

Biosensor immunoassays

Streptomycin assay

The performance of the SBP from the Qflex kit was compared with a previously developed monoclonal antibody against DHS (Mab 4G8 [19]) in combination with Fc4 of the coated biosensor chip docked into the Biacore 3000. According to the Qflex Kit Streptomycin handbook for milk assay, calibration graphs for milk were prepared using four calibrants (500, 250, 125 and 62.5 ng ml^{-1}), the SBP (five times diluted in HBS-EP) and 20 µl injections at a flow rate of 40 µl min^{-1}. The calibrants were 10 times diluted with the SBP solution using the Biacore 3000 control software. The regeneration solution (0.2 M sodium hydroxide + 20% acetonitrile) was injected for 1 min at a flow rate of 40 µl min^{-1}. Under these conditions, calibration graphs for reconstituted milk and buffer were compared. The average maximum responses

obtained with buffer and milk (n = 10) were 1030 ± 10 and 878 ± 32 RU, respectively, and the 50 % inhibition values were calculated as 120 and 340 ng ml^{-1}, respectively. This clearly demonstrated the negative influence of milk on the performance of the assay. The 10 blank milk samples were also analysed after the addition of Strep at the MRL level (200 ng ml^{-1}). This resulted in an average response of 546 ± 65 RU, which means that the inhibition of the maximum response due to the presence of Strep at the MRL level was only 38 %.

Using a 40 times dilution of the stock solution of the anti-DHS Mab, comparable maximum responses and sensitivities in buffer and milk were obtained as with the SBP. The regeneration conditions as used with the SBP worked well with this Mab also. To obtain a better sensitivity, the flow rate of the assay was lowered to 20 µl min^{-1} and the Mab stock solution was diluted 100 times. The calibration graphs obtained under these conditions in buffer and milk showed 50 % inhibition values of 60 and 140 ng ml^{-1}, respectively (see Fig. 1A), which is sensitive enough to control milk samples at the MRL level.

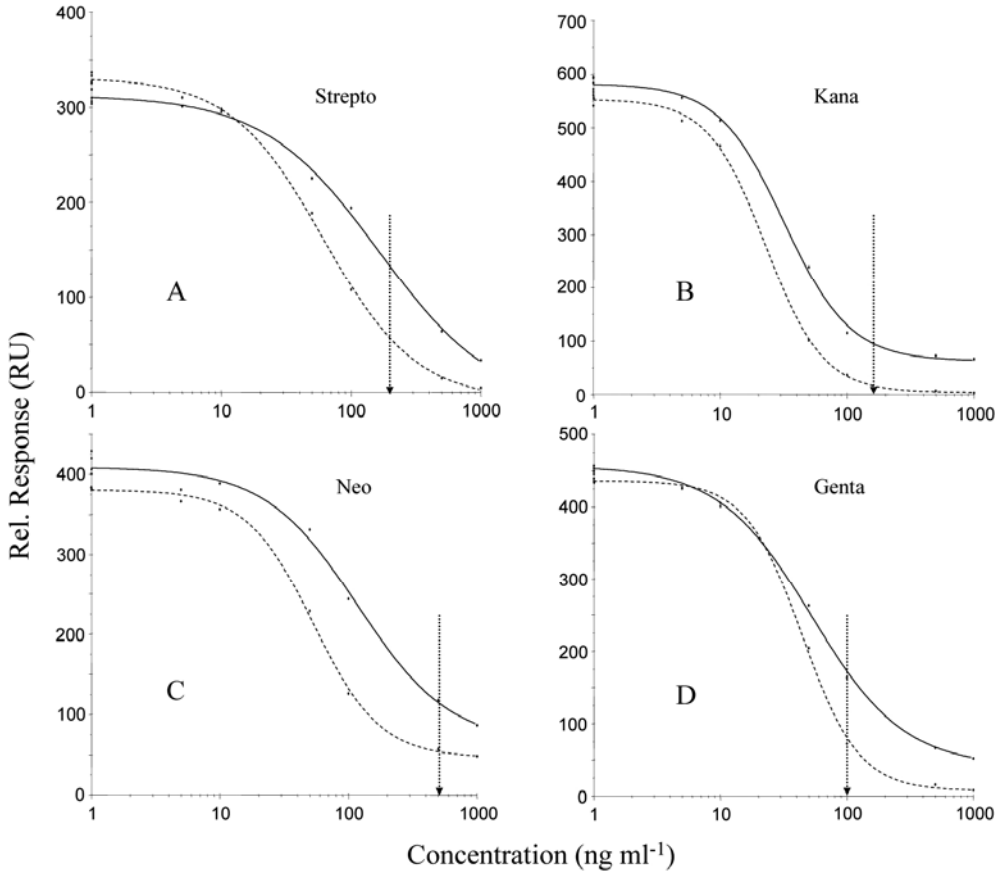

Figure 1. BIA calibration graphs for buffer (dotted lines) and reconstituted milk (solid lines) obtained in the individual Fcs: (A) Strep in Fc4; (B) Kana in Fc3; (C) Neo in Fc2; and (D) Genta in Fc1. The arrows indicate the MRLs in milk.

Kanamycin assay

The commercially available anti-Kana Pab was used in a 25000 times dilution in combination with Fc3, and the regeneration procedure as described above worked well with this combination. The calibration graphs obtained for buffer and reconstituted milk are shown in Fig. 1B. The 50 % inhibition values in buffer and milk were 20 and 40 ng ml^{-1}, respectively, which is sufficient to control milk samples at the MRL level of 150 ng ml^{-1}.

Neomycin assay

The commercially available anti-Neo Pab was used at 400 times dilution in combination with Fc2 and with the regeneration conditions as described above. The calibration graphs obtained for buffer and reconstituted milk are shown in Fig. 1C. The 50 % inhibition values for buffer and milk were 70 and 150 ng ml^{-1}, respectively, which is sufficient to control milk samples at the MRL level of 500 ng ml^{-1}.

Gentamicin assay

The previously developed Pab against gentamicin [7] was used at 1000 times dilution in combination with Fc1 and with the regeneration conditions described above. The calibration graphs obtained for buffer and reconstituted milk are shown in Fig. 1D. The 50% inhibition values for buffer and milk were 40 and 70 ng ml^{-1}, respectively, which is sufficient to control milk samples at the MRL level of 100 ng ml^{-1}.

Mixed antibody assay in buffer

Table 1. BIA in buffer. Responses (average of triplicate analyses) obtained in the four Fcs with the mixed antibodies (without and with the addition of the aminoglycosides at MRL levels) and with the individual antibodies (percentage of inhibition in parenthesis).

Antibody	**Added aminogl. (ng ml^{-1})**	**Response in RU (% of inhibition)**			
		Genta Fc1	**Neo Fc2**	**Kana Fc3**	**Strep Fc4**
Mixed antibodies	–	445 ± 8	323 ± 5	623 ± 13	336 ± 8
Anti-Genta	–	418 ± 3	4 ± 2	3 ± 2	0 ± 2
Anti-Neo	–	15 ± 0	310 ±0	63 ± 1	0 ± 0
Anti-Kana	–	2 ± 0	1 ± 0	551 ± 2	0 ± 0
Anti-DHS	–	0 ± 0	0 ± 0	0 ± 0	331 ± 0
Mixed antibodies	Genta (100)	70 ± 1 (84)	268 ± 2 (17)	578 ± 1 (7)	297 ± 3 (12)
Mixed antibodies	Neo (500)	424 ± 1 (5)	42 ± 1 (87)	554 ± 2 (11)	304 ± 1 (10)
Mixed antibodies	Kana (150)	422 ± 1 (5)	270 ± 3 (16)	72 ± 1 (88)	279 ± 25 (17)
Mixed antibodies	Strep (200)	432 ± 1 (3)	315 ± 1 (2)	607 ± 1 (3)	46 ± 1 (86)
Mixed antibodies	DHS (200)	431 ± 1 (3)	316 ± 1 (2)	608 ± 0 (3)	53 ± 0 (84)

A mixture of the four antibodies was prepared. Anti-Genta, anti-Neo, anti-Kana and anti-DHS were used at dilutions of 1000, 400, 25000 and 100 times, respectively. The four Fcs were serially connected and the maximum responses obtained in the four Fcs, with buffer as sample, are presented in Table 1. The individual antibodies were injected through the four Fcs also and the responses due to the interactions in the four Fcs are shown in Table 1. The anti-Genta, anti-Kana and anti-DHS gave specific responses in the corresponding Fcs (immobilized with Genta, Kana and Strep, respectively). The anti-Neo reacted also in Fc3 and slightly in Fc1. Standard solutions of the five aminoglycosides at MRL levels were analyzed with the mixed antibodies. As shown in Table 1, the maximum responses in the corresponding Fcs were strongly inhibited (from 84 to 88%). The addition of Strep and DHS resulted in a significant inhibition in Fc4 only. The addition of the other three aminoglycosides had an inhibiting effect in the non-corresponding Fcs also, which means that the specific antibody–antigen interactions were influenced by the other aminoglycosides.

Mixed antibody assay in reconstituted milk

At first, the 10 reconstituted milk samples were injected without the antibody mixture (1:10 diluted with buffer) to investigate the nonspecific binding (NSB) of milk components to the aminoglycosides-coated Fcs. As shown in Fig. 2, the lowest NSB was found in Fc4 and the highest in Fc3. However, compared with the maximum responses obtained after the injection of the mixed antibodies in blank milk, these NSBs were low (see Fig. 2).

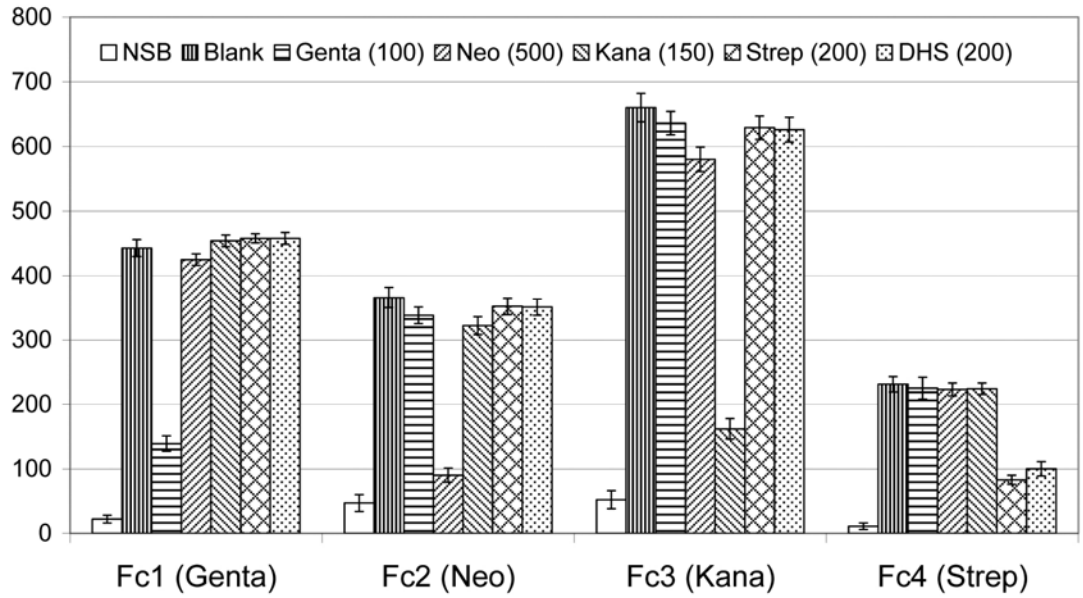

Figure 2. Average relative responses obtained by the mixed antibody assay with reconstituted milk samples (n = 10) in the four Fcs. The milk samples were analyzed without the antibody mixture (nonspecific binding (NSB)) and with the antibody mixture without (blank) and with the addition of the five aminoglycosides at MRL levels.

The addition of Genta at the MRL level (100 ng ml^{-1}) resulted in an average inhibition in Fc1 of 69 % and the inhibitions in the other Fcs were low (3–8 %). Using a calibration graph for Genta in milk (25, 50, 100 and 200 ng ml^{-1}), the limit of detection (LOD is equal to the concentration corresponding with the average response of blank milk samples minus three times the standard deviation) was calculated as 20 ng ml^{-1}.

The addition of Neo at the MRL level (500 ng ml^{-1}) resulted in an average inhibition in Fc2 of 75 % and the inhibition in the other Fcs were between 3 and 12 %. Using the calibration graph for Neo in milk (125, 250, 500 and 1000 ng ml^{-1}), the LOD was calculated as 40 ng ml^{-1}.

The addition of Kana at the MRL level (150 ng ml^{-1}) resulted in an average inhibition in Fc3 of 75 % and the inhibitions in the other Fcs were between 0 and 12 %. Using the calibration graph for Kana in milk (37.5, 75, 150 and 300 ng ml^{-1}), the LOD was calculated as 15 ng ml^{-1}.

The addition of Step and DHS at the MRL level (200 ng ml^{-1}) resulted in average inhibitions in Fc4 of 64 and 57 %, respectively, and the inhibitions in the other Fcs were between 0 and 5 %. Using calibration graphs for Strep and DHS in milk (50, 100, 200 and 400 ng ml^{-1}), the LODs were calculated as 30 and 60 ng ml^{-1}. The greater sensitivity of the anti-DHS Mab for Strep was reported before [19].

Table 2. Calculated concentrations (average of triplicate analyses) of the aminoglycosides in spiked reconstituted milk samples (at the levels: 0.25 MRL, 0.5 MRL, MRL and 2 MRL) using the four calibrants with mixed aminoglycosides (at the levels: 0.07 MRL, 0.22 MRL, 0.67 MRL and 2 MRL).

Aminoglycoside	**MRL**	**Calculated concentration (ng ml^{-1}) (percentage recovery)**			
		0.25 MRL*	**0.5 MRL***	**MRL***	**2 MRL***
Genta	100	17 ± 3 (68)	38 ± 9 (76)	77 ± 1 (77)	136 ± 5 (68)
Neo	500	90 ± 2 (72)	190 ± 5 (76)	434 ± 7 (89)	979 ± 6 (98)
Kana	150	25 ± 2 (67)	50 ± 4 (67)	77 ± 4 (51)	93 ± 1 (31)
Strep	200	52 ± 11 (104)	89 ± 3 (89)	176 ± 2 (88)	356 ± 1 (89)
DHS	200	50 ± 5 (100)	79 ± 1 (79)	139 ± 1 (70)	272 ± 11 (68)

* Level of spiking

Mixed calibration standards in the mixed antibody assay in reconstituted milk.

To investigate the possibility of using the combined assay as a quantitative assay and to reduce the amount of calibration standards, mixtures of four aminoglycosides (Strep, Neo, Kana and Strep) were prepared in reconstituted milk at different levels (0.07 MRL, 0.22 MRL, 0.67 MRL and 2 MRL). These mixed calibration standards were used with the mixed antibodies (1:10; v/v) and analyzed as described in the experimental section. The four calibration graphs obtained with the responses in the

four Fcs were used to calculate the concentrations of the aminoglycosides in blank and spiked milk samples. The concentrations calculated in the 10 blank milk samples were all below the lowest calibrations standards <0.07 × MRL. As shown in Table 2, in general, due to the influence of the different aminoglycosides in the mixed calibration standards on the calibration graphs in the four Fcs, the calculated concentrations of the spiked milk samples with the individual aminoglycosides were underestimated. Especially the higher concentrations of Kana were highly underestimated. At the higher concentrations, the calibration graph for Kana is quite flat (see Fig. 1B) and a small change in relative response has a great impact on the calculated concentrations.

CONCLUSIONS

The combined BIA used a mixture of four specific antibodies and four aminoglycosides immobilized onto the biosensor chip surface into the four serially connected Fcs of the Biacore 3000. This combined assay was found to be a qualitative assay for the detection of the five relevant aminoglycosides in reconstituted milk below their MRL levels and within 7 min. In this qualitative assay, five calibrants are recommended (blank milk sample and blank milk spiked with Genta, Neo, Kana and Strep at the MRL levels).

