SPECIAL GUEST EDITOR SECTION

Rapid Analysis of Coumarins Using Surface Plasmon Resonance

AOIFE LACY, LYNSEY DUNNE, BRIAN FITZPATRICK, STEPHEN DALY, and GARY KEATING
Dublin City University, National Centre for Sensor Research, School of Biotechnology, Dublin 9, Ireland
ANDREW BAXTER
XenoSense Ltd., The Innovation Centre, Northern Ireland Science Park, Queen's Rd, Queens Island, Belfast BT3 9DT, Northern Ireland
STEPHEN HEARTY and RICHARD O'KENNEDY[1]
Dublin City University, National Centre for Sensor Research, School of Biotechnology, Dublin 9, Ireland

Coumarin molecules are ubiquitous in nature. Several have come to prominence as potential clinical therapeutic candidates. The principal example is warfarin, which is a very widely prescribed anticoagulant. Other coumarin derivatives, such as aflatoxin B_1, are insidious contaminants in crop-derived foodstuffs. Extreme potency is a common feature of all biochemically active coumarins and, thus reliable methods for their rapid and sensitive detection are of paramount importance. Accordingly, this review examines the current methods used in the analysis of these molecules and compares them with immunoassay-based strategies. As a case study, we report on our experiences with using coumarin-specific polyclonal, monoclonal, and recombinant antibodies in conjunction with a surface plasmon resonance-based biosensor for analysis of coumarins. We chart the assay development process and demonstrate high sensitivity and reproducibility that compares favorably with established methodologies.

Coumarins derive their class name from the vernacular name of the tonka bean (*Dipterx odorata*), "Coumaru," and belong to a group of compounds known as the benzopyrones. They consist of a benzene ring joined to a pyrone. The general structure of the 4 main subtypes is shown in Figure 1.

Coumarin and coumarin derivatives have been very extensively studied because of their wide-ranging potential applications (1, 2). They have been used as anticoagulants, antitumor agents, bacteriostatic agents, and for the treatment of edema. In addition, they have many uses in analysis, e.g., study of metabolism, as fluorescent labels and enzyme substrates/inhibitors, in lasers and in ion measurements (2–6). Coumarins and their derivatives are also used as food flavorants and as perfume stabilizers. However, some coumarins exhibit potent toxicity and, in fact, several derivatives are used as rodenticides. In contrast to other less potent mycotoxins, the coumarin-based aflatoxins are deemed mutagenic and potentially carcinogenic and thus, no Tolerable Daily Intake (TDI) has been established.

Since 1954, coumarin is classified as a toxic substance by the U.S. Food and Drug Administration (FDA), following reports of possible hepatic tumor-inducing properties in rats (7). The FDA banned its use, classifying all coumarin-containing foods as 'adulterated.' Furthermore, coumarin was designated as a chemical carcinogen by the National Institute for Occupational Safety and Health as a result of tests performed on rodents. However, caution should be taken in extrapolating this information to human situations; various mutagenicity tests have shown that coumarin and its metabolites are nonmutagenic (2). Recent studies suggest that rats are inherently more susceptible to coumarin-induced hepatotoxicity (8, 9). This was originally attributed to the dominance of the 7-hydroxycoumarin metabolic pathway over the 3-hydroxycoumarin-branched pathway in humans. However, the European Food Safety Authority Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food has concluded that it is the balance between bioactivation and detoxification that dictates the inter- and intraspecies polymorphic susceptability to coumarin-induced hepatotoxicity (10). Thus, the Panel concluded that hepatotoxic responses should be taken into account in setting a TDI and that, in defining relevant safety factors to the 'no-observed-adverse-effect' level for hepatotoxicity, a factor of 10 should be included for potential interspecies variation, together with a factor of 10 for potential individual differences between humans. On this basis, a TDI of 0–0.1 mg coumarin/kg bulk weight has been proposed (10).

The clinical interest in coumarin and 7-hydroxycoumarin as anticancer agents arose from reports that these agents had achieved objective responses in some patients with advanced malignancies (11, 12). In addition, both coumarin and its derivatives have shown promise as potential inhibitors of cellular proliferation in vitro (1, 13, 14).

Guest edited as a special report on "Biosensors: Making Sense of Food" by Harvey Indyk.

[1] Author to whom correspondence should be addressed: e-mail: Richard.okennedy@dcu.ie

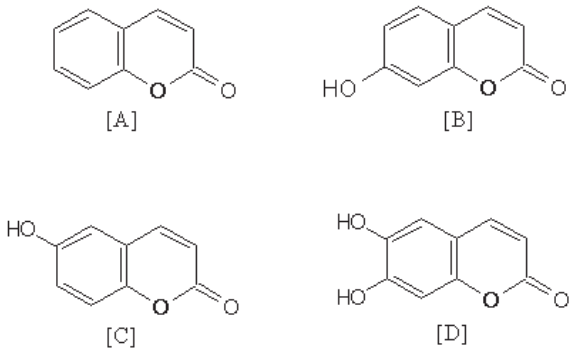

Figure 1. Structures of coumarin (A), 7-hydroxycoumarin (B), 6-hydroxycoumarin (C), and esculetin (D).

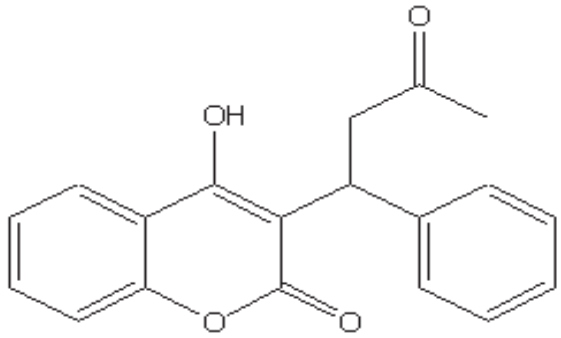

Figure 2. Structure of warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin).

