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Biosensor assay of sulfadiazine and sulfamethazine residues in pork

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Biosensor-based immunochemical screening assays for the detection of sulfadiazine (SDZ) and sulfamethazine (SMT) in muscle extract from pigs were developed. Samples were extracted with aqueous buffer and then centrifuged. This simple and straightforward preparation allowed up to 40 samples to be processed and analysed in 1 d. The limits of detection for the assays were found to be 5.6 ng g^{-1} for SDZ and 7.4 ng g^{-1} for SMT. These figures were well below the European and US legal limits for sulfonamides (100 ng g^{-1}). The precision (RSD) between runs was $< 8\%$ and the recovery was between 91 and 98%. The validation proved the assays to be accurate and the analysis of routine field samples showed good correlation with an established TLC screening procedure. No false negative or positive results were obtained with blank and spiked samples. The influence of cross-reacting metabolites on immunoassays was demonstrated by testing incurred tissue samples, collected from sulfonamide treated pigs after only a short withdrawal period. The quantitative results obtained by biosensor analysis were a combination of parent sulfonamide plus N^4-acetyl metabolite while the HPLC method used for confirmatory analysis detected only the parent sulfonamide. This gave rise to some false positive results and highlighted the need to use real samples in evaluating any assay thoroughly. False negative results were not obtained.

Introduction

The modern pig industry exists on an industrial scale and economic losses due to non-optimal growth or the outbreak of disease can be substantial. Sulfonamides are commonly incorporated into porcine feedstuffs for therapeutic and prophylactic reasons to minimise these losses.[1]

In Europe and the USA, an acceptable maximum residue limit (MRL) of 0.1 mg kg^{-1} total sulfonamide has been set in the target tissues of muscle, fat, liver and kidney.[2,3] Despite a recommended withdrawal period, sulfonamide residues are commonly detected in edible pig tissues.[4–6] This may be due to non-adherence of the withdrawal period prior to slaughter, cross-contamination between medicated and non-medicated feeds in mills or contact between treated and non-treated pigs.[7]

The determination of sulfonamide residues in pork generally requires the use of lengthy extraction procedures prior to analysis.[8] To overcome this, some workers employ the use of fluid matrices (serum, urine, bile) as predictors of sulfonamide levels in tissue.[9–11] The use of fluid matrices as predictors removes the need for time consuming extraction procedures, but, the correlation between sulfonamide concentrations in the chosen fluid and the chosen tissue requires extensive experimental proof. For most analysts, the setting of the MRL value of 0.1 mg kg^{-1} in edible tissue identifies muscle, liver or kidney as a preferred sample matrix.

Recently, immunobiosensor assays for the detection of sulfonamide residues have been described.[12–15] Sternesjö *et al.*[12,13] described the detection of sulfamethazine in milk and while Elliott *et al.*[14] and Crooks *et al.*[15] outlined the use of bile as a predictor for sulfonamide concentrations in tissue. All groups described the benefits of the technology in offering rapid and reliable analysis.

A fast analytical procedure, however, is of little real benefit if the sample preparation time is long. The aim of this study was to develop an immunobiosensor screening assay for sulfadiazine and sulfamethazine in pork muscle. Sample preparation was kept to a minimum to allow for high sample throughput with minimum effect on reliability, sensitivity or precision. The methods were validated and the results compared with those found using an established HPLC procedure.[16]

Experimental

Equipment

The assays were developed on a commercial optical biosensor, BIACORE Q (Biacore, Uppsala, Sweden). Instrument operation and data handling were performed with BIACORE Q Control Software. A Heidolph DIAX 900 homogenizer equipped with homogenizer tool 18G (KEBO Lab, Sweden), was used in the tissue preparation. Centrifugation was carried out in a Heraeus Biofuge pico instrument (Heraeus AB, Vasby, Sweden).

Chemicals and reagents

HBS-EP (0.01 M HEPES, pH 7.4, 0.15 M sodium chloride, 3 mM EDTA, 0.005% v/v polysorbate 20) used as instrument running buffer, Sensor Chip CM5, certified grade, and an Amine Coupling Kit [containing *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and 1 M ethanolamine, pH 8.5] were obtained from Biacore. Sulfadiazine (SDZ) and sulfamethazine (SMT) reference standards, minimum purity 99%, were purchased from Sigma (Poole, Dorset, UK). Water soluble amine

derivatives of SDZ and SMT, used for immobilization on the sensor chip, were obtained from Biacore. An SDZ rabbit polyclonal and SMT sheep polyclonal antibodies were produced at the Veterinary Sciences Division of DARDNI (Belfast, UK).[15] CM-dextran sodium salt was purchased from Fluka (Gillingham, Dorset, UK). All other chemicals were obtained from Sigma. Extraction buffer was prepared from 20 mM sodium phosphate buffer, pH 7.2, containing 9.0 g l^{-1} of sodium chloride and 0.3 l^{-1} of CM-dextran sodium salt.