Due to the significant interaction of the anti-Neo in other Fcs and some of the aminoglycosides into the different BIAs, the quantification in the combined assay resulted in underestimated concentrations (especially for Kana). For the more accurate quantification of the aminoglycosides in reconstituted milk, the individual BIAs, using the separate Fcs, should be applied.

Three of the Fcs (coated with Genta, Neo and Kana) were used for around 250 cycles (within 3 weeks) and no significant decreases in the assay performances were noticed in these Fcs. After 500 cycles in Fc4 (coated with the Strep derivative), the initial maximum response of the binding Mab was strongly reduced (by ca. 50 %). The instability of this coating should be improved to use this assay for high throughput screening or for a longer period.

Additional experiments have to be performed to prove that the combined assay is suitable for the detection of the aminoglycosides in whole milk samples and to investigate possible interferences from fat and somatic cell count.

ACKNOWLEDGEMENTS

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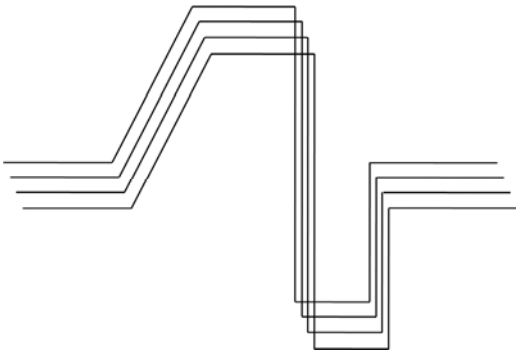
Part IV

Biosensor immunoassay development for fluoroquinolones



Chapter 9

Biosensor immunoassay for flumequine in broiler serum and muscle

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ABSTRACT

Flumequine (Flu) is one of the fluoroquinolones most frequently applied for the treatment of broilers in the Netherlands. For the detection of residues of Flu in blood serum of broilers, a biosensor immunoassay (BIA) was developed which was fast (7.5 min per sample) and specific (no cross-reactivity with other (fluoro)quinolones). This inhibition 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 to 800 ng ml^{-1}.
For the detection of Flu in muscle, an easy extraction procedure in buffer was selected and the measuring range was from 24 to 4000 ng g^{-1}. Average recoveries of 66 till 75% were found with muscle samples spiked at 0.5, 1 and 2 times the maximum residue limit (MRL in muscle = 400 ng g^{-1}) and the decision limit (CCα) and the detection capability (CCβ) were determined as 500 and 600 ng g^{-1}, respectively.
Incurred muscle samples were analyzed by the BIA and by LC-MS/MS and a good correlation was found (R^2 = 0.998). Serum and muscle samples from with Flu treated broilers were analyzed and the concentrations found in serum were always higher than those found in muscle (average serum/muscle ratio was 3.5) and this proved the applicability of the BIA in serum as predictor of the Flu concentration in muscle.

INTRODUCTION

“The development of chain oriented monitoring and surveillance in the poultry chain" was the title of a Dutch research project in which biosensor options for the on-site and simultaneous detection of pathogens and drug residues in broiler slaughterhouses were evaluated. Automated optical biosensors of the company Biacore AB were chosen and, to perform combined assays, the Biacore 3000, with four serially connectable flow channels (Fcs), was selected. Blood serum of broilers was chosen as the matrix for the detection of antibodies against pathogens (i.e. *Salmonella*) and drug residues with sulfonamides as the first model compounds. For the biosensor detection of sulfonamides in broiler serum, different approaches, ranging from specific to multi-sulfonamide assays, were followed [1]. In the finally selected multi-sulfonamide biosensor immunoassay (BIA), a mutant antibody (M.3.4) was used in combination with a sulfonamide-derivative coated on the surface of the biosensor chip in one of the flow channels (Fcs) [2]. The sample preparation consisted of a dilution in buffer only and with this BIA, all 17 sulfonamides tested, including the five sulfonamides registered for application in broilers, could be detected in broiler serum with limits of detection between 4 and 82 ng ml^{-1}. For the application in a combined assay with the detection of anti-salmonella, the biosensor conditions (sample dilution, sample buffer, running buffer and regeneration conditions) were adapted to the serological assay conditions (using lipopolysaccharides (LPS)-coated chips [3]). The adapted BIA was successfully applied during the analysis of samples from an animal experiment and during a survey in which 310 broiler serum samples of 31 different flocks were tested for anti-salmonella and sulfonamides [4].

However, other antibiotics can be used and for instance the quinolones are frequently applied veterinary drugs of which flumequine (Flu) is the most applied in broilers in the Netherlands. To establish safe limits for human consumption, the European Union (EU) established maximum residue limits (MRL) for Flu in chicken edible tissues of 1000, 800, 400 and 250 ng g^{-1} in kidney, liver, muscle and skin + fat, respectively [5].

For the development of a BIA, antibodies are necessary and different approaches for raising antibodies against (fluoro)quinolones were described previously [6-13]. Polyclonal [6,7] and monoclonal antibodies [8] were developed against sarafloxacin and cross-reactivities (CR’s) were observed with structurally related quinolones including difloxacin, enrofloxacin, norfloxacin, trovafloxacin and nalidixic acid. Monoclonal antibodies against enrofloxacin were highly specific [9] whilst polyclonal antibodies against enrofloxacin [10] showed CR with ciprofloxacin and antibodies against ciprofloxacin showed CR with enrofloxacin and norfloxacin [11]. An ELISA with polyclonal antibodies raised against norfloxacin, linked to ovalbumin via the secondary amine group as found in the piperazine moiety of norfloxacin, was able to detect nine different (fluoro)quinolones [12]. However, the CR towards Flu was 6% only. In the same study, highly specific ELISA’s were obtained with polyclonal

antibodies against ciprofloxacin, enrofloxacin, Flu and nalidixic acid. The Flu ELISA in milk showed 50% inhibition at 29 µg kg^{-1} and that is three to four times more sensitive than obtained with highly specific chicken egg yolk antibodies against Flu [13]. As shown in Figure 1, Flu has a deviating structure compared to the other quinolones. This explains the high specificity of the antisera against Flu.

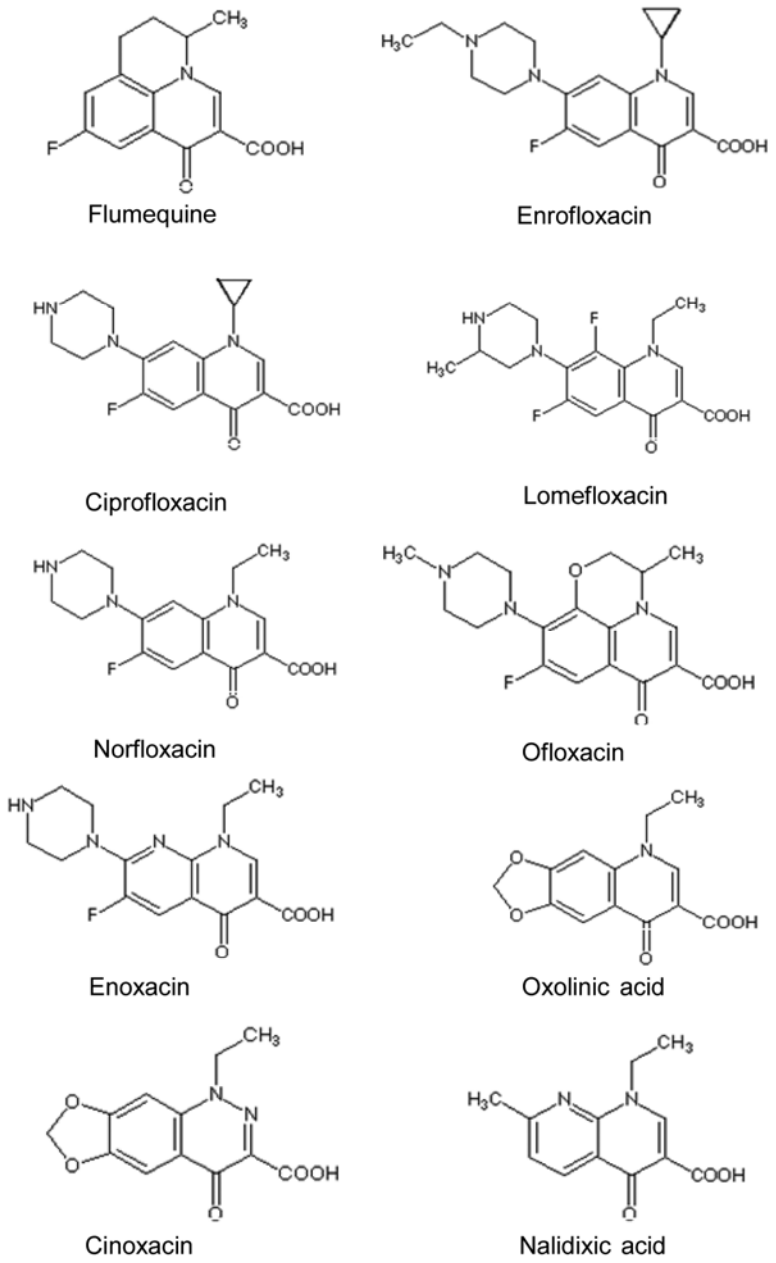

Figure 1. Molecular structures of the (fluoro)quinolones.

In the present study, because of the availability, antisera against Flu were raised in rabbits using two immunogens (Flu-bovine serum albumin (BSA) and Flu-keyhole limpet hemocyanin (KLH)). The antisera were tested in an ELISA and in the Biacore using a CM5 biosensor chip coated with Flu in which a previously described two-step immobilization procedure [10], with ethylene diamine as spacer, was applied. For the

detection of Flu in broiler sera, the easy sample preparation (dilution in anti-Flu containing buffer only) was adapted to the conditions previously described for the detection of anti-salmonella and sulfonamides [4]. Maximum residue limits (MRLs) for serum are not prescribed and the MRL in muscle of 400 ng g^{-1} was chosen as the minimum required performance limit (MRPL) in serum and, under these conditions, the decision limit (CCα) and the detection capability (CCβ) for Flu in serum were determined. The BIA was further applied during a survey with 310 broiler serum samples obtained from 31 different farms and sera from treated and untreated broilers were analyzed. For the application of the BIA in muscle, a simple extraction procedure was used, and CCα and CCβ were determined using blank muscle samples analyzed with and without the addition of Flu at the MRL level. The BIA results were compared with LC-MS/MS results using incurred samples. Serum and muscle samples obtained from broilers treated with Flu were analyzed and the ratio of Flu in both materials was determined to evaluate the applicability of serum levels as predictor for Flu levels in muscle.

MATERIALS AND METHODS

Materials

Piromidic acid was supplied by ICN Biochemicals (Ohio, USA) and marbofloxacin by Laboratoire Pharmaceutique Veterinaire (Lure Cedex, France). Enrofloxacin, ciprofloxacin and CM-dextran sodium salt were obtained from Fluka Chemie (Zwijndrecht, the Netherlands) and difloxacin from Abbott Laboratories (North Chicago, Illinois, USA). Isobutyl chloroformate, Tween-20, Tween-80, *N*,*N*-dimethylformamid (DMF), tributylamine (TBA), dimethylsulfoxide (DMSO), ethylene diamine (EDA) and acetonitrile were obtained from VWR International (Amsterdam, the Netherlands). EIA grade horseradish peroxidase (HRP) was from Roche Diagnostics (Mannheim, Germany). HBS-EP buffer (containing 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% Surfactant P20), CM5 biosensor chips and the amine coupling kit (containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride-NaOH (pH 8.5)) were supplied by Biacore AB (Uppsala, Sweden). Goat anti-rabbit IgG was obtained from Caltag Laboratories (Burlingame, CA). COSTAR® ELISA microtiter plates were obtained from Corning Incorporated (Corning, NY 14831, USA). Solutions of tetramethylbenzidine (TMB) peroxidase substrate and peroxidase were obtained from Kirkegaard and Perry Labs (Gaithersburg, MD, USA). Flumequine (Flu), ofloxacin, enoxacin, cinoxacin, oxolinic acid, norfloxacin, naldixic acid, lomefloxacin, bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), NHS, EDC, antifoam Y-

30 emulsion and all other reagents were supplied by Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands) unless stated otherwise.

Equipment

The Biacore 3000 and the Biacore Q were purchased from Biacore AB (Uppsala, Sweden). Both instruments have four flow channels (Fcs) and are fully automated with a capacity of 192 samples (two microtiter plates). The Biacore Q is dedicated to the qualitative or quantitative determination of analytes in food related products and can be used in combination with specially developed Qflex® Kits. A disadvantage of the Biacore Q is that only one of the four Fcs can be used at the same time. In the Biacore 3000, the four Fcs can be serially connected and simultaneously detected. During the development of the Flu BIA, one Fc was used only (a reference Fc was not applied) and therefore, both instruments were used, when available, during this study.
The Ultra Microplate Reader (Model EL_X 808) of Bio-Tek Instruments was supplied by Beun de Ronde (Abcoude, the Netherlands).

Preparation of the immunogens

For the coupling of Flu to BSA and KLH, the mixed anhydride procedure was followed. Here, by means of isobutyl chloroformate, the carboxyl group of Flu is converted to an acid anhydride what reacts with the amino groups of the proteins. Flu (5 mg) was dissolved in DMF (1 ml) and cooled to 4 ºC. TBA (5 µl) was added and after 10 min at 4 ºC, isobutyl chloroformate (5 µl) was added and stirred for 20 min at 4 ºC. A portion (0.25 ml) of this solution of activated Flu was added to a cooled solution of the proteins (10 mg BSA or KLH dissolved in 3 ml 0.1 M Na_2CO_3) and, after stirring for 3.5 h at 4 ºC, the solution was dialyzed at 4 ºC against PBS for 3 days. The molar ratios of Flu/proteins were determined by measuring the ultraviolet spectra of Flu, the proteins, and the conjugates and, using the absorbances at 250 nm, were calculated as 7.2 and 6.5 for BSA and KLH, respectively. The Flu-protein conjugate solutions were diluted in PBS until concentrations of 1 mg ml^{-1}.

Preparation of the HRP-conjugates

The Flu-HRP conjugate was prepared according to the activated succinimide ester procedure in which the carboxyl group of Flu is converted to a reactive succinimide ester by adding a mixture of EDC and NHS. The ester reacts spontaneously with primary amine groups of the HRP. Flu (6.3 mg) was dissolved in a mixture of DMF (1 ml) and PBS (0.2 ml) and to 1 ml of this solution, 0.5 ml of EDC (32 mg EDC in 1.4 ml of PBS), 0.5 ml of NHS (30 mg in 2.9 ml of PBS) and 1 ml of DMF were added. After 1 h of mixing at RT, 0.5 ml of this solution was added to a solution of HRP (5 mg dissolved in 1 ml of PBS). After overnight mixing at RT, the product was dialyzed at 4 ºC for four days against PBS and stored at -20 ºC until used.

Preparation of the antisera

For the preparation of polyclonal antisera, immunizations were performed at the Laboratory of Hormonology (Marloie, Belgium). Two rabbits (New Zealand White SPF) were immunized with the two Flu-protein conjugates (Flu-BSA in rabbit MH41 and Flu-KLH in rabbit MH42). Intradermal multisite injections were performed according to the laboratory protocol: first injection at day 0, second injection at day 14, third injection at day 28, and thereafter one injection every 4 weeks. The first injection consisted of 1 ml of the protein solution (0.5 mg protein in 0.5 ml PBS mixed (1:1; v/v) with Freund's complete adjuvant). In all subsequent injections, the complete adjuvant was replaced by Freund's incomplete adjuvant. The first bleeding was performed at day 0 before the first injection (pre-immune serum) and the second bleeding at day 38. The following bleedings were performed every 10 days after each injection. Due to a misunderstanding, the final bleeding was performed 4 months after the last injection. After collection, the blood samples were placed in vacutainer tubes at room temperature for 24 h. After that, the blood was centrifuged and the collected serum was stored at -80 °C until further use. From each of the two rabbits, small amounts (3 ml) of pre-immune serum and the first bleeding were obtained followed by five large bleedings (ca. 24 ml of serum each) and a final bleeding (72 ml of serum each). The sera obtained from the final bleedings (fbs) were used in the final BIA.