Warfarin (also known as coumadin) is consistently among the most prescribed drugs in the Western world. The emergence of warfarin as a drug of therapeutic importance can be attributed to the findings of Karl Paul Link and his collaborators at the University of Wisconsin during the late 1930s and early 1940s (15), and it was subsequently identified as 3,3′-methylbis-4-hydroxycoumarin, or dicoumarol.

More than 100 related structural compounds of dicoumarol were synthesized in order to identify and characterize the key structural determinant of anticoagulant efficacy (16, 17). Warfarin (3-[acetonylbenzyl]-4-hydroxycoumarin) was subsequently synthesized by Ikawa in 1944, with the name "warfarin" derived from *W*isconsin *A*lumni *R*esearch *F*oundation and coum*arin* (Figure 2). The anticoagulant activity of coumarins is directly related to their ability to interfere with the vitamin K-dependent post-translational modification of the essential blood clotting factors (II, VII, IX, and X) and warfarin is the therapeutic of choice for treatment of a variety of thromboembolic disorders, including atrial fibrillation, deep vein thrombosis, and threatened stroke. There is a great inter- and intra-individual pharmacokinetic and pharmacodynamic variability and this is reflected in the efficacious dose, which can differ greatly among individuals (18, 19). It is thus vital that warfarin medication is administered at an appropriately individualized dosage (20).

Aflatoxins

Aflatoxins were discovered in the 1960s following the deaths of several thousand turkey poults in England after their ingestion of contaminated Brazilian ground nut meal (1, 7, 11). Aflatoxins are highly toxic fungal secondary metabolites produced by *Aspergillus* spp. (1). Fungal contamination of foodstuffs and feeds, and of crops such as maize, cottonseed, peanuts, and tree nuts can occur during growth and storage. The molecular structures of the aflatoxin branch of the coumarin family are outlined in Figure 3. The most clinically significantly occurring aflatoxin is aflatoxin B_1 (AFB_1), which is produced by certain strains of *A. flavus* and *A. parasiticus*. Other aflatoxins, designated B_2, G_1, and G_2 are also produced, but AFB_1 is generally present in the largest amount and exhibits the greatest toxicity. Aflatoxin M_1 (AFM_1 or 4-hydroxy-AFB_1), a hydroxylated metabolite of AFB_1, is excreted in the milk of dairy cattle following consumption of aflatoxin-contaminated food. AFQ_1 and AFP_1 are metabolites of AFB_1 found in the mouse and rhesus monkey (1, 21).

AFB_1 is a highly potent carcinogen that has been implicated in the development of human hepatocellular carcinoma and is classified as a human carcinogen by The International Agency for Research on Cancer (22). In the United States, the Federal Food Drug and Cosmetic Act Sec. 402(a) established specific action levels set at 20 ppb total aflatoxins in food and 0.5 ppb for AFM in milk (23). The European Union (EU) has also established guidelines on maximum residue limits (MRL) for aflatoxins in a range of commodities (Table 1). However, several countries within the EU have proposed their own MRL with some degree of variation observed from country to country (Table 2; 24). These levels are currently undergoing review. Clinical symptoms of aflatoxosis noted in animals include gastrointestinal dysfunction, reduced reproductive ability, reduced feed utilization and efficiency, anemia and jaundice with the effects of aflatoxin poisoning varying from species to species (25). Cases of acute aflatoxin poisoning in humans have been reported sporadically, mainly in Africa and Asia. In the majority of reported cases, humans become exposed to aflatoxins by consuming contaminated food such as maize, rice, or cereal products. A broad range of symptoms are associated, including vomiting, abdominal pain, pulmonary edema, acute liver damage, loss of function of the digestive tract, convulsions, cerebral edema, and death, depending on the ingested level.

Biosensor-Based Analysis of Coumarins

The next sections of this paper emphasize the application of surface plasmon resonance (SPR)-based sensors for the detection of 7-hydroxycoumarin (26), warfarin (20), and

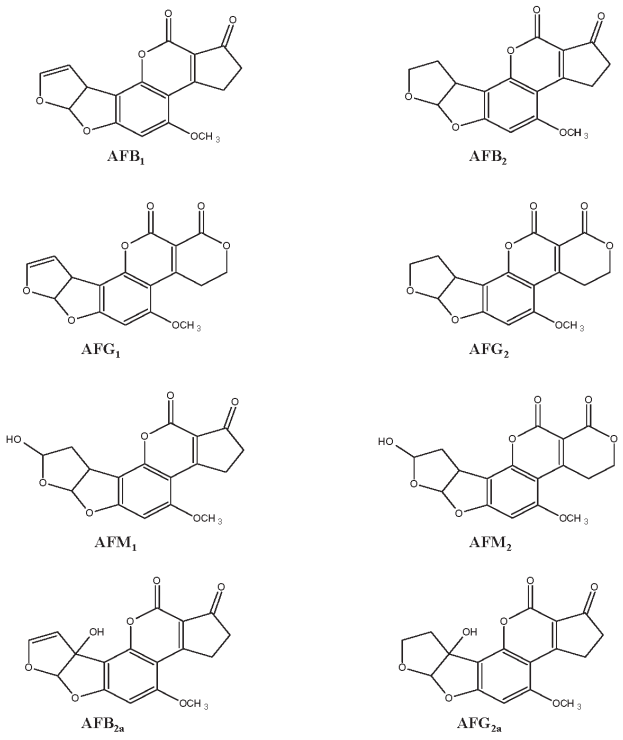

Figure 3. Chemical structure of aflatoxin B_1 (AFB_1) and its structural analogs: aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), aflatoxin M_2 (AFM_2), aflatoxin B_{2a} (AFB_{2a}), and aflatoxin G_{2a} (AFG_{2a}).

AFB_1 (27, 28), incorporating specifically generated polyclonal, monoclonal, or recombinant antibodies.