Antibody purification and cross-reactivity

The γ-globulin fractions of both antibodies were precipitated from the plasma of the respective animals using saturated ammonium sulfate, then dialysed extensively against 0.15 M sodium chloride (saline) containing 15 mM sodium azide before sterile filtration through a 0.22 μm filter (Millipore, Bedford, MA, USA). The antibody solutions were then diluted in HBS-EP to the assay concentrations. A cross-reactivity profile of the SDZ antibody was determined by construction of calibration curves for SDZ, the N^4-acetyl-SDZ metabolite and for other sulfonamides under assay conditions. The midpoint relative response value of the SDZ calibration curve was used to calculate the midpoint concentration (IC_{50}) for SDZ and then for the cross-reacting compounds. Cross-reactivity was then calculated as a percentage relative to SDZ. A similar profile was constructed for the SMT antibody.

Immobilization

Immobilization of the amine derivatives of SDZ and SMT was carried out according to the method of Johnsson *et al.*[17] Briefly, a Sensor Chip CM5, certified grade, was activated with a 7 min pulse of 0.2 M EDC, 50 mM NHS solution at a flow rate of 10 μl min^{-1}. The sulfonamide derivative was dissolved in 50 mM borate buffer, pH 8.5, to a final concentration of 0.5 mM before coupling to the activated surface for 7 min at a flow rate of 10 μl min^{-1}. The surface was then deactivated with a 3 min pulse of 1 M ethanolamine, pH 8.5, at a flow rate of 10 μl min^{-1}.

Production of incurred positive material

The treatment of pigs with sulfamethazine or sulfadiazine medicated meal was performed under normal farm conditions. Drinking water was available *ad libitum*.

Landrace pigs (n = 10) of finishing weight (approximately 30 kg) were fed sulfamethazine in their ration at a therapeutic rate of 100 mg kg^{-1} d^{-1} for 14 d. After 84 and 102 h withdrawal, five animals were removed for slaughter.

Another group (n = 12) were fed sulfadiazine in their ration at a rate of 100 mg kg^{-1} d^{-1} for 14 d. After 6 and 24 h withdrawal from medication, six animals were removed for slaughter.

At *post-mortem*, tissue samples from the gluteal muscle mass of each animal were removed and stored at −20 °C. All samples were analysed by biosensor and by HPLC within 3 weeks.

In addition to the positive incurred tissues, 70 randomly selected, 'real' field samples were obtained from the Swedish National Food Administration for analysis.

Sample preparation

Fat-free pork muscle (1 g) was weighed into 50 ml centrifuge tubes. Extraction buffer (9.0 ml) was added and then the muscle was homogenized for 15 s. The homogenizing tool was cleaned in between each sample by running for 5 s in de-ionized water and then the tool was wiped off with a paper-towel. An aliquot of the muscle extract was centrifuged at 13 000 rpm for 10 min and the supernatant was used in the analysis.

Calibration curve

The calibration curve was constructed from four calibration points run in duplicate using the four-parameter fit algorithm in the software of BIACORE Q. The calibration points were prepared in negative muscle extract according to the following procedure. Stock standard solutions with a concentration of 270 μg ml^{-1} were prepared by dissolving 29.13 and 29.37 mg of the sodium salt of SMT and SDZ, respectively, in 100 ml of de-ionized water. Each stock standard solution was then diluted 1 to 100 in water to an intermediate solution with a concentration of 2.7 μg ml^{-1}. This solution was then diluted 1 to 10 in negative muscle extract to a second intermediate solution, 270 ng ml^{-1}, which in turn was diluted 1 to 10 with negative muscle extract to the first calibration point, 27 ng ml^{-1}. Solutions for the rest of the calibration points, 9, 3 and 1 ng ml^{-1}, were prepared by a serial dilution 1 to 3 with negative muscle extract.