ELISA

Microtiter plates were coated overnight with 100 µl aliquots of goat anti-rabbit IgG [5 µg ml^{-1} in 50 mM sodium carbonate (pH 9.6)] at 4 °C. After coating, the plates were washed three times with washing buffer (PBS (pH 7.2) to which 0.05 % Tween-20 and 0.004 % antifoam were added) with a microplate washer. Aliquots of 50 µl of diluted standard or sample or buffer (during titer evaluations) were added to the wells, followed by 25 µl of appropriately diluted Flu-HRP in PBS and finally 25 µl of in PBS diluted anti-Flu. The plate was incubated for 1 h at 4 °C and after washing (three times with washing buffer), the bound peroxidase was assessed by adding 100 µl aliquots of a tetramethylbenzidine (TMB) peroxidase substrate system. After incubation in the dark for 20-30 min at RT, the reaction was stopped by adding 100 µl aliquots of 1 M phosphoric acid and the colored product of the peroxidase reaction was measured at 450 nm in the microplate reader.

Stock solutions of the quinolone standards (1 mg ml^{-1}) were prepared in methanol which contained 2 mM NaOH.

Preparation of the biosensor chip

For the immobilization of Flu via its carboxyl group to the carboxylated sensor surface of the CM5 biosensor chip, a previously described two-steps procedure applied for enrofloxacin was followed [10].

First, the chip surface was bench top activated with 50 µl of a mixture of 0.4 M EDC and 0.1 M NHS (1:1; v/v) during 15 min at RT. The chip surface was washed with water and dried under a stream of nitrogen gas. To the chip, 50 µl of 0.1 M EDA (pH of 8.5) was added and after 15 min incubation at RT, the chip was washed with water and dried under a stream of nitrogen gas.

Then, Flu was esterified with NHS in the presence of EDC. Flu (3-4 mg) was dissolved in 0.3 ml DMSO and 1.2 ml of sodium carbonate buffer (1.59 g Na_2CO_3 + 2.93 $NaHCO_3$ in 1 L water, adjusted to pH 9.6) was added. Of this solution, 100 µl was mixed with 100 µl 0.4 M EDC and 100 µl 0.1 M NHS and after an incubation of 1 h at RT, this mixture (50 µl) was added to the activated chip. After an incubation of 1 h at RT, the chip was washed with water, dried under a stream of nitrogen and docked into the Biacore 3000.

Biosensor Immunoassay (BIA)

After the immobilization of Flu to the chip surface of the CM5 chip, the chip was docked into the Biacore 3000 or the Q. HBS-EP with 0.05% Tween-80 was used as the running buffer with a flow rate of 20 µl min^{-1} and an injection volume of 40 µl was applied. For the regeneration, 10 µl of 0.2 M HCl was injected, followed by 20 µl of 0.2 M NaOH + 20% acetonitrile, both at a flow rate of 20 µl min^{-1}. A complete cycle between two sample injections took 7.5 min.

Samples and preparation

Samples

Broiler serum samples (n=310) from 31 different flocks of 31 different Dutch farms (10 sera from each farm) used during the survey and serum and muscle samples from 100 broiler chickens (75 blank and 25 treated with Flu) were obtained from the Animal Sciences Group (ASG; Lelystad, the Netherlands). The 25 incurred samples were obtained via an animal experiment performed at the ASG in which 3-week-old Ross 308 broilers (n=25) were treated with Flu (Flumequine 50% water-soluble powder of Dopharma (Raamsdonksveer, the Netherlands)) through the drinking water for five consecutive days with an intended dose of 30 mg kg^{-1} total body weight. To obtain incurred samples with high concentrations of Flu, animals were slaughtered directly after the treatment with Flu.

Sample preparation for broiler serum samples

Chicken serum (20 µl) was pipetted into a 96 well microtiter plate and 40 µl of sample buffer (HBS-EP to which 5 g l^{-1} CM-dextran sodium salt, 0.3 M sodium chloride and 0.05% Tween 80 were added) and 40 µl of diluted antiserum (final bleeding of MH41 which was diluted 25 times in sample buffer) were added. For the calculations of concentrations of Flu in serum, a calibration graph was prepared by adding 40 µl of standard solutions of Flu in sample buffer to the 20 µl of a blank sample and 40 µl of diluted antiserum. The standard solutions added were 400; 200; 100; 50; 25; 12.5; 6.25 and 3.125 ng ml^{-1}, which resulted in final concentrations of 160; 80; 40; 20; 10; 5; 2.5 and 1.25 ng ml^{-1} in the well and 800; 400; 200; 100; 50; 25; 12.5 and 6.25 ng ml^{-1} of broiler serum.

Sample preparation for muscle samples

Broiler muscle was grinded and 1 g was extracted with 5 ml of sample buffer during a 10 min head over head mixing. After centrifugation (3500 rpm during 10 min), 20 µl of the extract was pipetted into a well of a 96 wells microtiter plate and 40 µl of diluted antiserum (25 times diluted in sample buffer) and 40 µl of buffer were added. For the calculations of concentrations of Flu in muscle, a calibration graph was prepared by adding 40 µl of standard solutions of Flu in sample buffer to the 20 µl of a blank muscle extract and 40 µl of diluted antiserum. The standard solutions added were 400; 200; 100; 50; 25; 12.5; 6.25 and 3.125 ng ml^{-1}, which resulted in final concentrations of 160; 80; 40; 20; 10; 5; 2.5 and 1.25 ng ml^{-1} in the well and 4000; 2000; 1000; 500; 250; 125; 62.5 and 31.2 ng g^{-1} of broiler muscle.

RESULTS AND DISCUSSION

Evaluation of Flu antisera in the ELISA

The sera obtained from the different bleedings of the two rabbits (MH41 and MH42) were compared for optimum dilution in the ELISA (final dilution (fd) resulting in an absorbance op 1.0). All bleedings, except for the final bleeding (fb), were taken at 4 weeks intervals and 10 days after the booster injections. The fbs were, due to a misunderstanding, taken 4 months after the last booster injection. The highest titers (1/100000) were obtained with the large bleedings taken before the fbs. The titers of the fbs were two (MH41) and five times (MH42) lower.

The dilutions of the antisera (fbs) and HRP dilutions, as well as sensitivity towards Flu and specificity were comparable using antiserum MH 41 (against Flu-BSA) and MH 42 (against Flu-KLH). The antisera and the Flu-HRP were used in final dilutions (fd) of 6000 and 100000 times, respectively. A calibration curve of Flu in buffer (PBS) showed a 50% inhibition at 5 ng ml^{-1}. This is a comparable sensitivity as

obtained with the last large bleedings and as obtained in a direct competitive ELISA [12] with polyclonal antibodies raised in sheep (50% inhibition at 30 ng g^{-1} of kidney using a 10% kidney homogenate) and 100 times more sensitive than obtained in assay buffer in an indirect competitive ELISA [13] using polyclonal antibodies against Flu extracted from chicken eggs (IgY). The specificity was tested with standard solutions of different (fluoro)quinolones and no cross-reactivity (<0.1%) was found with enrofloxacin, oxolinic acid, lomefloxacin, ofloxacin, enoxacin, norfloxacin, cinoxacin, naldixic acid, piromidic acid, difloxacin, marbofloxacin and ciprofloxacin. This specificity was also described with the other polyclonal antisera against Flu [12-13] and is explainable by the deviating structure of Flu (Figure 1).

BIA

Preparation and testing of the biosensor chip

The total surface (0.5 cm^2) of the CM5 chip was bench top coated with Flu and, after washing with three 5 µl injections of 50 mM NaOH, the average absolute response measured in the four Fcs was 21900 ± 380 RU which was approximately 1600 RU higher compared with a blank untreated chip surface.

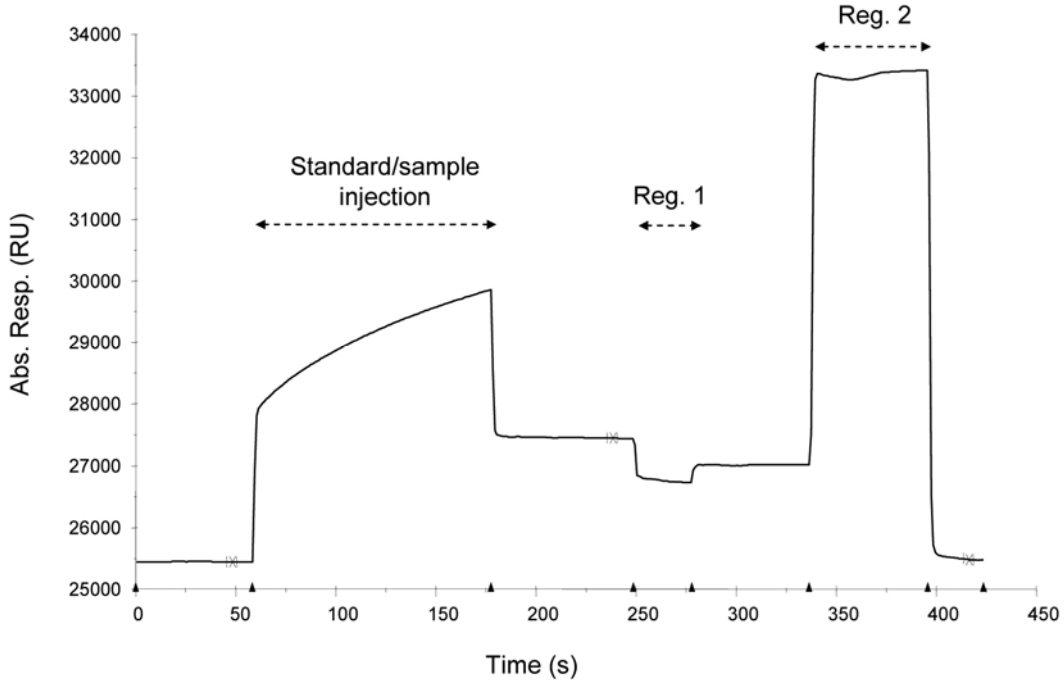

Figure 2: Typical sensorgram showing a complete cycle involving an injection of a zero standard solution followed by the double regeneration procedure (0.5 min injection with 0.2 M HCl followed by a 1 min injection with 0.2 M NaOH + 20% acetonitrile).

Of the two rabbits (MH 41 and 42), the three last bleedings (bleeding 4, 5 and fb) were tested in the Biacore. Just as with the ELISA, the bleedings 4 and 5 could be used more diluted than the fbs (fds of 1000-2000 versus 400 for the fb). Injecting (2

min at a flow rate of 25 µl min^{-1}) the fbs of MH 41 and 42 in a fd of 400 times resulted in maximum responses of 700-800 RU. In the final protocol, the antiserum (fb of MH41) was even less diluted (fd = 62.5 times) to obtain a robust assay with high maximum relative responses (>2000 RU, see Figures 2 and 3). Compared with the ELISA, the BIA under these conditions, consumes about 100 times more of the antiserum. The higher consumption of antibodies in a BIA compared to an ELISA (about 10 times) was described previously for a sulfonamide assay [14] and the higher consumption of antibodies is, in general, considered as a disadvantage of such fast assays. For the removal of the bound antibodies and re-use of the chip, different reagents were tested and the best results were obtained by the injection of 0.2 M HCl (0.5 min) followed by a 1 min injection of 0.2 M NaOH + 20% acetonitrile. As shown in Figure 2, the second regeneration step (Reg.2) had much more effect than the first regeneration step (Reg.1). However, both regeneration steps were necessary for a stable baseline during a series of injections of standards and samples.
A calibration curve of Flu in sample buffer showed 50% inhibition at 2.5 ng ml^{-1} (Figure 3) and, compared with the ELISA, this is two times more sensitive.

BIA in broiler serum

Prior to the injection into the Biacore, broiler serum was diluted in sample buffer. For the possible future combination with the other assays in broiler serum (e.g. anti-salmonella and sulfonamides), a previously applied sample buffer, with additives to reduce non-specific binding (CM-dextran sodium salt, a high concentration of sodium chloride and Tween 80), was used.

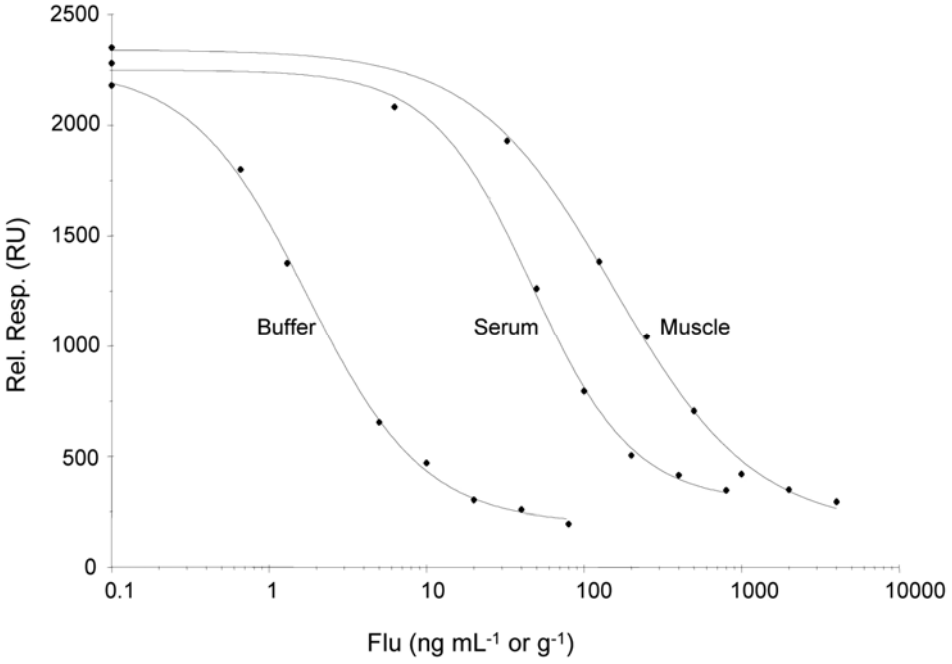

Figure 3. Calibration graphs of Flu in sample buffer, broiler serum and muscle.

Just as with the sulfonamide assay, broiler sera were five times diluted in this sample buffer. To evaluate the performance of the BIA in broiler serum, calibration curves of Flu were prepared in blank broiler serum. In the final format, a two-fold serial dilution series was prepared starting from 800 ng ml^{-1} of broiler serum. As shown in Figure 3, compared with the calibration curve of Flu in buffer, the presence of chicken serum had an effect on the calibration curve (shift to the right) which only partly could be explained by the dilution step (factor of 5). Because of this matrix effect, quantification of Flu in serum should be performed with the help of calibration curves in broiler serum. Using such calibration curves, the limit of detection (LOD = the concentration at the average relative response minus 3 times the standard deviation (S.D.)) of 15 ng ml^{-1} of broiler serum was determined after analysing 21 blank broiler serum samples. This resulted in a measuring range of 15 - 800 ng ml^{-1} of broiler serum.

Survey

Broiler sera (n=310) were obtained from 31 different flocks of 31 different Dutch farms (10 sera from each farm). Except for one sample (containing 130 ng ml^{-1}), all samples obtained from the 31 farms were found negative (< LOD of 15 ng ml^{-1}). Another 116 serum samples from the same flock of which the positive sample came from and which were taken at the same time, were analyzed and in 77 samples, the concentrations were found below the LOD and in the other 39 samples, the concentrations varied from 15 to 50 ng ml^{-1}.