We previously reviewed the use of antibodies in sensor applications, including SPR-based systems (24, 29, 30), and the principles of such sensors are well established (31–33). Currently there is an extensive range of sensitive analytical methods available for the quantitative determination of warfarin and its metabolites in animal and human biological samples. The common detection formats range from liquid chromatographic (LC) methods to phosphorescence-based measurements (Table 3). However, the majority of these techniques cannot be applied for direct analysis of biological samples without extensive sample pretreatment.

LC-mediated detection of warfarin is predominantly carried out by using a reversed-phase configuration. A number of investigators have used derivatization schemes to enhance the detectability of warfarin by ultraviolet (UV) and fluorescence detection methods and to allow for resolution of warfarin enantiomers. Advances in column chromatography have facilitated the development of various columns incorporating β-cyclodextrin molecules capable of enantiomeric resolution of the warfarin in biological specimens.

The current trend in anticoagulant therapy is towards lower-intensity treatment (52) and more streamlined management of patient medication. Thus, there is a need to develop newer, more sensitive analytical techniques capable of detecting lower concentrations of warfarin in biological fluids, or for the accurate determination of the physiologically free fraction of warfarin in plasma.

Immunoassays facilitate the quantitative measurement (direct or indirect) of a specific interaction between antibody and cognate antigen (the analyte). Typically, immunoassay data yields a sigmoidal shape calibration curve that is best fitted with a 4-parameter logistic model. Consequently, the linear working range of immunoassays can be slightly limited when compared to equivalent chromatographic methods. In addition, notwithstanding rigorous assay validation (53, 54), the nature and batch of the antibody used can be a source of significant variance in assay performance. However, the development of monoclonal and recombinant techniques has successfully addressed these complications. Similarly, recent advances in genetic engineering of antibodies and antibody fragments with enhanced affinities ($K_D \approx 10^{-15}$ M; 55) will allow the determination of even lower analyte concentrations in complex matrixes without the need for extensive sample pretreatment. Furthermore, the sample throughput potential of immunoassays is substantially greater than comparable chromatographic techniques, due ostensibly to the requirement for extensive sample pretreatment and derivatization steps associated with the latter method.

SPR-Based Immunoassays for 7-Hydroxycoumarin

Preliminary work on the development of SPR-based assays for coumarins focused on specific detection of 7-hydroxycoumarin, using a Biacore 1000™ biosensor and in-house generated polyclonal antibodies (26). The simplest biosensor-based immunoassay configurations use a sensor chip surface-coated with specific antibody that facilitates

Table 1. Maximum levels for aflatoxins in a variety of food types established as guidelines by the EU set out in Commission Regulation (EC) No. 466/2001

Product	B_1, ppb	$B_1 + B_2 + G_1 + G_2$, ppb	M_1, ppb
Ground nuts, nuts and dried fruit, for direct human consumption	2	4	—
Ground nuts, nuts and dried fruit, subject to sorting or physical treatment	8	15	—
Cereals for direct human consumption	2	4	—
Milk	—	—	0.05

Table 2. Maximum limits for aflatoxin B_1 in foods established by various countries within the EU (24)

Country	Maximum limit of AFB_1, ppb	Food
Finland	2	All
Germany	2	All
The Netherlands	5	All
Belgium	5	All
Portugal	25	Peanuts
	5	Children's food
	20	Others
Austria	1	All
	2	Cereals, nuts
Switzerland	1	All
	2	Maize, cereals
Spain	5	All
Luxemburg	5	All
Ireland	5	All
Denmark	5	All
Greece	5	All

Table 3. Techniques and detection limits for warfarin analysis

Detection method	LOD/LOQ, ng/mL	Reference
Thin-layer chromatography	(Primarily qualitative detection limits not reported)	Breckenridge and Orme (34)
Radioimmunoassay	pg range	Cook et al. (35)
Phosphorescence	300–4000	Capitán-Vallvey et al. (36)
Capillary electrophoresis	200–20 000	Gareil et al. (37)
LC–total:	LOD about 0.2	Lee and Schwartz (38)
Fluorescence	1–100 (free)	Steyn et al. (39)
	500–10 000 (total)	Steyn et al. (39)
	6.0–450	King et al. (40)
UV	40–800	Fasco et al. (41)
	100–5000	De Vries et al. (42)
	100–1000	Chan and Woo (43)
LC–enantiomeric:		
Fluorescence	25–2500	Boppana et al. (44)
UV	LOD about 20	De Vries and Schmitz-Kummer (45)
	LOD about 20	Takahasi et al. (46)
	12.5–2500	Ring and Bostick (47)
SPR-based immunoassay	0.5–500 (monoclonal)	Fitzpatrick and O'Kennedy (20)
	10–2000 (polyclonal)	
Amperometric biosensor	1500 ng/mL–150 μg/mL	Hutt et al. (48)
Gas chromatography/ Mass spectrometry (GC/MS)	LOD about 2	Bush et al. (49)
	LOD about 10	Kunze et al. (50)
	LOD about 25	Maurer and Arlt (51)

direct binding of complementary antigen, reported as a proportional binding response signal. However, direct covalent attachment of antibodies can denature the antigen-binding region of the antibody. In addition, the random nature of the direct coupling strategy can render many of the antibody molecules suboptimally oriented, with many of the antigen-binding sites inaccessible to analyte molecules. Controlled orientation of homogenous antibody layers has been achieved using protein A/G affinity-capture with highly reproducible surface-binding capacity over 120 binding-regeneration cycles (32). The principle disadvantage of this strategy is the requirement to constantly replenish the test antibody between each individual binding cycle, which can be costly and may exacerbate possible batch-to-batch variation. The coumarin-specific assay configurations used, as illustrated for warfarin (Figure 4), were, in fact, dictated by the low molecular weight nature of the analyte; low molecular weight entities fail to generate appreciable SPR binding responses and therefore, can only be measured indirectly, using an inhibition format.