Analysis cycle

The sample/calibrant solution (40 μl) was mixed with an equal volume of the relevant antibody solution inside the instrument. The mixture was then injected for 1 min over the sensor surface at a flow rate of 40 μl min^{-1}. The response from antibody binding to the sulfonamide derivative immobilised on the surface was measured, after which the surface was regenerated using a 1 min pulse of 20% acetonitrile in 0.2 M sodium hydroxide solution.

Results and discussion

The screening assays for SMT and SDZ were designed as inhibition assays. A fixed concentration of antibody is mixed with the sample and then injected over a sensor surface immobilized with an analyte derivative. Excess antibody will bind to the surface and give a response, relative to the baseline (Fig. 1). The response is inversely related to the amount of analyte present in the sample.

In the initial studies, interference from the sample matrix was observed, but, the effect was very constant from sample to sample. This problem was overcome by preparing the calibration solutions in negative muscle extract. The calibration curve was constructed from four calibration points run in duplicate using a four-parameter equation (Fig. 2). Responses from unknown samples were evaluated against the calibration curve to obtain the concentration in ng ml^{-1}. This value was then multiplied with a dilution factor of 10 to obtain results in ng g^{-1}

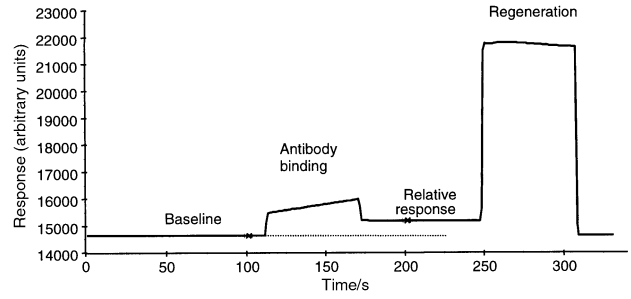

Fig. 1 Sensogram from one analysis cycle showing the binding of antibody and the regeneration of the sensor surface.

muscle. The concentration interval of the calibration curve was chosen to cover the maximum residue limit of 100 ng g^{-1} sulfonamide in edible tissue.

The detection limits of the assays were determined from the value of three standard deviations below the mean response for 10 known negative field samples and were found to be 7.4 and 5.6 ng g^{-1} for SMT and SDZ, respectively. These values are well below the EU and US MRL value of 100 ng g^{-1}. The specificities for the two assays were determined under assay conditions, *i.e.*, in muscle extract, for the respective main metabolites and a number of other sulfonamides (Table 1). Both assays show a moderate cross-reactivity towards sulfamerazine and in the case of the SDZ assay also towards sulfathiazole and sulfamethoxypyridazine. The N^4-acetyl metabolites yield a high cross reactivity in their respective assays.

The precision was determined by spiking known negative samples with sulfonamide at 0.5, 1 and 1.5 times the MRL concentration. The sulfonamide solution was left to soak into the muscle for 0.5 h before adding the extraction buffer. Precision within a run was determined from five different samples and was found to be 3.8% (50 ng g^{-1}), 3.1% (100 ng g^{-1}) and 1.5% (150 ng g^{-1}) for SMT and 5.1% (50 ng g^{-1}), 1.8% (100 ng g^{-1}) and 2.5% (150 ng g^{-1}) for SDZ. Precision between runs was determined from four runs with 12 different samples (three analysed in each run) and was found to be 3.3% (50 ng g^{-1}), 6.7% (100 ng g^{-1}) and 7.4% (150 ng g^{-1}) for SMT and 8.4% (50 ng g^{-1}), 3.5% (100 ng g^{-1}) and 3.8% (150 ng g^{-1}) for SDZ.

Recovery was calculated from all runs made with spiked negative samples. The recoveries were found to be 91% (50 ng g^{-1}), 94% (100 ng g^{-1}) and 92% (150 ng g^{-1}) for SMT and 92% (50 ng g^{-1}), 98% (100 ng g^{-1}) and 97% (150 ng g^{-1}) for SDZ.

The robustness of the biosensor methods was checked by monitoring the relative response of the same sample extract over 48 repeated analysis cycles (Fig. 3). Minimal drift of the response was observed. Moreover, the sensor chips were re-used up to four times during one month, running approximately 200 sample analysis cycles without any decrease in response.