BIA in broiler muscle

For the detection of Flu in muscle, an easy sample preparation procedure was selected. The homogenized muscle sample was extracted with buffer (1 g + 5 ml) and further diluted (five times) in an anti-Flu containing buffer of which an aliquot was injected into the Biacore. In Figure 3, a calibration graph, obtained with fortified muscle extract, with a 50% inhibition at about 200 ng g^{-1} is shown. Blank muscle samples obtained from 21 different broilers were analyzed and the average background concentration found was 6 ± 6 ng g^{-1}, resulting in an LOD of 24 ng g^{-1} (average + 3 times S.D.). The same samples (*n*=21) were spiked with Flu at 200, 400 and 800 ng g^{-1} and analyzed spread over 3 days by one technician and the average concentrations found were 150 ± 30, 265 ± 61, and 527 ± 135 ng g^{-1}, respectively and the average recoveries ranged from 68 till 75%.

Considering the BIA for muscle as a quantitative screening method and using the data obtained with the samples fortified at the MRL (400 ng g^{-1}), the decision limit (CCα) and the detection capability (CCβ) were calculated according to the validation procedures prescribed in the European Commission Decision 2002/657 [15] as 500 ng g^{-1} (MRL plus 1.64 times the corresponding S.D. (1.64 x 61 ng g^{-1})) and 600 ng g^{-1}

(CCα + 1.64 x 61 ng g^{-1}), respectively. For a screening assay, only CCβ, which is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1-β, is mandatory [15].

By mixing the proper amounts of incurred and control materials, muscle samples of broilers were prepared with concentrations of Flu of approximately 0.5, 1 and 2 times the MRL (400 ng g^{-1}). The preparation of these materials and the results obtained by LC-MS/MS analyses, averages of 10 randomly selected and in duplicate analysed samples (see Table 1), were presented during the 5th International Symposium on Hormone and Veterinary Drug Residue Analysis (May 16-19, 2006, Antwerp, Belgium) [17]. In Table 1, the results obtained with the LC-MS/MS method (considered as reference method in which data are corrected for recovery by the application of a calibration curve in the matrix) are compared with the BIA results (average of four analyses each in which data were not corrected for recovery) and, although the average concentrations found by the BIA were 20% lower (at one and two times MRL) than found by LC-MS/MS (explainable by the 66-75 % recovery in the BIA), a high correlation (R^2 = 0.9982) was found.

Table 1: Average concentrations of Flu as found by the BIA (n=4) and by LC-MS/MS (n=10) in incurred muscle samples.

Incurred samples MRL*	RESULTS (ng g^{-1})	
	BIA	LC-MS/MS
0.5	81 ± 16	82 ± 6
1	248 ± 6	312 ± 12
2	447 ± 48	562 ± 23

* Mixed muscle samples from treated and untreated broilers aiming for levels of 0.5, 1 and 2 times MRL.

Animal experiment

Using the normal sample preparation (five times diluted sera), the serum samples from the treated broilers (n=22) were analyzed and in 85% of the samples concentrations outside the range of the calibration curve (>800 ng ml^{-1}) were found. The sera were diluted an extra 10 times with blank broiler serum and the concentrations found (corrected for the dilution) varied from 500 to 2400 ng ml^{-1} with an average of 1740 ± 550 ng ml^{-1}. The concentrations in the corresponding muscle samples, as measured with the BIA, varied from 85 to 645 ng g^{-1}, with an average of 495 ± 132 ng g^{-1}. The data obtained in serum and muscle samples were not corrected for recoveries. As shown in Figure 4, all serum samples had higher concentrations of Flu than the corresponding muscle samples with an average serum/muscle ratio of 3.5. However, the correlation (y=2.9314x + 292) between the concentrations of Flu found in the individual sera (y-axe) and muscle samples was rather low (R^2= 0.50). This was

probably caused by the fact that all samples were obtained at the same time directly after treatment and by possible differences in intestinal absorption of Flu by the individual broilers.

From literature, the serum/tissue ratio of Flu is unknown. For two other quinolones (danofloxacin and enrofloxacin) in orally treated broiler chickens, steady state tissue concentrations markedly exceeded plasma concentrations [16]. The average concentrations in muscle were 2.1 and 2.5 times higher compared to levels in plasma (showing averages of 120 (for danofloxacin) and 520 ng ml^{-1} (for enrofloxacin)). Previously, high serum/muscle ratios in broilers were reported [4] for sulfamethoxazole (6.2) and sulfadiazine (8.7).

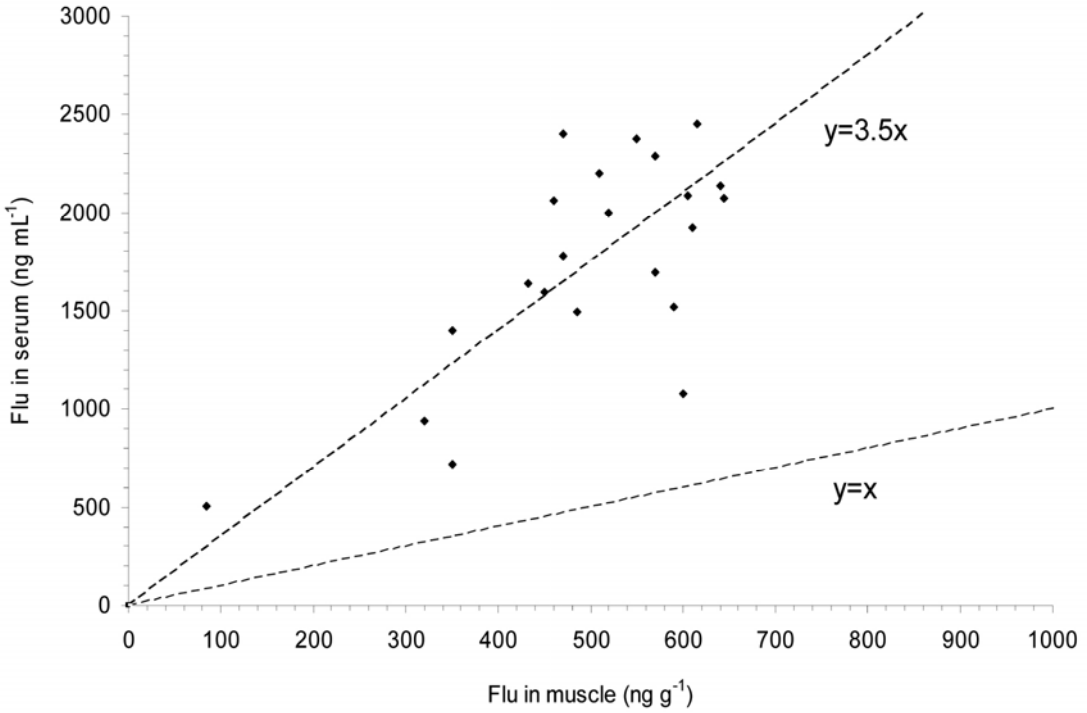

Figure 4. Correlation between Flu concentrations in broiler serum and muscle, as determined with the BIA, showing the variation of the serum versus muscle concentrations around the average serum/muscle ratio of 3.5 (y=3.5x) and showing that this ratio was above 1 (y=x) for all broilers.

Flu in serum as predictor of Flu levels in muscle

MRLs for Flu are established for the edible parts of broilers only. However, according to the project aim, an action limit in serum should be established to guarantee Flu levels in muscle below the MRL of 400 ng g^{-1}. Because all sera from the animal experiment contained higher concentrations than the corresponding muscle samples, the MRL in muscle of 400 ng g^{-1} was chosen as the minimum required performance limit (MRPL) in broiler serum. From 21 different blank broiler serum samples spiked at MRPL level, the decision limit CCα in broiler serum was calculated as 460 ng ml^{-1}

(MRPL plus 1.64 times the corresponding S.D.) and the detection capability CCβ was calculated as 520 ng ml^{-1} (CCα + 1.64 times the corresponding S.D.).
In broiler serum samples from the animal experiment (n=22), the concentration of Flu ranged from 500 till 2400 ng ml^{-1} of which 21 samples were non-compliant (>CCβ) and one sample was compliant with a concentration just below CCβ (506 ng ml^{-1}). The Flu concentration in the corresponding muscle sample was 85 ng g^{-1} (no false compliant result). Three of the 21 muscle samples of which the corresponding sera samples were non-compliant (>CCβ) contained Flu concentrations below the MRL of 400 ng g^{-1}, which results in a percentage of false non-compliant results of 14.

CONCLUSIONS

The polyclonal antiserum-based BIA for Flu in broiler serum and muscle is a quantitative screening assay and it proved 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, easy to apply and with suitable measurements ranges (15 to 800 ng ml^{-1} for serum and 24 to 4000 ng g^{-1} for muscle) which can simply be adapted by changing the sample (extract) volumes.
Concentrations of Flu in sera of treated broilers (obtained directly after treatment) were higher than the concentrations found in the corresponding muscle (with an average ratio of 3.5). The MRL in muscle was chosen as the MRPL in serum and the CCβ in serum, calculated as 520 ng ml^{-1}, was proposed as action level. In the future, such an action level should be confirmed by analyzing serum and muscle samples in which different withdrawal times are applied.
Although the conditions of the Flu BIA were adapted to previously prescribed conditions for the assays for sulfonamides and anti-salmonella, a combined assay still has to be tested. Such a combined assay should also include an assay which detects other allowed quinolones (danofloxacin, difloxacin, enrofloxacin and ciprofloxacin). Such a group-specific antiserum for (fluoro)quinolones was developed and applied in an SPR biosensor assay for the determination of fluoroquinolone residues in egg, chicken muscle and fish [18]. The application of the combination of both biosensor assays was described in a dual SPR biosensor immunoassay-directed identification of fluoroquinolones in chicken muscle by liquid chromatography electrospray quadrupole time-of-flight mass spectrometry [19]. The application of the combined assay in broiler serum is ongoing.

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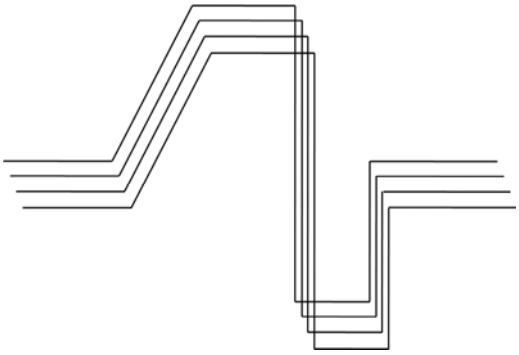
Chapter 10

Conclusions and future perspectives



CONCLUSIONS

Searching for an assay which could detect most of the relevant sulfonamides, the approach in which antibodies were raised against the generic structure of the sulfonamides was partly successful. Monoclonal antibodies (Mabs) were obtained which recognized different sulfonamides, however, they were less sensitive for other sulfonamides. The use of mutated recombinant antibodies (Rabs), after the modification of the single chain variable fragment (scFv) molecules of the wild-type Mab 27G3, resulted in improved immunoassays. The applications of two genetically modifications (mutants A.3.5 and M.3.4) and the wild-type Mab 27G3 were evaluated in the biosensor using a CM5 sensor chip coated with the sulfonamide-derivative (TS, see Chapter 5) and chicken serum as sample material (Haasnoot et al., 2005a). The improvements in inhibition, due to the presence of different sulfonamides added to chicken serum at the 100 ng ml^{-1} level, are shown in Figure 1 for the three antibodies.

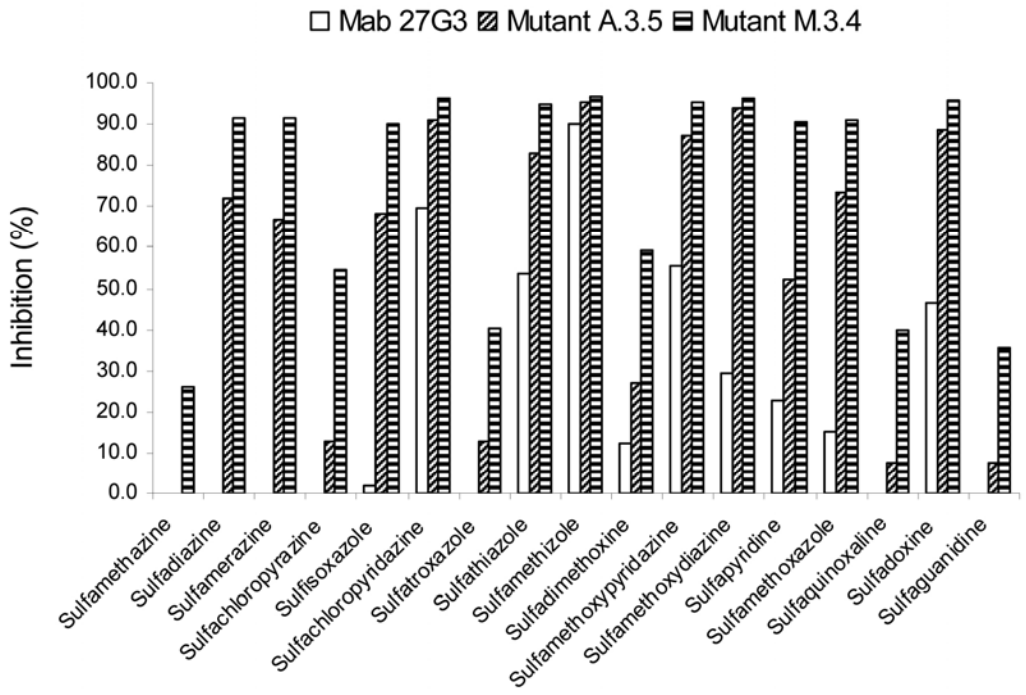


Figure 1. Inhibition (%) of the maximum responses, obtained with a BIA, due to the presence of different sulfonamides (100 ng ml^{-1}) added to a blank chicken serum using the wild-type Mab 27G3 and mutants A.3.5 and M.3.4. Chicken serum (20 µl) was diluted with 100 µl of the antibody-containing buffer of which 50 µl was injected at a flow rate of 20 µl/min (Haasnoot et al., 2005a).

With the wild-type Mab 27G3 and mutants A.3.5 and M.3.4, the number of sulfonamides showing significant inhibitions of the response (>20%) were 7, 12 and 17, respectively. The performance of the best mutant was compared in a biosensor immunoassay (BIA) format with other multi-sulfonamide antibodies and it was found to be the most sensitive towards most of the sulfonamides.

In comparison to Pabs and Mabs, the with the phage display technique obtained Rabs can be developed faster, in a more automatic process and with reduced or no consumption of laboratory animals (Brichta et al., 2005) and the Rab technology should be considered as a promising tool for future antibody development. However, in my experience, it took four years (the full Ph.D.-period of Dr. Korpimäki (University of Turku, Finland)) to develop and improve the anti-sulfonamide Rabs, which might be the reason why the number of available Rabs is still limited. The Rab-based BIAs were successfully applied to the detection of sulfonamides in serum and levels were higher and correlated well with levels in tissue as measured with LC-MS/MS (Haasnoot et al., 2005b).

Such a biosensor was also used for the detection of serum antibodies against *Salmonella* in chicken blood, in which recombinant DNA antigens were immobilized on the chip surface, and the results obtained suggested that this approach could be used for detecting past or present infections with a range of pathogens in animals (Jongerius-Gortemaker et al. (2002)). A comparable fast assay (testing in minutes) was developed for the detection of antibodies directed to Salmonella sero-groups B and D in porcine blood sera in a routine setting (Achterberg, et al., 2005) and was marketed (GE Healthcare) in combination with high-throughput equipment (8-channel Biacore Q100) with a capacity of up to eight samples in four to five minutes and a 96-well plate within one hour. Of one of the sulfonamide assays (using mutant M.3.4), the conditions were successfully adapted to the serological assay conditions (higher salt and carboxymethylated-dextran (CM-dextran) concentrations) by which the antibody concentration had to be increased as well (Haasnoot et al., 2005b). Under these conditions, the assay still worked perfectly well and this indicated the possibility for the simultaneous detection of anti-pathogens and residues of animals drugs which is an interesting combination for future research to broaden the application area in food safety control systems.