Thus, initial studies were conducted using a CM5 carboxymethylated dextran hydrogel to which bovine serum albumin (BSA)-conjugated 7-hydroxycoumarin was covalently attached [about 200 response units (RUs) in total] using EDC/NHS chemistry (20). Serum samples were spiked with 7-hydroxycoumarin at predefined concentrations and then preincubated with the polyclonal antibody at a predetermined optimal dilution. The sample preparations were subsequently passed over the immobilized conjugate, concomitantly generating a response that was inversely proportional to the coumarin level in the sample. The binding response was displayed as a real-time sensorgram, with arbitrary RUs. The assay exhibited impressive recovery levels of 98–103% and a detection range of 0.5–80 μg/mL. This compared favorably with other available analytical methods (Table 3). However, a gradual reduction in the surface-binding capacity of the conjugate-immobilized surface became apparent following a series of 60 sequential binding-regeneration cycles (data not shown). This appeared to be due to the loss of conjugate from the surface and suggested that an alternative immobilization strategy was required.

A number of excellent in-house-generated monoclonal antibodies directed against warfarin were available. The advantage of using monoclonal antibodies relates to their uniformity and homogeneity, thereby reducing the likelihood

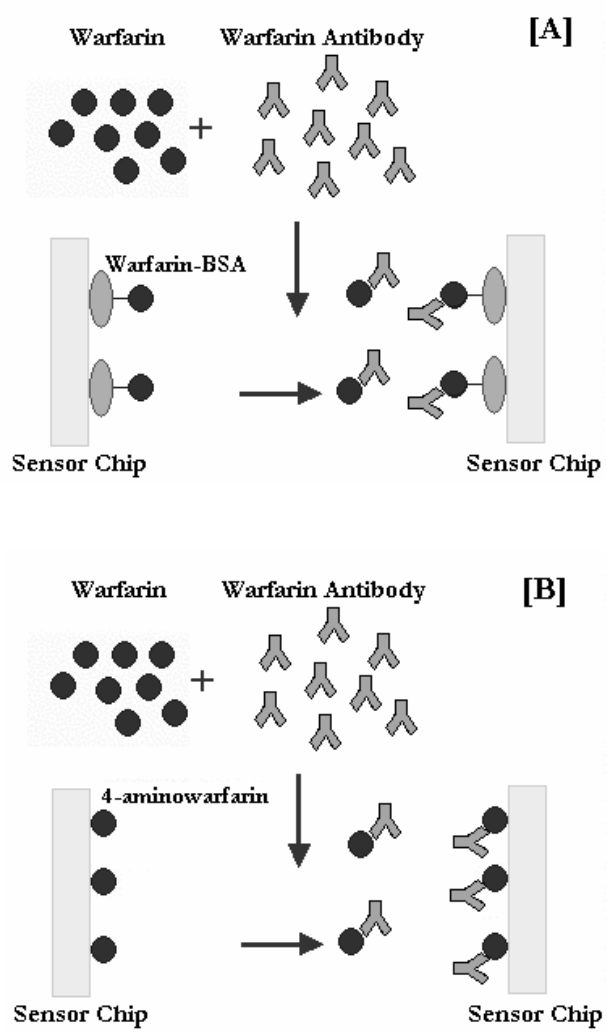

Figure 4. Schematic of the assay formats used for the detection of warfarin. Mixtures of antibody and analyte are injected over the sensor chip surface functionalized with either warfarin-BSA (A) or 4′-aminowarfarin (B). Competition between the immobilized and free warfarin for binding to the antibody takes place. The greater the level of free warfarin, the lower the quantity of antibody available for binding to the chip, resulting in a reduction in response recorded.

of significant batch-to-batch variation. The selected antiwarfarin monoclonal antibody was exquisitely specific and demonstrated no background cross-reactivity towards either the dextran surface or BSA carrier conjugate. However, in order to counteract the apparent deterioration of the conjugated warfarin surface, 4′-aminowarfarin was synthesized and directly coupled to a CM5 chip surface. It was possible to completely dissociate the protein G-purified antiwarfarin monoclonal antibody from this surface using quite mild regeneration pulses (10–25 mM HCl). The surface regeneration studies revealed that the directly immobilized 4′-aminowarfarin sensor chip surface was essentially unlimited with respect to re-usability and demonstrated no decrease in antibody-binding capacity over the course of >80 cycles, with a repeatability of antibody binding of 0.89% relative standard deviation (RSD_r; Figure 5). Such surfaces were in fact used for >1000 cycles (20). It must also be noted that, although equivalent amounts of both BSA-conjugated warfarin and 4′-aminowarfarin were immobilized, the directly coupled 4′-aminowarfarin surface exhibited greater surface binding activity. This can be explained by the divergent molecular weight ratios between the BSA-conjugated warfarin (84 kDa) and the unconjugated 4′-aminowarfarin molecule (318 Da), which resulted in increased epitope loading density on the directly coupled 4′-aminowarfarin surface. Consequently, a 20-fold increase in the monoclonal antibody dilution was sufficient to generate a binding response on the 4′-aminowarfarin surface that was comparable to that achieved for a nominal antibody dilution on the BSA-warfarin conjugated surface.

Development of an Inhibition SPR-Based Immunoassay for Warfarin

The feasibility of the biosensor-based assay for warfarin was initially validated (56) in a standardized control matrix of Hepes-buffered saline (HBS). Dilutions of warfarin were prepared in HBS buffer ranging from 0.03 to 5000 ng/mL. The protein G-purified antiwarfarin monoclonal antibody preparations were mixed with the corresponding dilution of warfarin using the instrument autosampler and allowed to equilibrate for a period of 5–10 min. The samples were then passed in random order, over the derivatized chip surface.