The results obtained from the analysis of the SMT and SDZ incurred material by biosensor and by HPLC are given in Table 2. The MRL value of 100 ng g^{-1} was used to identify positives. For SMT, based on the confirmatory HPLC result, one animal fell below the MRL value after 84 h withdrawal. After 102 h withdrawal, all five animals had SMT concentrations below the MRL value. The biosensor assay results showed all these animals to contain violative SMT levels (above the MRL). It should be observed that of these six animals, five contained SMT residues above the limit of detection (LOD) of the HPLC method (50 ng g^{-1}) but below the MRL value. For SDZ, all 10 animals screened as positive (above the MRL) using the biosensor assay. The HPLC method confirmed six of these as containing violative levels (above the MRL), *i.e.*, the six animals slaughtered after only 6 h withdrawal. After 24 h withdrawal sulfonamide levels, determined by the HPLC method, had fallen below the LOD. There were no false negative results obtained during the incurred sample studies.

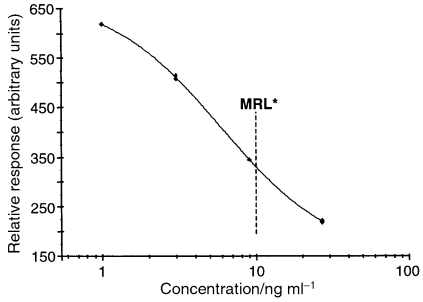

Fig. 2 Calibration curve for sulfamethazine (10 ng ml^{-1} corresponding to 100 ng g^{-1} pork muscle).

Table 1 Cross-reactivity profiles for both antibodies determined under assay conditions

	Cross-reactivity (%)	
Substance	Anti-SMT	Anti-SDZ
Sulfamethazine (SMT)	100	< 1
N^4-Acetyl-SMT	160	< 1
Sulfadiazine (SDZ)	< 1	100
N^4-Acetyl-SDZ	< 1	230
Sulfamerazine	17	11
Sulfathiazole	< 1	8.7
Sulfamethoxypyridazine	< 1	7.8
Sulfaquinoxaline	< 1	< 1
Sulfadoxine	< 1	< 1
Sulfapyridine	< 1	< 1

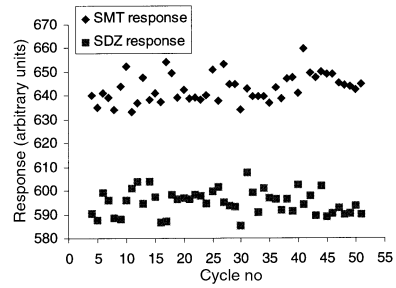

Fig. 3 Response from repeated analyses cycles. Note: the first three cycles are used for system conditioning.

Table 2 Results for the sulfonamide incurred samples

Sulfamethazine			Sulfadiazine		
Withdrawal/h	BIACORE muscle/ng g^{-1}	HPLC muscle/ng g^{-1}	Withdrawal/h	BIACORE muscle/ng g^{-1}	HPLC muscle/ng g^{-1}
84	> 270	120	6	> 270	390
	> 270	160		> 270	710
	> 270	140		> 270	470
	> 270	130		> 270	470
	170	70		> 270	370
				> 270	410
102	139	60	24	260	< 50
	201	80		111	< 50
	109	< 50		136	< 50
	264	80		151	< 50
	132	60		105	< 50
				> 270	< 50

Quantitatively, the biosensor method always gave higher concentrations of each sulfonamide than the relative HPLC result. The most likely explanation for this is the cross-reactivity profile of the antibodies used in the biosensor assays. Both antibodies displayed high cross-reactivity with the respective N^4-acetyl metabolites (Table 1). A significant amount of each N^4-acetyl sulfonamide must be present in the muscle samples at the periods of withdrawal.[18] The biosensor assay will detect these metabolites in addition to the parent sulfonamide to give a combined concentration. The HPLC method only detects the parent sulfonamide. These data highlight the importance of using real incurred samples in evaluating any assay as opposed to the more widely used method of spiking blank samples. A number of real 'field' samples (70) were also analysed using the biosensor assays and a TLC procedure used for routine screening.[19] All samples were found to be negative by both methods. These data indicate that when applying the assays in routine control programmes, problems with false positives are unlikely to be encountered.

The applied sample preparation procedure for the biosensor assays is simple and straightforward. In less than 3 h, 40 samples can be processed, allowing results to be obtained in a single working day. An additional advantageous feature of this development is that as the sample preparation is identical for both assays, the muscle extract from one sample can be used for concentration measurements of both analytes.

In summary, two robust and reliable assays have been developed for the most commonly used sulfonamides. These tests are capable of detecting residue concentrations at the EU and US legal limits in a manner which lends itself to satisfy the demands of routine testing laboratories.

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