The successful combination of immunoassays was already demonstrated in this thesis with the simultaneous detection of five aminoglycosides in milk in which four different assays were combined and with the flumequine BIA which was successfully coupled to a multi-fluoroquinolones BIA (Marchesini et al., 2007a).

In conclusion, serum and milk are suitable sample materials for the biosensor detection of antibiotics in the food chain. Such assays are fast, robust, automated, easy to handle, and require simple sample preparations (dilutions in antibody-containing buffer). However, the applied four-channel biosensor systems are expensive, too limited in multiplexing and antibodies are too specific for the simultaneous detection of antibiotics from different groups and the systems are therefore less suitable for control agencies and food industries to provide an increased and more efficient control on food contaminants (such as antibiotics) in the food chain. Cheaper alternatives and more extended multiplex systems need to be explored in which the

knowledge obtained and unique reagents prepared in the present research will likely be of great value and are used at present to study the application of some of the other promising systems described hereafter in the future perspectives.

FUTURE PERSPECTIVES

Biosensors

The application of a low-cost SPR-based prototype biosensor system (Spreeta^{TM}) has been described previously (Marchesini et al., 2007b) in which the sensitivities with inhibition assays for endocrine disruptors were comparable to those obtained with a Biacore 3000. However, this system was less robust and built with a single flow-channel only. Alternative eight-channel SPR sensor instruments were developed and used for the detection of low molecular weight endocrine-disrupting compounds (Dostálek et al., 2007) and an environmental contaminant in a miniaturized and portable format (Kim et al., 2007), which are interesting approaches for future research. Beyond that, an SPR system with the possibility for the simultaneous detection of 20 spots in 4 flow-channels (Biacore A100) is available (GE Healthcare). The SPR imaging (iSPR) technology takes multiplex SPR analysis a step further.

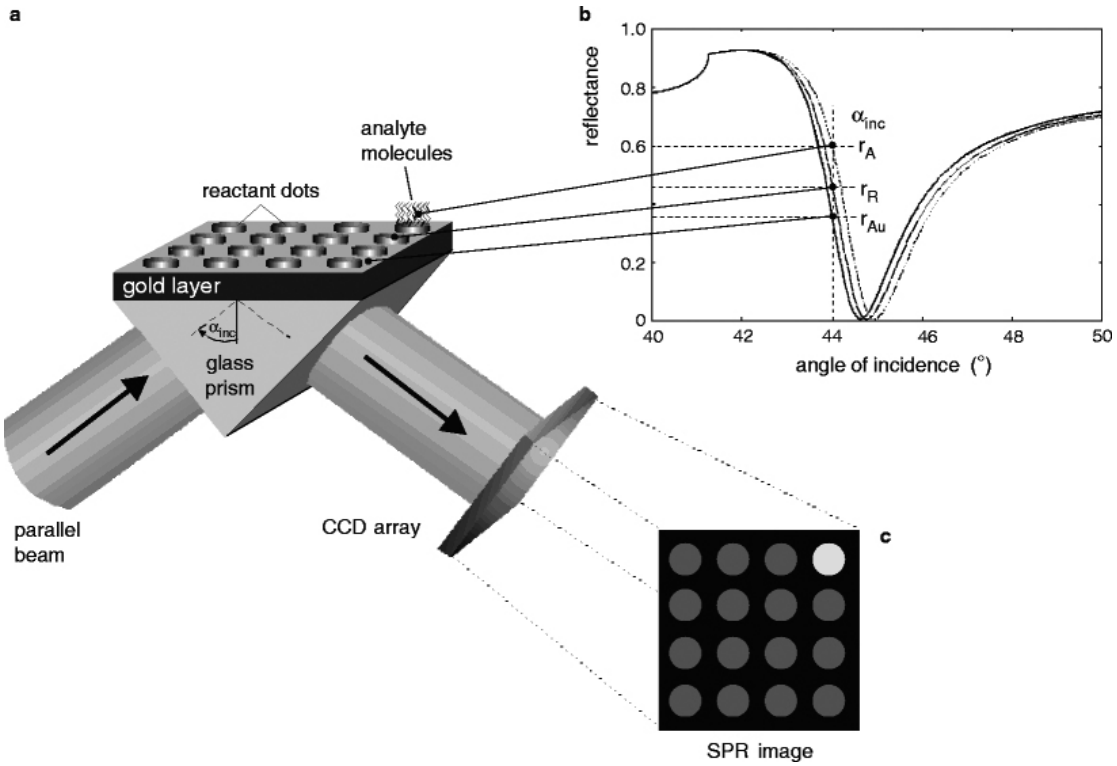


Figure 2. a) Schematic diagram of SPR imaging. b) Calculated SPR reflectance curve for a pure gold surface, a reactant dot and adsorbed analyte molecules on a reactant dot. c) The contrast of the SPR image is based on the different reflectance $r_A > r_R > r_{AU}$. (Steiner, G., 2004).

With iSPR (Figure 2), broad-beam monochromatic polarized light from a laser diode (at a specific wavelength) illuminates the whole functionalized area of the biosensor chip surface. The high resolution CCD video camera provides real-time difference images across the array format with up to hundreds of active spots and it captures all of the local changes at the surface of the biochip. There are several commercial iSPR instruments available, e.g. Biacore Flexchip (GE Healthcare), SPRi-Plex™ (Genoptics Bio interactions), ProteOn™ XPR 36 (Bio-Rad laboratories), SPRimager®II ARRAY system (GWC Technologies) and IBIS iSPR (IBIS Technologies B.V.). The instruments differ in optics, fluidics, sample handling and surface preparation. Rebe Raz et al. (2008) applied the IBIS iSPR and developed an inhibition immunoassay for gentamicin and neomycin which displayed a sensitivity at the low ng ml^{-1} level which is comparable with assays performed in the Biacore 3000. By combining more immunoassays on one sensor chip, such instruments will be highly relevant for multi-analyte screening of various food contaminants.
Another interesting automated multiplex biochip array technology (Evidence®) is marketed by Randox (www.randox.com) in which 25 immunoassays can be measured simultaneously using chemiluminescent signals and spots are measured with a CCD camera. They supply arrays for growth-promoters and antimicrobials but a major disadvantage is that it is a closed system which is not suitable for assay development. The different immunoanalytical microarray systems based on fluorescence, chemiluminescence, electrochemical, and label-free microarray readout systems have recently been reviewed by Seidel & Niessner (2008) and they also included an interesting and commercially available suspension microarray on a flow cytometer.

Flow cytometry

This promising and evolving suspension microarray is the Multi Analyte Profiling (xMAP®) technology (Luminex, 2007), which is an open system suitable for assay development. This technology uses small carboxylated polystyrene microspheres (5.6 μm beads), which are internally dyed with a red and an infrared fluorophore. By varying the ratio of the two fluorophores, up to 100 different color-coded bead sets can be distinguished, and each bead set can be coupled to a different biological probe. In combination with flow cytometry, it is possible to simultaneously measure up to 100 different biomolecular interactions in a single well. The carboxylated bead surface allows simple chemical coupling of capture reagents such as antibodies or drug-protein conjugates. This technology was already applied for the detection of sulfonamides in milk (Keizer de, et al., 2008) and in blood serum, meat drip and eggs (Bienenmann-Ploum, et al., 2008) using the Rab M.3.4 and for the simultaneous detection of aminoglycosides and sulfonamides in milk and blood serum (Haasnoot, et al., 2008). These experiments demonstrate that this technology is suitable for multiplexing of antibiotic assays in food materials and it is a serious option for future

multiplex applications. A five-fold increase in multiplexing capabilities can be obtained with the FlexMAP 3D™ platform of Luminex, using a third fluorophor, which offers 500-plex capability and runs three times faster compared with the Luminex100 or -200 systems. More relevant to food diagnostics is the development of the new Luminex multiplex platform called MagPix™ which is a low-cost, compact, rugged, diagnostic and environmental testing xMAP analyzer which moves away from a flow cytometry-based system to an instrument based on their already existing magnetic bead array (MagPlex™) analyzed on a magnet in a 2D readout with inexpensive Light Emitting Diodes (LEDs) and a CCD imager. It is expected to be launched in 2010 and this development will make future multiplexing faster, cheaper and more robust and applicable in the food chain.

Nanoparticles

The former described particles used in flow cytometry are on a microscale and nanoparticle research is currently an area of intense scientific investigations, due to a wide variety of potential applications in biomedical, optical, and electronic fields. Nanoparticles (NPs) are defined as particles having one or more dimensions in the sub-100 nanometer range (BSI, 2005). In analytical biochemistry, they are used as biosensor response enhancers (Guo and Dong, 2009) and as labels in clinical and food diagnostics due to their unique characteristics as the high surface-to-volume ratio and the size-dependent optical or magnetical properties.

Gold NPs (25 nm) were used by Mitchell and Lowe (2009) as response enhancers (by increased mass and gold plasmon coupling effect) in an SPR BIA for the detection of steroids. The biosensor chips were coupled with the steroids via oligoethylene linkers and the responses obtained with the anti-steroid primary antibodies were about 13-fold enhanced with a secondary antibody labeled with the gold NPs. Due to this enhancement, the primary antibody concentration could be reduced which resulted in a 12.5-fold increase in sensitivity. Yuan et al. (2008) reported about the use of larger gold NPs (40 nm) for signal enhancement on a mixed self-assembled monolayer (mSAM) sensor surface which resulted in 21.5 fold increase in signal and, due to a large reduction in antibody concentration, in a 30 times more sensitive assay for chloramphenicol.

The use of gold NPs and other new nanodiagnostic tools (e.g. quantum dots (QDs)) for diagnostic applications promise increased sensitivity, multiplexing capabilities, and reduced costs (Azzazy et al., 2006). The nanometer sized colloidal gold particles are frequently used as labels in lateral flow tests (Wang et al. (2007)) because of their ability to adhere proteins (e.g. antibodies) and their intense red color which is due to localized SPR (LSPR) a common phenomenon for nanometer-sized metallic structures (Hutter and Fendler, 2004). Localized surface plasmons (LSPs) are charge density oscillations confined to metallic NPs. Excitation of LSPs by an electric field

(light) at an incident wavelength where resonance occurs results in strong light scattering, in the appearance of intense surface plasmon (SP) absorption bands, and an enhancement of the local electromagnetic fields. The frequency (i.e. absorption maxima or color) and intensity of the SP absorption bands are characteristic of the type of material (gold, silver or platinum), and highly sensitive to the size, size distribution, and shape of the nanostructures, as well as to the environments which surround them. The fact that the color of metallic NPs depends markedly on the refractive index of the surrounding medium has been exploited for sensing applications. LSPR sensing is based on a simple optical extinction measurement, is not temperature sensitive, and requires only common laboratory equipment. Molecular interaction analysis of gold NPs on a solid transparent substrate (glass), to avoid the possibility of aggregation, have been reported by several groups. Such an LSPR-based immunosensor was developed for the detection of casein in milk (Hiep et al., 2007) in which anti-casein antibodies were immobilized to gold-capped silica NPs on a glass slide substrate and the binding of casein could be monitored by the peak absorbance intensity increments at around 520 nm using a UV-Vis spectrophotometer. Kreuzer et al. (2008) developed an LSPR-based biosensor for the detection of stanozolol using gold colloids (100 nm), coated with a stanozolol-protein conjugate, chemically sized on an activated glass substrate. Binding of anti-stanozolol antibodies was observed by a shift of the resonance wavelength (with a maximum of 13 nm) and the detection limit of the system was calculated as 2.4 nM or 0.7 ng ml^{-1}. Gold-silica core nanoshells, spherical silica core surrounded by a gold shell of a few nanometers in thickness, with different core/shell ratios result in different optical resonances and can be used for multiplexing (Prodan, et al., 2003). Advantages of the LSPR devices were described as the simplicity of the optical configuration, easy fabrication, the great potential for miniaturization, simple handling, low-cost, short assay times, and high sensitivity.

Advances in nanomaterials have produced a new class of fluorescent labels which is more suitable for multiplex detection by conjugating semiconductor nanocrystals, also known as quantum dots (QDs), with biorecognition molecules (Chan et al., 2002). These QDs (2-8 nm) are atom clusters comprising a core, shell, and coating. The core is made up of a few hundred to a few thousand atoms of a semiconductor material often composed of atoms from group II-VI (e.g. CdSe, CdTe, CdS, and ZnSe) or group III-V elements (e.g. InP and InAs) in the periodic table. A semiconductor shell (typically zinc sulfide) surrounds and stabilizes the core, improving both the optical and physical properties of the material. An amphiphilic polymer coating then encases the core and shell, providing a water-soluble surface that can be modified. For some of these QDs, this amphiphilic inner coating is covalently modified with a functionalized polyethylene glycol (PEG) outer coating to reduce nonspecific binding.

By varying the size and composition of QDs, the emission wavelength can be tuned and the broad absorption spectra, useful for the simultaneous excitation of different-sized QDs, and narrow symmetric emission spectra make QDs very well suited to optical multiplexing (Algar et al., 2009). More multicolor optical coding for biological assays has been achieved by embedding different-sized QDs into polymeric microbeads at precisely controlled ratios (Han et al., 2001). Other advantages of QDs are excellent brightness, negligible photobleaching, fairly high quantum yields, and photostability. These extraordinary fluorescence properties can be attributed to the unique fluorescence mechanism of semiconductor materials which fluoresce through the formation of excitons, or Coulomb-correlated electron-hole pairs, upon absorption of a photon of light. Compared with the excited state of a fluorophore, this exciton typically exhibits a much longer lifetime (up to about 200 nanoseconds). For diagnostic multiplex applications, a set of seven amine-, or carboxyl-derivatized or streptavidin-labeled Qdot® nanocristals with a broad excitation and narrow symmetric emission properties (maxima at: 525, 565, 585, 605, 655, 705 and 800 nm) is commercially available (www.invitrogen.com).

Highly monodisperse (1.57 ± 0.21 nm), ball-shaped and alkyl-functionalized silicon NPs (Si NPs) have been synthesized on a gram scale (Rosso-Vasic et al., 2008). Next to the size-dependent optical properties, the kind of surface influenced the photoluminescent properties as well. This phenomenon was the basis for a Dutch project (Functionalized silicon nanoparticles in multiplex diagnostics platforms (Nanoplex); http://www.onderzoekinformatie.nl/en/oi/nod/onderzoek/OND1334082/) [2008-2012] which will set a new test format by directly measuring multiplex biointeractions in solution (omitting flow cytometry) using (changes in) the different emission addresses of the Si NPs, both defining the type of biointeraction and the intensity.

Although QDs have proven to be suitable labels in bioanalysis, their application in quantitative immunoassays is still limited. Ding et al (2006) developed a competitive fluorescence-linked immunosorbent assay (cFLISA) in a microtiter plate for the detection of sulfamethazine in chicken muscle tissue extracts using a commercially available QD (QD 655 (Quantum Dot Corp, Hayward, CA, USA) as the fluorescent label coupled to the secondary antibody. The same QD was used for the detection of enrofloxacin in chicken muscle tissue (Chen et al., 2009) and the high emission amplitude of the QD (655 nm) led to significant improvements in the signal to noise ratios of the final detected signals. Nichkova et al (2007) described the application of two commercially available QDs as labels in an immunoassay microarray for the simultaneous microscopic detection of two biomarkers of exposure to two major classes of compounds: pyrethroid insecticides and triazine herbicides. Goldman et al. (2004) prepared bioinorganic conjugates of highly luminescent nanocrystals (CdSe-ZnS core-shell QDs) and antibodies to develop a four-plex immunoassay in a

microtiter plate for the simultaneous detection of four toxins (cholera toxin, ricin, shiga-like toxin 1 and staphylococcal enterotoxin B) in a single well using QDs with emission maximums of 510, 555, 590 and 610 nm.