Normalized binding responses (R/Ro) were used to construct a calibration curve of normalized response versus warfarin concentration (ng/mL) and fitted using a 4-parameter logistic model available in BIAevaluation™ 3.1 software (Figure 6). Inter- and intraday precision studies were conducted and, based on the degrees of precision, sensitivity, and recovery recorded, a direct comparison could be made with respect to each immobilization format and particular antibody preparation. The RSD values for the assay were typically of the order of 4%, except as expected towards the asymptotes of the spline curves, where the degree of precision decreased to about 9% at the higher limit of quantitation (LOQ). The variation for the complete curve, including the lower limit of quantitation (LLOQ) 0.97 ng/mL, fell comfortably within the current recommended validation guidelines for immunoassay procedures (53). It was concluded that the monoclonal antibody designated mAb4-2-25, when injected over a directly immobilized 4′-aminowarfarin drug surface, performed with the highest degrees of trueness and precision.

These results suggest that directly immobilized drug molecules should be used whenever target molecules with suitable reactive groups (e.g., the amine moiety on 4′-aminowarfarin) are available for direct coupling to the chip surface. In this instance, such surfaces showed exceptional stability with the immobilized ligand, and low variation was observed over the course of binding studies (<4%).

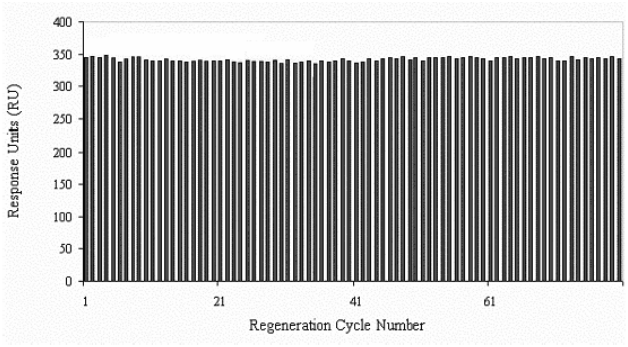

Figure 5. A series of 80 consecutive regeneration cycles of a 4 min binding of monoclonal antibody (*mAb4-2-25*) to the directly immobilized 4′-aminowarfarin drug surface. The surface was regenerated with one 30 s pulse of 25 mM HCl. The binding response demonstrated a repeatability of 0.89% RSD_r over the course of 80 cycles and no decrease in the measured binding response over the course of the regeneration study (i.e., cycle 1 = 345.1; cycle 80 = 343.0). Directly immobilized drug surfaces were essentially unlimited with respect to antibody binding capacity and can be used for >1000 cycles (24).

The performance of the optimized assay was further evaluated in a real sample matrix (urine). The ionic composition of urine can demonstrate considerable variation, depending on an individual's liquid volume intake. Thus, the antibody sample was prepared in HBS buffer of twice the normal ionic strength (i.e., 300 mM NaCl) to compensate for the potential of wide interindividual variability in the salt composition of urine. Warfarin dilutions were prepared at concentrations ranging from 0.97 to 500 ng/mL in the sample urine solution. A working stock of monoclonal antibody, at a predetermined optimal dilution, was mixed with 20 μL warfarin-spiked urine and allowed to equilibrate for 5 min in the autosampler rack before being passed over either the 4′-aminowarfarin- or warfarin-BSA-coated surface in random sequence. The assay sensitivity and precision, as reflected by the RSD and recovery values (Table 4) compared favorably with data obtained by LC analysis.

The limit of detection (LOD) approached 0.97 ng/mL. LC-based analysis proved to be much less sensitive (LOD = 5.0 ng/mL). Approximately 99% of warfarin present in plasma is protein-bound (24). Therefore, initially there should be relatively little (1%) warfarin free in plasma and in urine. Hence, the Biacore assay using antiwarfarin monoclonal antibody is sufficient for the detection of warfarin in the concentration range 0.97–250 ng/mL. If a higher range of detection is required (e.g., monitoring an increase in warfarin dose), antiwarfarin polyclonal antibodies can be used, as the working range of a Biacore assay for the detection of warfarin in urine was demonstrated to be from 10 to 5000 ng/mL (20).

Aflatoxin Sampling and Analysis

Aflatoxin concentrations in grains or nuts can vary from <1 to >12 ppb, with the aflatoxin highly concentrated in individual kernels (25). Therefore, it is essential to select an analytical sample for analysis that is truly representative of the consignment. Polar solvents such as methanol, chloroform, and acetonitrile are used to extract the aflatoxins from food and animal feed. Sample cleanup using solid-phase extraction techniques and immunoaffinity columns may also be required to complement the traditional methods of detection for AFB_1, which include various LC configurations and mass spectrometry (MS; 57, 58). These conventional analytical techniques offer good resolution, a high degree of precision, reproducibility, and sensitivity, with respect to the detection of aflatoxins. However, these methods are further complicated by their absolute requirement for extensive sample cleanup, rendering them more time-consuming and costly. Therefore, attention has focused on immunoanalytical techniques incorporating AFB_1-specific antibodies, which offer increased sensitivity and specificity for AFB_1 detection.

Antibodies (both monoclonal and polyclonal) have proved valuable in the development of traditional radioimmunoassays (RIAs; 59, 60) and enzyme-linked immunosorbent assays (ELISAs; 23, 61, 62) for detection of AFB_1. In addition, a number of novel immunoassay formats that offer improvements in sensitivity and suitability for routine and field analysis have been described (63–66).

Development of SPR-Based Inhibition Assays for the Detection of AFB_1

We have developed a number of immunoassays for aflatoxin using SPR. Initially they involved the use of an

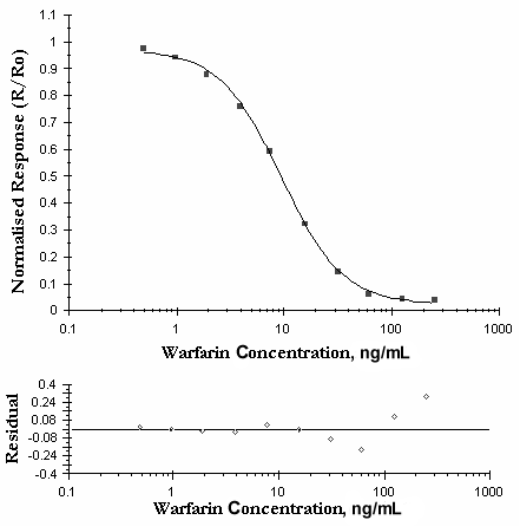

Figure 6. Interday assay calibration curve (4′-aminowarfarin chip) for the detection of warfarin in urine using an antiwarfarin monoclonal antibody. A 4-parameter equation was fitted to the data set using BIAevaluation 3.1. software. Each point on the curve is the mean of 3 replicate measurements analyzed over 3 days. Residual plots for the calibration curves are included (for each calibration curve), which illustrate the goodness of the fit of the applied 4-parameter equations and supports the % recovery findings.

Table 4. Intraday assay repeatability and percentage recoveries for the antiwarfarin monoclonal antibody used for the detection of warfarin in urine

Warfarin concn, ng/mL	Mean back calculated warfarin concn from calibration curve, ng/mL	RSD_r, %	Recovery, %
250.00	214.00	7.17	116.82
125.00	111.00	4.88	112.61
61.20	70.00	0.95	87.43
31.25	33.00	0.41	94.70
15.60	15.63	0.54	99.81
7.80	7.30	0.17	106.85
3.91	4.01	0.35	97.51
1.90	2.10	0.13	90.48
0.97	1.00	0.33	97.00

aflatoxin-BSA conjugate immobilized on the sensor chip in conjunction with polyclonal or monoclonal antibodies (27). The assay format was similar to that previously described for 7-hydroxycoumarin and warfarin. Although this format worked very well, exhibiting a LOD of 3 ng/mL (27), it was determined that performance could be improved by the use of high affinity genetically derived single-chain fragment variable (scFv) antibody fragments (Figure 7), rather than whole antibodies. The scFv antibody fragments directed against AFB_1 were isolated from a phage display library (67), constructed using a method previously described by Krebber et al. (68). The selected AFB_1-specific scFvs were expressed as both monomeric and dimeric entities. Both scFv types were separately evaluated in competitive ELISAs and then transferred to an SPR-based inhibition assay format (67, 69). For reasons previously discussed, in relation to the warfarin-specific biosensor-based immunoassay, it was decided to use an aflatoxin-derivatized chip surface (prepared by XenoSense Ltd., Belfast, Northern Ireland), in preference to the aflatoxin-BSA conjugate-immobilized surface. Free AFB_1 standards, ranging in concentration from 0.375–12 ng/mL for the monomeric scFv and 0.19–24 ng/mL for the dimeric scFv, were prepared in phosphate-buffered saline containing 5% (v/v) methanol.

Intraday variability studies confirmed the monomeric scFv had a range of detection for free AFB_1 spanning 0.375–12 ng/mL, with associated repeatability <0.61% RSD_r. The dimeric scFv had a range of detection of 0.19–24 ng/mL, with repeatability <3.37% RSD_r. The interday assay variation was then estimated by performing the assay over 3 separate days, and indicated a range of detection of free AFB_1 of 0.375–12 ng/mL for the monomeric and 0.19–24 ng/mL for the dimeric scFv, respectively. This was a significant improvement over the sensitivity achieved using polyclonal antibody (27) and underlines the advantage of using affinity-selected recombinant scFvs in conjunction with SPR-based inhibition assay formats. The interday reproducibility with the monomeric scFv ranged between 1.9 and 4.18% RSD_r and between 3 and 11.53% RSD_r for the dimeric scFv, respectively, thereby confirming the precision of the assay. These results demonstrate the applicability of these assays for detecting aflatoxins at very stringent control levels. Overall these assay formats, incorporating novel recombinant antibodies, were highly sensitive, robust, and reproducible.

Conclusions

The results presented in this review describe the successful development and validation of SPR-based immunoassays for the detection of coumarins in biological samples. The advantages of using biosensors such as Biacore include improved sample-to-result time, versatility, and amenability to automation. It is evident that anti-warfarin monoclonal antibodies can successfully quantitate warfarin in plasma and urine samples, with excellent correlation between the SPR

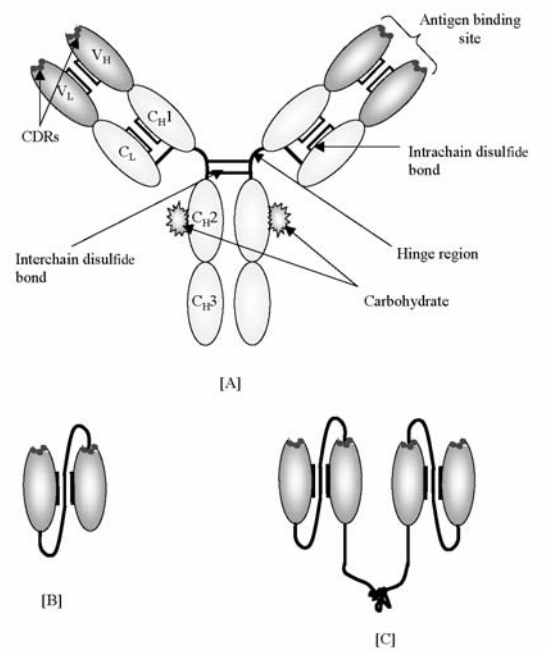

Figure 7. Diagrammatic representation of an immunoglobulin G (IgG) molecule (A) and both monomeric (B) and dimeric (C) scFvs. The IgG molecule is composed of 2 identical light chains and 2 identical heavy chains. The light chains comprise a variable (V_L) and constant (C_L) domain. The heavy chain consists of 1 variable (V_H) and 3 constant (C_H1, C_H2, and C_H3) domains with a hinge region connecting the C_H1 and C_H2 regions. The heavy and light chains are connected via disulfide bonds. Disulfide bonds are also present in the constant and variable regions. The complementarity determining regions (CDRs) within the variable domains confer antigenic specificity and contain considerable amino acid sequence variation. The monomeric scFv consists of a variable heavy and light chain domain stabilized with a serine-glycine linker, and the dimeric scFv comprises 2 scFv fragments dimerized via a double helix.

inhibition assay and LC protocols more commonly used for the detection of warfarin in human plasma (20).