Artificial "antibodies"

The most critical and time-consuming reagent in an immunoassay is the antibody and artificial "antibodies" such as molecularly imprinted polymers (MIPs) and aptamers are described as potential alternatives. These alternatives can be prepared in vitro, avoiding the need for animals, and against non-immunogenic and toxic targets.

MIPs have been demonstrated to be a promising class of synthetic receptors that can be tailored to meet specific end-use recognition requirements. MIP technology is a general strategy of synthesis that allows preparation of polymeric materials with "memory" of a particular molecule (analyte). Most modern imprinting is performed through a process called non-covalent imprinting in which a template molecule interacts with a functional monomer to form a pre-polymerization complex in the presence of a solvent. The solvent is known as the porogen because of its role in pore forming during polymerization. A cross-linking monomer is added to the pre-polymerization complex to form a scaffold around the binding site during polymer synthesis. The template is removed from the polymer and the result is a highly cross-linked polymer with exposed imprinted cavities capable of analyte binding. The serious problem of interferences in analysis by bleeding of residual template (analyte) was successfully solved by the use of analogs as templates (Urraca et al., 2006). For antibiotics, HPLC, as for sulfadimethoxine (Hung et al., 2008), and molecularly imprinted solid-phase extraction (MISPE), as for fluoroquinolones (Turiel et al., 2007), are the analytical techniques where MIPS have found most applications (Fernández-González et al., 2006). They are usually synthesized in bulk, ground, sieved, sedimented to remove fine particles and packed in columns. MIP-based SPE columns for the selective pre-concentration of the antibiotics chloramphenicol and fluoroquinolones (with high recoveries for sarafloxacin, norfloxacin, enrofloxacin, ciprofloxacin, lomefloxacin and ofloxacin) are commercially available via the company MIP Technologies (http://www.miptechnologies.com). While the selectivity, high capacity, robustness, low costs, and easy handling and synthesis of MIPs make them an ideal choice for the development of sensing devices, their use for antibiotic sensing is an unexplored field that deserves further research. In residue analysis, MIPs are most frequently described for mycotoxin detection (Urraca et al., 2006 and Appell et al., 2008). Yu and Lai (2005) described an SPR sensor (Spreeta) chip surface which was covered with a thin molecularly imprinted polypyrrole (MIPPy) film by electrochemical polymerization. The same procedure was followed be Choi et al (2009) for the preparation of a MIPPy film with zearalenone on bare gold biosensor chips. These chips were successfully applied for the direct detection of ochratoxin A

in wheat and wine extracts and zearalenone in corn extracts, which are interesting examples for future research with MIPs in the optical biosensors described in this thesis.

Aptamers are oligonucleotides (DNA or RNA) with the ability to bind to non-nucleic acid target molecules such as peptides, proteins and antibiotics with high affinity and specificity. They are isolated from combinatorial libraries of synthetic single-stranded nucleic acids of 10^{14}-10^{15} DNA or RNA molecules containing a random region flanked on both sides with fixed primer sequences for amplification. This isolation by exponential enrichment, via an in vitro iterative process of adsorption, recovery and reamplification is known as systematic evolution of ligands by exponential enrichment (SELEX). The enriched library is cloned and sequenced, and individual sequences are chosen for their best affinity and specificity. The final selected aptamer(s) can now be produced in sizeable quantities by chemical syntheses. The high affinity of aptamers for their targets is given by their capability of folding upon binding their target molecule. They can incorporate small molecules into their nucleic acid structure or integrate into the structure of larger molecules such as proteins. Thanks to their unique characteristics and chemical structure, aptamers offer themselves as ideal candidates for use in analytical devices and techniques. The mainly clinical applications are reviewed (Tombelli et al., 2005; Hamula et al., 2006 and Mairal et al., 2008). Aptamers have been selected for binding to several antibiotics (tetracyclines, aminoglycosides, chloramphenicol and viomycin (Lorenz and Schroeder, 2006) however they were used to study the mode of interaction of these antibiotics with RNA and not in a screening assay. Stead et al. (2008) described the first aptamer-based and food control-related screening assay for the detection of malachite green in salmon tissue extracts. The assay uses an RNA-based aptamer with an affinity for malachite green of which the complex showed a circa 2000 times increase in fluorescence signal and this detection principle was used for the spectrofluorimetric detection. They described a rapid complex formation (within 10 min) and the complex was found to be stable for circa 18 hours. It should be mentioned that malachite green was known to bind to RNA and DNA at forehand since it has been used as an interacting dye for many years. A further broadening of aptamers into an area as food control will have to address the new challenge of complex matrices. However, just as with antibodies, MIPs and aptamers are relatively target specific.

Bio-effect related assays

For the broader detection of compounds or bio-effect related detections, the application of whole cells or other biomolecules (transport proteins or receptors) might be more interesting for future applications in food diagnostics. For the detection of tetracyclines in poultry tissue, such a whole-cell-based bioassay in a 96-well

microtiter plate format, based on a genetically engineered luminescent bacterial strain containing the regulation unit of tetracycline resistance factor, has been applied (Virolainen et al., 2008). This assay is better suited for high-throughput analysis and has the potential to displace growth inhibition assays. However, such bioassays are not yet described for other antibiotics although the use of the multidrug-binding repressor protein (QacR) from *Staphylococcus aureus* (Schumacher et al., 2001) might be an interesting approach for the future development of a multi-drug biosensor. Currently, cellular biosensors, based on various microbial species containing reporters which are specifically induced via selected promoters, are more widely used in pharmaceutical drug discovery and in environmental biology (Urban et al., 2007) and for monitoring environmental chemical contaminants (Patel, 2006) and this will also influence the development of new bioassays for the detection of antibiotics in food.
Whole cell-based assays need incubation times of a few hours and by the use of their functional ingredients (e.g. receptors) in multiplex diagnostic platforms, faster functional binding assays can be developed. Such fast receptor-based lateral flow tests are already commercially available for the detection of beta-lactams in combination with tetracyclines (e.g. Twin sensor^{TM} of Unisensor (Angleur, Belgium)). The combination of a receptor assay for β-lactams antibiotics with antibody-based assays for groups of sulfonamides and fluoroquinolones in an ELISA format has also been described as an interesting option for multi-analyte detection (Adrian et al., 2008).

According to an EU definition (2002/657/EC), screening methods are used to detect the presence of a substance or class of substances at the level of interest with the capability for a high sample throughput. They are used to sift large numbers of samples for potential non-compliant results and they are specifically designed to avoid false compliant results. Of the technologies described above, only two of the flow cytometers have high-throughput capacities (see Table 1). In my opinion, different potential end-users (e.g. farmers, inspectors, food industries, or control laboratories), require different strategies for screening (from low- to high-throughput, handheld to automated on-line devices and from single analyte to multiplex systems) and the suitability of the different technologies for the different end-users are presented in Table 1. The dip sticks or lateral flow tests are most suitable for farmers and inspectors, because of the lack of investment in equipment, easy format and performance and speed (minutes per sample), but they are most limited in multiplex capacity and qualitative results (compliant or suspected non-compliant) are obtained. All other suggested technologies are quantitative when calibrations curves are used for the calculations of concentrations. Multiplex capacities of more than 100 (like the FlexMap 3D and iSPR) are not very useful and realistic in food control. Future multiplexing will probably consist of combinations of 5 to 25 assays and most technologies are capable of handling that. The nanoparticle-based assays are limited

in multiplex capacity (<10) but some (e.g. LSPR) require less investment in equipment compared with the other technologies of which the cost-prices of the flow cytometers are estimated as <25 and 50 k€ for the MagPix and Luminex 200, respectively.

Table 1. The possible multiplex capacity (MC (the estimated theoretically maximum number of assays in the multiplex format)), sample throughput (TP (high (H), medium (M) or low (L))), costs of investment in equipment (Inv. (high (H) >100 k€, medium (M) 25-50k€, low (L) <25 k€, or no costs (N)))), application format (AF (handheld (H), automated with on-site possibility (A), or not automated (N))) and end-users of the multiplex technologies described in the future perspectives.

Technology	**MC**	**TP**	**Inv.**	**AF**	**End-user**		
					Farmer/ Inspector	**Food industry**	**Control labs**
Biosensors							
- Multichannel	20	M	H	A	-	+	+
- iSPR	>100	M	H	A	-	+	+
- Evidence	25	M	H	A	-	+	+
Flow cytometry							
- Luminex 200	100	H	M	A	-	+	+
- FlexMAP 3D	500	H	H	A	-	-	+
- MagPix	50	M	M	A	-	+	+
Nanoparticles							
- LSPR	<10	M	L	N	-	+	+
- QDs	<10	M	M	N	-	+	+
Dip sticks	4	L	N	H	+	+	-

- not or less suitable, + suitable

The developments described above bring great promise for future custom-made and cost-effective screening assays in the different areas of the control of the food chain. The knowledge obtained from the research described in this thesis about the use of biosensors, chip surface coupling chemistries, assay formats, assay and regeneration conditions for the different antibiotic assays and for one in combination with the detection of anti-salmonella, sample behavior, antibiotic concentrations in serum, plasma and the different tissues, etc., and the unique reagents (Pabs, Mabs and Rabs and antibiotic-derivatives and conjugates) prepared within this research are of great value and are used at present to study the application of the other promising multiplex systems based on iSPR and flow cytometry.

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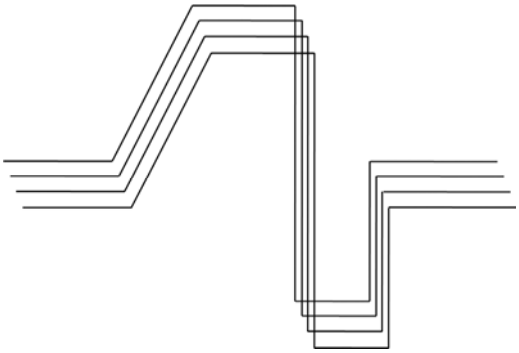
Summary



The food chain is threatened by various hazards and the presence of residues of antibiotics, used during cattle-breeding, is one of the serious risks for consumers. In food analysis for antibiotics, screening technologies are powerful tools that provide a rapid screen of large numbers of samples when conventional analytical methods are too cumbersome. Due to their simplicity and high-throughput capacity, immunoassays are applicable for screening in the food chain. However, in general, they are very specific, and only suitable for the detection of one or two antibiotics, which seriously limits their application. New antibodies and assay formats with multiplex capacity might give new possibilities for control agencies and food industries for increased and more efficient controls on food contaminants.

Surface Plasmon Resonance (SPR)-based optical biosensors with four serially connectable flow channels have been launched with claims as being suitable for fast (in minutes) four-plex assays, providing easy (label-free) detection and fully automated operation and the option for the direct detection of small molecules (as antibiotics).

The aim of the research described in this thesis was to evaluate the application of these new optical biosensors as possible control instruments in the food chain with multiplex immunoassays for antibiotics as a model. To reduce the number of tests, the development and application of group-specific antibodies (with sulfonamides as model compounds) was explored together with their improved performances after protein engineering. Their application in biosensor immunoassays was investigated, together with the possibility to predict sulfonamide levels in tissue from levels in blood serum and the combined application with serological assays for anti-pathogens. The other antibiotics (aminoglycosides and flumequine) were used as models to explore different biosensor immunoassay formats and to study the interference of immunoassays in a multiplex format and the foreseen matrix interferences from protein-rich products as milk, meat extract and blood serum. The overall result should be an answer to the question: “Can biosensors fulfill the needs of control agencies and food industries to provide an increased and more efficient control on food contaminants (such as antibiotics) in the food chain?”

In the introduction **(Chapter 1)**, the background of antibiotics, their analysis, the applied biosensors and the different types of antibodies are described. **Part I** presents the development of multi-sulfonamide antibodies by the development of polyclonal (Pabs) and monoclonal antibodies (Mabs) against the generic ring structure of sulfonamides (**Chapter 2)**. Hereto, a sulfathiazole derivative (abbreviated as TS) was synthesized and linked to carrier proteins in such a way that the aromatic amino group, common to all sulfonamides, was distal to the proteins. Mice were immunized with the TS-protein conjugates, and four different Mabs were obtained after the fusion of spleen cells of one of the mice with myeloma cells and the screening of the produced hybridomas supernatants. With an optimized ELISA protocol, the best of these Mabs

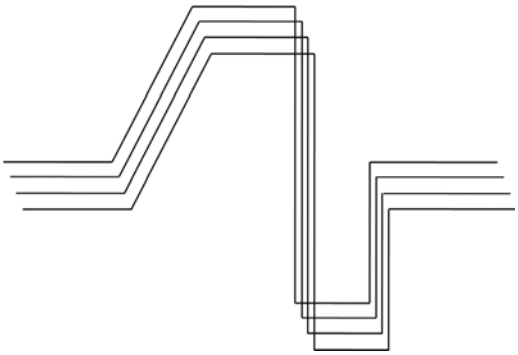
(Mab 27G3) showed 50% inhibition with eight structurally different sulfonamides at concentrations less than 100 ng ml^{-1} or 5 ng/well. Although this was a uniquely broad specificity with a never seen sensitivity, the sensitivity towards the different sulfonamides varied too much to call it a generic sulfonamide ELISA. In **Chapter 3**, the preparation of Pabs against eight different sulfonamides is described. Here, the aromatic amino group, common to all sulfonamides, was used for linking to carrier proteins and, in ELISA's, these antibodies showed high sensitivities and specificities. Their performances were compared with multi-sulfonamide Pabs raised against the generic structure of sulfonamides by the immunization with two sulfonamide derivatives (abbreviated as TS and PS) coupled to carrier proteins in such a way that the common structure was distal to the proteins. These Pabs recognized several structurally different sulfonamides. ELISA's with the anti-TS Mabs (from Chapter 2) were found to be much more sensitive towards different sulfonamides as compared with another Mab described in literature. However, the sensitivity towards some important sulfonamides, such as sulfamethazine (= sulfadimidine), was low.

Part II describes the development of multi-sulfonamide biosensor immunoassays (BIAs) based on broad-specificity Mabs and recombinant antibodies (Rabs) derived from one of them. In **Chapter 4,** the development of BIAs in chicken serum is described and different antibodies (one Mab and two Pabs), all raised against similar sulfamethazine-carrier protein conjugates, were used. Compared with the specific Pabs, the Mab 21C7-based BIA resulted in a better sensitivity for sulfamethazine detection and it was found suitable for the detection of 8 sulfonamides in 10 times diluted chicken serum with limit of detection (LODs) between 7 and 20 ng ml^{-1}. In **Chapter 5**, the performance of this unique Mab was compared with another multi-sulfonamide Mab supplied in a commercial kit (Qflex Kit Sulfonamides) and with a mutant antibody (M.3.4), which was derived previously from the anti-TS Mab. Each of these antibodies showed interactions with all 17 sulfonamides tested and one (Mab 21C7) was sensitive for the *N*4-acetyl metabolites also. The mutant M.3.4-based assay was found to be the most sensitive towards most of the sulfonamides, whereas the Qflex Kit Sulfonamides detected the five sulfonamides registered for application in poultry in the Netherlands within the narrowest measurement range. In **Chapter 6**, the application of mutant antibodies (A.3.5 and M.3.4) in BIAs for the detection of sulfamethoxazole and sulfadiazine (most applied sulfonamides in broilers in the Netherlands) in serum and plasma samples from treated broilers is described. The assays were fast (5 min per sample), the sample preparation was easy (dilution in antibody-containing buffer only) and an equal sensitivity for the two sulfonamides was obtained with LODs in serum and plasma below 10 ng ml^{-1} (for the A.3.5-based BIA). The concentrations found with the BIA in serum and plasma of the treated broilers were comparable and higher than the concentrations found in tissue by LC-MS/MS. To predict the concentrations of the two sulfonamides below the maximum

residue limit (MRL) of 100 ng g^{-1} in the tissue with the highest level (skin+fat), the proposed action level of the multi-sulfonamide BIA in serum is 130 ng ml^{-1}.
The latest mutant antibody (M.3.4), with a better sensitivity towards more sulfonamides, was applied during a survey in which sera of 1 out of 31 different flocks were found positive with low concentrations (25 ± 21 ng ml^{-1}) of sulfamethoxazole. This BIA was performed under the conditions applied in the serological assay for the detection of three anti-salmonella serotypes which broadens the future application area.
In **Part III,** the development of BIAs for aminoglycosides is described, starting with the direct assay, using antibody-coated biosensor chips, for gentamicin and (dihydro)streptomycin (**Chapter 7**). These BIAs even worked in milk far below the MRLs. In that chapter, the direct assay format for (dihydro)streptomycin was compared with the inhibition assay format using a streptomycin (STREP)-protein conjugate immobilized on the chip surface, and the sensitivities in buffer were comparable for both drugs in both formats. With milk, interferences, probably due to the nonspecific binding of milk proteins to the protein-coated sensor chips, were observed in both BIAs. These interferences could be reduced by the application of ultrafiltration as sample pretreatment or by the use of a reference flow channel. In **Chapter 8**, the development of a single BIA for the simultaneous detection of five aminoglycosides in reconstituted skimmed milk is described. Four aminoglycosides (gentamicin, neomycin, kanamycin and a streptomycin derivative) were directly (without coupling to proteins) immobilized onto the sensor surface in the four flow channels that were serially connected, and these flow channels were used in combination with a mixture of four specific antibodies. Milk samples were diluted ten times in the antibodies-containing buffer and the LODs in reconstituted milk were between 15 and 60 ng ml^{-1}, which was far below the MRLs (varying from 100 to 1500 ng ml^{-1}).
In **Chapter 9** the development of a specific flumequine BIA is described. Such an assay was useful because flumequine is one of the fluoroquinolones most frequently applied for the treatment of broilers in the Netherlands and - at that time – could not be detected at the desired level with a multi-fluoroquinolone BIA. This assay was validated as a quantitative assay for the performance in broiler muscle. Applying an easy extraction procedure with buffer, the measuring range was from 4 to 4000 ng g^{-1} (MRL = 400 ng g^{-1}) and the decision limit (CCα) and the detection capability (CCβ) were determined as 500 and 600 ng g^{-1}, respectively. Serum and muscle samples from flumequine-treated broilers were analyzed and the concentrations found in serum were always higher than those found in muscle (average serum/muscle ratio was 3.5). This proved the applicability of the BIA in serum as predictor of the flumequine concentration in muscle.

In conclusion (**Chapter 10**), blood serum and milk are suitable sample materials for the biosensor detection of antibiotics in the food chain. Such assays are fast, robust, automated, easy to handle, and require simple sample preparations (dilutions in antibody-containing buffer). However, the applied four-channel systems are expensive, too limited in multiplexing and the antibodies are too specific for the simultaneous detection of more antibiotics from different groups. Therefore, cheaper alternatives and more extended multiplex systems need to be explored, in which the knowledge obtained in the present research will likely be of great value. Possible alternatives - ranging from low-cost, imaging and localized SPR-based systems to other multiplex biochip and suspension microarray systems as well as nanoparticle-based detections and the use of artificial “antibodies” (molecularly imprinted polymers (MIPs) and/or aptamers) or whole cell- or their functional ingredients-based assays - are described in the future perspectives (Chapter 10). This potential brings great promise for future custom-made and cost-effective screening assays in the different areas and at different control points in the food chain.

Samenvatting



De voedselketen wordt bedreigd door verscheidene gevaren en de aanwezigheid van restanten van antibiotica, gebruikt in de veeteelt, is één van de serieuze risico's voor consumenten. In voedselanalyse voor antibiotica zijn screeningsmethoden sterke instrumenten die het mogelijk maken om snel grote aantallen monsters te analyseren als conventionele analytische methoden te bewerkelijk zijn. Door hun eenvoud en grote doorvoercapaciteit zijn immunologische testen toepasbaar voor het screenen in de voedselketen. Over het algemeen zijn zij echter erg specifiek en daardoor alleen geschikt voor het tegelijkertijd detecteren van maar één of twee antibiotica waardoor hun toepassing wordt gelimiteerd. Nieuwe antilichamen en testvormen met multiplex capaciteiten zouden nieuwe mogelijkheden kunnen bieden voor controle-instanties en voedselindustrieën om meer intensieve en efficiëntere controles op voedselcontaminanten te kunnen gaan toepassen.

Op Surface Plasmon Resonance (SPR) gebaseerde optische biosensoren met vier serieel koppelbare vloeistofstroomkanalen zijn op de markt gebracht met claims als het geschikt zijn voor snelle (in minuten) vierplex testen, het verzorgen van een makkelijk (labelvrije) detectie en een volledig geautomatiseerde werking en de mogelijkheid voor de directe detectie (zonder labels) van kleine moleculen (zoals antibiotica).

Het doel van dit proefschrift was om deze nieuwe optische biosensoren te evalueren voor hun mogelijke toepassing als controle-instrument in de voedselketen met multiplex immunologische testen voor antibiotica als een model. Om het aantal testen te verminderen, werd de ontwikkeling en toepassing van groepspecifieke antilichamen (met sulfonamiden als modelverbindingen) onderzocht samen met hun verbeterde verrichtingen na modificaties middels `protein engineering`. Hun toepassing in immunologische biosensortesten en om hiermee sulfonamideniveaus in weefsel te voorspellen aan de hand van niveaus in bloedserum alsmede de combinatie met serologische testen voor antipathogenen werden ook onderzocht. De andere antibiotica (aminoglycosiden en flumequine) werden als modellen gebruikt om de verschillende vormen van immunologische biosensortesten te onderzoeken. Onderzoek naar interferenties van dergelijke testen in een multiplex vorm en de voorziene matrixinterferenties van eiwitrijke producten zoals melk, vleesextract en bloedserum werd ook uitgevoerd. Het eindresultaat zou een antwoord moeten zijn op de vraag of het mogelijk is om met biosensoren de behoeften van controle-inspecties en voedselindustrieën, aan een meer intensieve en efficiënte controle op de aanwezigheid van voedselcontaminanten (zoals antibiotica) in de voedselketen, te kunnen verwezenlijken.

In de inleiding (**Hoofdstuk 1**) worden de achtergronden van antibiotica, de analysemogelijkheden, de toegepaste biosensoren en de verschillende typen aan gebruikte antilichamen beschreven. **Deel I** presenteert het onderzoek naar de ontwikkeling van multi-sulfonamide antilichamen waarbij polyklonale (Pabs) en

monoklonale antilichamen (Mabs) tegen de ringstructuur van sulfonamiden zijn opgewekt (**Hoofdstuk 2**). Hiervoor werd een sulfathiazolderivaat (afgekort als TS) gesynthetiseerd en zodanig aan een dragereiwit gekoppeld dat de aromatische aminegroep, aanwezig in alle sulfonamiden, van het eiwit af was gericht. Muizen werden geïmmuniseerd met het TS eiwit conjugaat en na de fusie van de miltcellen van één van de muizen met myelomacellen, en het screenen van de geproduceerde hybidomasupernatanten, werden vier verschillende Mabs verkregen. Met een geoptimaliseerd ELISA protocol liet het beste Mab (Mab 27G3) een 50% inhibitie zien met acht structureel verschillende sulfonamiden bij concentraties minder dan 100 ng ml^{-1} of 5 ng/test. Hoewel dit een unieke brede specificiteit was met een op dat moment niet eerder vertoonde gevoeligheid, was die gevoeligheid voor de verschillende sulfonamiden te variabel om het een generieke sulfonamide ELISA te noemen. In **Hoofdstuk 3** wordt het maken van Pabs tegen acht verschillende sulfonamiden beschreven. Hier werd de in alle sulfonamiden aanwezige aromatische aminegroep gebruikt voor het koppelen aan dragereiwitten. In ELISA´s lieten deze antilichamen een grote gevoeligheid en een hoge specificiteit zien. Hun prestaties werden vergeleken met multi-sulfonamiden Pabs opgewekt tegen de generieke structuur van sulfonamiden door het immuniseren met twee sulfonamidederivaten (afgekort als TS en PS) zodanig gekoppeld aan dragereiwitten dat deze gemeenschappelijke structuur van het eiwit af gericht was. Deze Pabs herkenden verschillende structureel verschillende sulfonamiden. De ELISA´s met de anti-TS Mabs uit hoofdstuk 2 waren gevoeliger voor verschillende sulfonamiden indien vergeleken met andere in de literatuur beschreven Mabs. Echter, de gevoeligheid voor enkele belangrijke sulfonamiden, zoals sulfadimidine, was laag.

Deel II beschrijft de ontwikkeling van multi-sulfonamiden biosensor immunoassays (BIAs) gebaseerd op multi-sulfonamiden Mabs en recombinant antilichamen (Rabs) afgeleid van één van hen. In **Hoofdstuk 4** wordt de ontwikkeling van dergelijke biosensortesten in kippenserum beschreven waarbij verschillende antilichamen (één Mab en twee Pabs), allemaal opgewekt tegen sulfadimidine en gebruik makend van gelijke immunogenen, werden gebruikt. Vergeleken met de specifieke Pabs, resulteerde de op Mab 21C7 gebaseerde biosensortest in een betere gevoeligheid voor de detectie van sulfadimidine. Deze test werd geschikt bevonden voor het detecteren van 8 sulfonamiden in tien keer verdunde kippenserum met detectielimieten (LODs) tussen 7 en 20 ng ml^{-1}. In **Hoofdstuk 5** wordt de werking van dit unieke Mab vergeleken met een andere multi-sulfonamiden Mab geleverd in een commerciële kit (Qflex Kit Sulfonamiden) en met een gemuteerd antilichaam (M.3.4) die eerder was afgeleid van de anti-TS Mab. Ieder van deze antilichamen lieten interacties zien met all 17 geteste sulfonamiden en één (Mab 21C7) was ook gevoelig van *N*4-acetyl metabolieten. De op mutant M.3.4 gebaseerde test werd als het meest gevoelig voor de meeste sulfonamiden beoordeeld terwijl de Qflex Kit Sulfonamiden de vijf in

Nederland voor pluimvee geregistreerde sulfonamiden detecteert binnen het kleinste meetbereik. In **Hoofdstuk 6** wordt de toepassing van gemuteerde antilichamen (A.3.5 en M.3.4) in biosensortesten voor de detectie van sulfamethoxazol en sulfadiazine (de meest gebruikte sulfonamiden bij slachtkuikens in Nederland) in serum en plasma van behandelde slachtkuikens beschreven. De testen waren snel (5 min per monster), de monstervoorbewerking was eenvoudig (alleen verdunning in een antilichamen-bevattende buffer) en voor de twee sulfonamiden werd een gelijke gevoeligheid verkregen met LODs in serum en plasma beneden 10 ng ml^{-1} (voor de A.3.5-gebaseerde test). De met de biosensortest gevonden concentraties in serum en plasma van behandelde slachtkuikens waren vergelijkbaar en hoger dan de met LC-MS/MS gevonden concentraties in weefsel. Om te voorspellen dat de concentraties van de twee sulfonamiden in het weefsel waarin de hoogste gehalten voorkomen (vel + vet) beneden het MRL niveau van 100 ng g^{-1} zullen liggen, wordt een actieniveau van de multi-sulfonamiden test in serum voorgesteld van 130 ng ml^{-1}.

Het later ontwikkelde gemuteerde antilichaam (M.3.4), met een betere gevoeligheid voor meer sulfonamiden, werd toegepast tijdens een survey. Hierin werden sera van 1 van de 31 verschillende onderzochte koppels positief bevonden met lage concentraties aan sulfamethoxazol (25 ± 21 ng ml^{-1}). Deze test werd uitgevoerd onder aangepaste condities, zoals toegepast in de serologische test voor het detecteren van drie anti-salmonella serotypen, waardoor het toekomstige toepassingsgebied wordt uitgebreid.

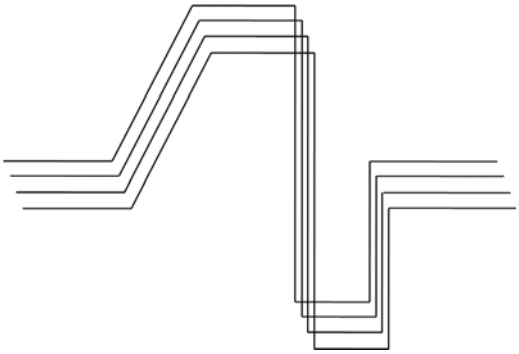
In **Deel III** wordt de ontwikkeling van BIAs voor aminoglycosiden beschreven. Hierbij is begonnen met de directe test, gebruik makend van met antilichamen beladen biosensorchips, voor gentamicine en (dihydro)streptomycine (**Hoofdstuk 7**) die zelfs werkzaam waren in melk ver beneden de MRL´s. In dat hoofdstuk wordt de directe test voor (dihydro)streptomycine vergeleken met de inhibitietest. Daarbij is een streptomycine-eiwitconjugaat op het chipoppervlak geïmmobiliseerd en de gevoeligheden in buffer waren vergelijkbaar voor beide geneesmiddelen in beide testvormen. Met melk werden interferenties waargenomen in beide testvormen die vermoedelijk werden veroorzaakt door niet specifieke binding van melkeiwitten aan de met eiwit gecoate chips. Deze interferenties konden worden gereduceerd door het toepassen van ultrafiltratie als monstervoorbewerking of door het gebruik van een referentiekanaal. In **Hoofdstuk 8** wordt de ontwikkeling van één test voor het gelijktijdig kunnen meten van vijf aminoglycosiden in gereconstrueerde magere melk beschreven. Vier aminoglycosiden (gentamicine, neomycine, kanamycine en een streptomycinederivaat) werden direct (zonder koppeling aan eiwitten) op het sensoroppervlak geïmmobiliseerd in de vier vloeistofkanalen die serieel werden gekoppeld en gebruikt werden in combinatie met een mengsel van vier specifieke antilichamen. Melkmonsters werden tien maal verdund in een antilichamen bevattende buffer en de detectiegrenzen in gereconstrueerde melk lagen tussen 15 en

60 ng ml^{-1}. Deze gevoeligheden waren ver beneden de MRL´s (variërend van 100 tot 1500 ng ml^{-1}).

In **Hoofdstuk 9** wordt de ontwikkeling van een specifieke BIA voor flumequine beschreven. Een dergelijke test was nuttig omdat flumequine één van de fluorquinolonen is die het meest frequent worden gebruikt voor de behandeling van slachtkuikens in Nederland en, op dat moment, niet op het gewenste niveau kon worden gedetecteerd met een multi-fluorquinolonen BIA. Deze test werd gevalideerd als een kwantitatieve test voor de uitvoering in vlees van slachtkuikens. Na het toepassen van een makkelijk uitvoerbare extractieprocedure met buffer was het meetbereik van 4 tot 4000 ng g^{-1} (MRL= 400 ng g^{-1}). De beslissingsgrens (CCα) en het detectievermogen (CCβ) werden bepaald op respectievelijk 500 en 600 ng g^{-1}. Serum en vleesmonsters van met flumequine behandelde slachtkuikens werden geanalyseerd en de gevonden concentraties in serum waren altijd hoger dan die gevonden in spierweefsel (gemiddelde serum/weefsel verhouding was 3,5). Dit bewees de toepassing van de BIA in serum als voorspeller voor de concentraties aan flumequine in spierweefsel.