Recent advances in genetic engineering, with the development of phage-displayed libraries and various in vitro affinity maturation techniques, have demonstrated the potential of generating antibodies of extremely high affinities to almost any molecule (30). This approach was successfully applied to the detection of AFB_1, providing the ability to accurately and quantitatively detect subpicogram quantities for food quality and environmental studies.

Acknowledgments

We thank Enterprise Ireland, the Higher Educational Authority Program for Research in Third Level Institution, the North/South Collaboration Fund, Science Foundation Ireland and Fusion for their support.

References

(1) Murray, R.D.H., Mendez, J., & Brown, S.A. (1982) *The Natural Coumarins, Occurrence, Chemistry, and Biochemistry*, Wiley, Chichester, UK
(2) O'Kennedy, R., & Thornes, R.D. (1997) *Coumarins: Biology, Applications and Mode of Action,* Wiley, Chichester, UK
(3) Lacy, A., & O'Kennedy, R. (2004) *Curr. Pharm. Design* **10**, 3797–3811
(4) Katerinopoulos, H.E. (2004) *Curr. Pharm. Design* **10**, 3835–3852
(5) Pochet, L., Frederick, R., & Masereel, B. (2004) *Curr. Pharm. Design* **10**, 3781–3796
(6) Fylaktakidou, K.C., Hadjipavlou-Litina, D.J., Litinas, K.E., & Nicolaides, D.N. (2004) *Curr. Pharm. Design* **10**, 3813–3833
(7) Egan, D., O'Kennedy, R., Moran, E., Cox, D., Prosser, E., & Thornes, R.D. (1990) *Drug Metab. Rev.* **22**, 503–529
(8) Born, S.L., Caudill, D., Fliter, K.L., Lefever, F.R., & Purdon, M.P. (2002) *Drug Metab. Dispos.* **30**, 483–487
(9) Lake, B.G. (1999) *Food Chem. Toxicol.* **37**, 423–453
(10) European Food Safety Authority (2004) *EFSA J.* **104**, 1–36
(11) Thornes, R.D. (1997) in *Coumarins: Biology, Applications and Mode of Action*, R. O'Kennedy & R.D. Thornes (Eds), Wiley, Chichester, UK, pp 255–265
(12) Ebbinghaus, S.W., Mohler, J.L., & Marshall, M.E. (1997) in *Coumarins: Biology, Applications and Mode of Action*, R. O'Kennedy & R.D. Thornes (Eds), Wiley, Chichester, UK, pp 209–239
(13) Egan, D., James, P., Cooke, D., & O'Kennedy, R. (1997) *Cancer Letts.* **118**, 201–211
(14) Cooke, D., & O'Kennedy, R. (1999) *Anal. Biochem*. **274**, 188–194
(15) Link, K.P. (1943–1944) *Harvey Lecture Series*, **39**, 162–216
(16) Campbell, H., Smith, W., Roberts, W., & Link, K. (1948) *J. Biol. Chem,* **138**, 1–20
(17) Cooke, D., Fitzpatrick, B., O'Kennedy, R., McCormack, T., & Egan, D. (1997) in *Coumarins: Biology, Applications and Mode of Action*, R. O'Kennedy & R.D. Thornes (Eds), Wiley, Chichester, UK, pp 303–332
(18) Bertola, J., Mazoyer, E., Bergmann, J., Drouet, L., Simoneau, G., & Mahe, I. (2003) *Thromb. Res.* **109**, 287–291
(19) Linder, M. (2001) *Clin. Chim. Acta* **308**, 9–15
(20) Fitzpatrick, B., & O'Kennedy, R. (2004) *J. Immunol. Methods* **291**, 11–25
(21) Dalezios, J.I., Wogan, G.N., & Weinreb, S.M. (1971) *Science* **171**, 584–585
(22) Ward, C.M., Wilkinson, A.P., Bramham, S., Lee, H.A., Chan, H.W.S., Butcher, G.W., Hutchings, A., & Morgan, M.R.A. (1990) *Mycotoxin Res.* **6**, 73–83
(23) Hussein, H., & Brasel, J. (2001) *Toxicology* **167**, 101–134
(24) Creppy, E. (2002) *Toxicol. Lett*, **127**, 19–28
(25) Gourama, H., & Bullerman, L. (1995) *J. Food Prot.* **58**, 1395–1404
(26) Keating, G.J., Quinn, J., & O' Kennedy, R. (1999) *Anal. Lett*. **32**, 2163–2176
(27) Daly, S.J., Keating, G.J., Dillon, P.P., Manning, B.M., O'Kennedy, R., Lee, H.A., & Morgan, M.A. (2000) *J. Agric. Food Chem.* **48**, 5097–5104
(28) Dunne, L., Daly, S., Baxter A., Haughey, A., & O'Kennedy, R. (2005) *Spectrosc. Lett.* **38**, 229–245
(29) Killard, A.J., Deasy, B., O'Kennedy, R., & Smyth, M.R. (1995) *Trends Anal. Chem*. **14**, 257–267
(30) Leonard, P., Hearty, S., Brennan, J., Dunne, L., Quinn, J., Chakraborty, T., & O'Kennedy, R. (2003) *Enzyme Microb. Technol.* **32**, 3–13
(31) Jönsson, U., Fagerstam, L., Ivarsson, B., Lundh, K., Löfas, S., Persson, B., Roos, H., Rönnberg, L., Sjölander, S., Stenberg, E., Stahlberg, R., Urbaniczky, C., Östlin, H., & Malmqvist, M. (1991) *BioTechniques* **11**, 620–627
(32) Quinn, J., Patel, P., Fitzpatrick, B., Manning, B., Dillon, P., Daly, S., O'Kennedy, R., Alcocer, M., Lee, H., Morgan, M., & Lang, K. (1999) *Biosens. Bioelectron.* **14**, 587–595
(33) Malmborg, A.C., & Borrebaeck, C.A.K. (1995) *J. Immunol. Methods* **183**, 7–13
(34) Breckenbridge, A., & Orme, W. (1973) *Clin. Pharmacol. Ther.* **14**, 955–961
(35) Cook, C., Ballentine, D., Seltzman, T., & Tallent, C. (1979) *J. Pharmacol. Exp. Pharm.* **210**, 391–398
(36) Capitán-Vallvey, L.F., Deheldel, M.K.L., & Avidad, R. (1999) *Arch. Environ. Contam. Toxicol*. **37**, 1–6
(37) Gareil, P., Grammond, J., & Guyon, F. (1993) *J. Chromatogr.* **615**, 317–325
(38) Lee, M., & Schwartz, R. (1981) *Ann. Intern. Med*. **94**, 140–141
(39) Steyn, J., Van Der Merwe, H., & De Kock, M. (1986) *J. Chromatogr.* **378**, 254–260
(40) King, S.-H.P., Joslin, M.A., Raudibaugh, K., Pieniaszek, Jr, H.J., & Benedek, I.H. (1995) *Pharm. Res.* **12**, 1874–1877
(41) Fasco, M., Piper, L., & Kaminsky, L. (1977) *J. Chromatogr.* **131**, 365–373
(42) De Vries, J., Harenberg, J., Walter, E., Zimmerman, R., & Simon, M. (1982) *J. Chromatogr. Biomed. Appl.* **231**, 83–92
(43) Chan, K., & Woo, K. (1988) *Methods Find. Exp. Clin. Pharmacol.* **10**, 699–703
(44) Boppana, V., Schaefer, W., & Cyronak, M. (2002) *J. Biochem. Biophys. Methods* **54**, 315–326
(45) De Vries, J., & Schmitz-Kummer, E. (1993) *J. Chromatogr.* **614**, 315–320
(46) Takahashi, H., Kashima, T., Kimura, S., Muramoto, N., Nakahata, H., Kubo, S., Shimoyama, Y., Kajiwara, M., & Echizen, H. (1997) *J. Chromatogr.* **701**, 71–80

(47) Ring, P.R., & Bostick, J.M. (2000) *J. Pharm. Biomed. Anal*. **22**, 573–581
(48) Hutt, A., Hadley, M., & Tan, S. (1994) *Eur. J. Drug Metab. Pharmacokinet.* **3**, 241–251
(49) Bush, E., Low, L., & Trager, W. (1983) *Biomed. Mass Spectrosc.* **10**, 395–398
(50) Kunze, K., Wienkers, W., Thummel, K., & Trager, W. (1996) *Drug Metab. Dispos.* **24**, 414–421
(51) Maurer, H., & Arlt, J. (1998) *J. Chromatogr.* **714**, 181–195
(52) Lodwick, A. (1999) *Clin. Appl. Thromb Hem.* **5**, 208–215
(53) Findlay, J.W.A., Smith, W.C., Lee, J.W., Nordblom, G.D., Das, I., DeSilva, B.S., Khan, M.N., & Bowsher, R.R. (2000) *J. Pharm. Biomed. Anal.* **21**, 1249–1273
(54) Bruno, J. (1998) *BIAjournal* **2**, 9–11
(55) Boder, E.T., Midelfort, K.S., & Wittrup, K.D. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 10701–10705
(56) Wong, R.L., Mytych, D., Jacobs, S., Bordens, R., & Swanson, S.J. (1997) *J. Immunol. Methods* **209**, 1–15
(57) Hunt, D., Bourdon, A., Wild, P., & Crosby, N. (1978) *J. Sci. Food Agric.* **29**, 234–238
(58) Rosen, R., Rosen, J., & DiProssimo, V. (1984) *J. Agric. Food Chem.* **32**, 276–278
(59) Langone, J., & VanVunakis, H. (1976) *J. Nat. Cancer Inst.* **56**, 591–595
(60) Korde, A., Pandey, U., Banerjee, S., Sarma, H., Hajare, S., Venkatesh, M., Sharma, A., & Pillai, M. (2003) *J. Agric. Food Chem*. **51**, 843–846
(61) Candlish, A., Stimsin, W., & Smith, J. (1985) *Lett. Appl. Microbiol.* **1**, 57–61
(62) Aldao, M., Carpinella, M.C., Corelli, M., & Herrero, G.G. (1995) *Food Agric. Immunol.* **7**, 307–314
(63) Schneider, E., Usleber, E., Martlbauer, E., Dietrich, R., & Terplan, G. (1995) *Food Addit. Contam.* **12**, 387–393
(64) Carlson, M., Bargeron, C., Benson, R., Fraser, A., Phillips, T., Velky, J., Groopman, J., Strickland, P., & Ko, H. (2000) *Biosens. Bioelectron*. **14**, 841–848
(65) Niessen, M., Wichers, J., Lee, H., Alcocer, M., Morgan, M., & van Amerongen, A. (1998) in *Proceedings of European Research Towards Safer and Better Food*, Karlsruhe, Germany, Oct. 18–20
(66) Pal, A., & Dhar, T. (2004) *Anal. Chem*. **76**, 98–104
(67) Daly, S., Dillon, P., Manning, B., Dunne, L., Killard, A., & O'Kennedy, R. (2002) *Food Agric. Immunol.* **14**, 155–274
(68) Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H.R., & Pluckthun, A. (1997) *J. Immunol. Methods* **201**, 35–55
(69) Brennan, J., Dillon, P., & O'Kennedy, R. (2003) *J. Chromatogr. B* **18**, 217–227