In **Hoofdstuk 10** wordt geconcludeerd dat bloedserum en melk geschikte monstermaterialen zijn voor het met de biosensor kunnen meten van antibiotica in de voedselketen. Dergelijke testen zijn snel, robuust, geautomatiseerd, makkelijk toe te passen en vereisen eenvoudige monstervoorbewerkingen (verdunningen in een antilichamen bevattende buffer). Echter, de toegepaste vierkanaals systemen zijn duur, te beperkt m.b.t. multiplexen en de antilichamen zijn te specifiek voor het gelijktijdig kunnen meten van meer antibiotica. Daarom moeten goedkopere alternatieven en meer uitgebreide multiplexsystemen worden onderzocht waarbij de in het huidige onderzoek verkregen kennis van grote waarde kan zijn. Mogelijke alternatieven, die variëren van goedkope, “imaging” en “localized” SPR systemen tot andere multiplex biochip en suspensie microarray systemen, als ook op nanodeeltjes gebaseerde detecties en het gebruik van kunstmatige “antilichamen” (MIPs en aptameren) of op hele cellen of hun functionele ingrediënten gebaseerde testen worden beschreven in de vooruitblik naar de toekomstige ontwikkelingen (Hoofdstuk 10). Deze vooruitblik laat goede mogelijkheden zien voor de ontwikkeling van klantspecifieke en kostenefficiënte screeningmethoden voor de verschillende aandachtsgebieden en controlepunten in de voedselketen.

Dankwoord



Dit proefschrift is mede tot stand gekomen omdat het ontbreken ervan een handicap oplevert in een nieuwe werksituatie waarin het begeleiden van AIO's meer standaard is dan een uitzondering. Omdat niet de gangbare route van een masteropleiding en AIO-traject is gevolgd, is het fundament voor dit proefschrift terug te voeren op een 38 jaar durende carrière. En, waar moet je dan beginnen met bedanken? Wel, allereerst natuurlijk bij mijn vrouw Annie voor haar grote steun die ik heb mogen ontvangen gedurende mijn gehele carrière binnen het Rijks Zuivel Station (RZS) en het RIKILT van adjunct-laborant naar senior onderzoeker en clusterleider. Er zijn vele uren in avonden en weekeinden geïnvesteerd in opleiding en werk maar er werd altijd soepel mee omgegaan. Annie, bedankt en ik hoop nog op vele gezellige jaren samen in een goede gezondheid. Mijn zwager Jan Guldemond wil ik bedanken omdat hij destijds dacht dat een laboratoriumomgeving voor mij wel eens geschikt zou kunnen zijn en voor het contactleggen met het RZS.
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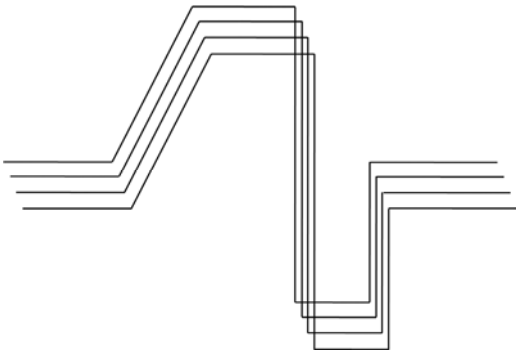
About the author



CURRICULUM VITAE

Willem Haasnoot was born on the 14th of August 1953 in Leiden, the Netherlands. In 1970 he obtained his secondary school diploma (MULO-B in Voorschoten) after which he sailed the world's seas for 6 months as an ordinary seaman employed by the Dutch Royal Nedlloyd. Unfortunately, due to his glasses, a planned carrier as a steersman proved to be no option. In March 1971, he was employed as an assistant laboratory worker at the Government Dairy Station in Leiden and in September 1971 he started with a 2-year lasting part-time (evenings) preparatory higher professional education (VHBO) for laboratory technicians (Delftse Analisten Cursus) which was successfully ended in 1973. His compulsory national service (18 months) ended in December 1974, with the rank of sergeant in the Royal Netherlands Army Signal Corps, after which he returned to the Government Dairy Station to perform composition analyses on dairy products. A 3-year part-time (evenings) higher professional education for laboratory technicians (HBO-A: van Leeuwenhoek Instituut in Delft) was successfully finished in 1977. In 1979 he moved to Wageningen to work as a technician specialized in chromatography and mycotoxins and to contribute to the formation of the new State Institute for Quality Control of Agricultural Products (RIKILT). In 1982 he graduated from the 3-year lasting part-time (evenings) higher professional education (HBO-B, chemical direction of the O.L.A.N. in Arnhem), after which he started as a senior technician within the department of Protein Chemistry of RIKILT. In the years 1984 and 1985, he studied chemistry as a part-time student at the Utrecht University. This study was not completed because the combination of work, a family - extended with two daughters in 1978 and 1980 - and a study was not manageable. From 1987 to 1992, he was a senior technician in the department of Biopharmaceutical Analyses (BFA) of RIKILT where the research was focused on the development of immunochemical methods for the detection of residues of growth promoters. Since 1992, he is responsible for the immunochemical research within RIKILT; first in the function of scientific researcher, and from 2002 also officially in the function as leader of the group Biomolecular Detection. The research was initially focused on the development of immunochemical methods (ELISA's, immunoaffinity chromatography (IAC), immunofiltration (IF), strip tests, etc.) for the detection of growth promoters and was gradually enlarged to assays for animal drugs, contaminants and proteins. Since 2000, optical biosensors play an important role in his research and parts of the results have been used to compile this thesis.

CURRICULUM VITAE

Willem Haasnoot werd op 14 augustus 1953 in Leiden geboren. In 1970 behaalde hij het MULO-B diploma te Voorschoten waarna hij als lichtmatroos in dienst van de Koninklijke Nedlloyd gedurende 6 maanden de wereldzeeën bevoer. Een geplande carrière als stuurman op de grote vaart zat er, als brildragende, helaas niet in. In maart 1971 begon hij als adjunct-laborant bij het Rijks Zuivel Station (RZS) te Leiden en startte hij met de tweejarige avondopleiding VHBO aan de Delftse Analisten Cursus welke in 1973 met succes werd afgerond. Zijn achttien maanden durende militaire dienstplicht eindigde in december 1974, met als laatste rang sergeant bij het wapen der Verbindingsdienst, waarna hij weer terugkeerde bij het RZS voor het uitvoeren van samenstellingsonderzoek aan zuivelproducten. Een driejarige avondopleiding (HBO-A aan het van Leeuwenhoek Instituut te Delft) werd in 1977 met succes afgerond. In 1979 verhuisde hij naar Wageningen om als analist met de specialisaties chromatografie en mycotoxinen mee te werken aan de vorming van het Rijkskwaliteits Instituut voor Land- en Tuinbouwproducten (RIKILT). In 1982 werd de driejarige opleiding HBO-B (chemische afdeling) aan de avondschool voor Laboratorium personeel te Arnhem-Nijmegen (O.L.A.N.) met succes afgerond waarna hij startte als laboratorium hoofdassistent binnen de afdeling Eiwitchemie van het RIKILT. In de jaren 1984 en 1985 volgde hij de deeltijdopleiding scheikunde aan de Rijksuniversiteit Utrecht. Deze opleiding werd niet afgerond omdat de combinatie van werken, een gezin - uitgebreid met twee dochters in 1978 en 1980 - en een studie niet was vol te houden. Van 1987 tot 1992 was hij laboratorium hoofdassistent binnen de afdeling Biofarmaceutische Analyse (BFA) waar onderzoek werd uitgevoerd naar de ontwikkeling van (immunochemische) methoden voor het aantonen van residuen van groeibevorderende stoffen. Sinds 1992 is hij verantwoordelijk voor het immunochemisch onderzoek binnen het RIKILT, eerst in de functie van wetenschappelijk onderzoeker, en vanaf 2002 ook officieel als leider van de cluster Biomoleculaire Detectie. Dit onderzoek was in eerste instantie gericht op het ontwikkelen van immunochemische methoden (ELISA’s, immunoaffiniteits-chromatografie (IAC), immunofiltratie (IF), striptesten, etc.) voor de detectie van groeibevorderaars en werd later uitgebreid met testen voor diergeneesmiddelen, contaminanten en eiwitten. Sinds 2000 hebben optische biosensoren een belangrijke rol gespeeld bij het onderzoek en delen van de hiermee verkregen resultaten zijn gebruikt voor het samenstellen van dit proefschrift.

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Book chapters:

1. Baumgartner, S., Fürtler-Leitzenberger, I., Drs, E., Molinelli, A., Krska, R., Immer, U., Schmitt, K., Bremer, M.G.E.G., ***Haasnoot, W***., Danks, Ch., Romkies, V., Reece, P., Wilson, Ph., Miening, M., Weller, M., Niessner, R., Corsini, E., Mendonça, S. (2008). European Survey for hidden allergens in food – A case study with peanut and hazelnut. Chapter 23 in *ACS Symposium Series No. 1001/Food Contaminants: Mycotoxins and Food Allergi*es (MW Trucksess, PM Scott, and EM Herman, Eds.) pp 370-381.
2. ***Haasnoot, W***, Bienenmann-Ploum, M, Korpimäki, T, Cazemier, G., Pré du, J., Kohen, F. (2005) Biosensor detection of sulfonamides: from specific to multi-sulfonamide assays. In Rapid Methods for biological and chemical contaminants in food and feed. (A. van Amerongen, D. Barug and M. Lauwaars Eds.), Wageningen Academic Publishers, pp 321-337.
3. ***Haasnoot, W***., Schilt, R. (2000) Immunochemical and Receptor Technologies (Chapter 5) of the book *"Residue Analysis in Food-Principles and Applications"* (ed. Michael O'Keeffe), Harwood Academic Publishers, Amsterdam.
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Non-peer-reviewed publications:

1. Bremer, M.G.E.G., Smits, N.G.E., ***Haasnoot, W***., Nielen, M.W.F. (2008) Immunological detection of illegally administered somatotropins and its biomarkers in cattle. *Proceedings of the EuroResidue VI Conference*, 19-21 May, 2008, Egmond aan Zee, the Netherlands, pp. 1279-1284.
2. ***Haasnoot, W.,*** Eekelen, H.D.L.M., Bienenmann-Ploum, M.E., Gerçek, H., Nielen, M.W.F. (2008) Multiplex flow cytometric immunoassay for drug residues using the xMAP technology. *Proceedings of the EuroResidue VI Conference*, 19-21 May, 2008, Egmond aan Zee, the Netherlands, pp. 431-436.
3. Marchesini, G.R., Hooijerink, H., ***Haasnoot, W***., Nielen, M.W.F. (2008) SPR biosensor screening assays coupled to bio-affinity-directed analytical identification of bioactive substances. *Proceedings of the EuroResidue VI Conference*, 19-21 May, 2008, Egmond aan Zee, the Netherlands, pp. 93-98.
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5. Gerçek, H., Delahaut, P., ***Haasnoot, W***., Nielen, W.M.F. (2008) Flow cytometric immunoassay for fluoroquinolones in chicken serum. *Proceedings of the EuroResidue VI Conference*, 19-21 May, 2008, Egmond aan Zee, the Netherlands, pp. 303-308.
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method for porcine fat. *Proceeding of the EuroResidue V Conference*, 10-12 May, 2004, Noordwijkerhout, the Netherlands, pp. 644-648.

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10. Baumgartner, S., Drs, E., Krska, R., Bremer, M.G.E.G., ***Haasnoot, W.***, Banks, J., Reece, P., Danks, C., Tomkies, V., Kiening, M., Weller, M.G., Schmitt, K., Immer, U., Corsini, E., Wilson, P., Scarniet, I. (2003) ALLERGENTEST the Development of Rapid Easy to Use Tests for the Detection of Peanut and Hazelnut Protein and Allergens in Food. *Proceedings of Euro Food Chem XII Conference*, 24-26 September, Brugge, Belgium, pp. 638-641.
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12. Voirin, G., Sigrist, H., ***Haasnoot, W.***, Skinner, N., Liley, M. (2000) A fluorescence waveguide sensor for the detection of antibiotic residues in milk. *Proceedings of the EuroResidue IV Conference*, 8-10 May 2000, Veldhoven, the Netherlands, pp. 1108-1112.
13. Verheijen, R., ***Haasnoot, W.*** (2000) Development of one step strip tests for residue analysis. *Proceedings of the EuroResidue IV Conference*, 8-10 May 2000, Veldhoven, the Netherlands, pp. 1103-1107.
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22. Courtheyn, D., Verheye, N., Bakeroot, V., Dal, V., Schilt, R., Hooijerink, H., Bennekom van, E.O., ***Haasnoot, W***., Stouten, P., Huf, F.A. (1993) Detection of corticosteroids in animal feed and premixes: A new category of growth promotors. *Proceedings of the EuroResidue II Conference*, 3-5 May, 1993, Veldhoven, the Netherlands, pp. 251-256.
23. ***Haasnoot, W***., Morais Ezkerro, S., Keukens, H.J. (1993) Applications of immunochemical selectivity for screening and confirmation of animal drug residues in biological samples. *Proceedings of the EuroResidue II Conference*, 3-5 May, 1993, Veldhoven, the Netherlands, pp. 347-351.
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31. ***Haasnoot, W.***, Elenbaas, H.L., Venema, D.P. (1986) Applications of fast protein liquid chromatography in food control, *Proceedings 2^{e} FPLC symposium*, pp. 15-25.
32. ***Haasnoot, W***., Elenbaas, H.L. (1986) FPLC als hulpmiddel bij de controle van agrarische produkten. *LAB/ABC*, december, 20-24.

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34. ***Haasnoot, W***., Elenbaas, H.L. (1985) Fast ion exchange chromatography on cheese proteins, *Proceedings 1^{e} FPLC Symposium*.
35. ***Haasnoot, W***., Stouten, P., Elenbaas, H.L. (1984) De enzymatische bepaling van 3-hydroxyboterzuur in eieren en eiprodukten. *De Ware(n)-chemicus*, 14, 40-49.

Training and supervision plan (2000-2009)

VLAG graduate school

Discipline specific activities

Courses

- Luminex Fundamental Assay Techniques, Wageningen, 2007.
- Introductie tot LC-MS, Hyphen MassSpec Wageningen, 2006
- Bio interaction analysis, Breda, 2000

Conferences

- International Symposium on Food Safety, Daejeon, South-Korea, 2009.
- NWO-Studiegroep Analytische Scheikunde, Lunteren, 2008.
- EuroResidue VI, Egmond aan Zee, 2008.
- 30th Mycotoxin Workshop, Utrecht, 2008.
- xMAP Conference, Amsterdam, 2007.
- NSTI Nanotech 2007, Santa Clara, CA, USA, 2007.
- Rapid Methods Europe 2007, Noordwijkerhout, 2007.
- xMAP conference, Amsterdam, 2006.
- 232nd ACS National Meeting, San Francisco, USA, 2006.
- The Ninth World Congres on Biosensors, Toronto, Canada, 2006.
- Rapid Methods Europe 2005, Noordwijk aan zee, 2006.
- EuroResidue V, Noordwijkerhout, 2004.
- Rapid Methods Europe 2004, Noordwijk aan zee, 2004.
- VIIth Int. Conference on Agri-Food Antibodies, Uppsala, Sweden, 2003.
- 4th Int. symposium on hormone and veterinary drug residue analysis, Antwerp, Belgium, 2002.
- Bull Seminar 2001, Maastricht, 2001.
- Brokerage Event Snelle Detectiemethoden, Ede, 2000.
- EuroResidue IV, Veldhoven, 2000.

General courses

- Advanced Course Guide to Scientific Artwork, Wageningen, 2009.
- Het Persoonlijk Efficiency Programma, Wageningen, 2004.
- Leidinggeven aan Professionals, Rheden, 2003.
- Excel, Wageningen, 2001.
- Several other general courses (e.g. scientific writing, project management, word, windows, etc.) were followed earlier than the year 2000.

Optionals

Active participation (meetings/presentations) in EU-projects:

- EU-NANODETECT [2008-2012]
- EU-CONffIDENCE [2008-2013]
- EU-BIOCOP [2005-2009]
- EU-AFRAMILK [2001-2004]
- EU-MICROQUAL [2000-2003]

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