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Screening method for the detection of a range of nitrofurans in avian eyes by optical biosensor

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ABSTRACT

An immunobiosensor assay was developed for the multi-residue screening of a range of nitrofuran compounds in avian eyes. A polyclonal antibody which binds at least 5 of the major parent nitrofurans was raised in a rabbit after inoculation with a nitrofuran mimic-protein conjugate. Sample homogenates were extracted into 0.1 M hydrochloric acid and subjected to clean-up by solid phase extraction and micro-centrifugation prior to biosensor analysis. Validation data obtained from the analysis of 21 fortified samples has shown that the method has a detection capability (CCβ) of less than 1 ng eye^{-1} for nitrofurazone (NFZ). In addition, cross-reactivity data and the analysis of a smaller number of fortified samples have shown that the method will also detect a range of other major parent nitrofurans including furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFA) and nifursol (NFS). Intra-assay variation (n = 10) was calculated at 12.9% and 10.1% at concentrations of 1 ng eye^{-1} and 2 ng eye^{-1} NFZ respectively. Inter-assay variation (n = 3) was determined to be 10.8% and 4.7% at the same NFZ concentrations respectively. The cross-reactivity profile and validation data for the detection of these nitrofurans are presented together with the results obtained following the analysis of a small number of incurred samples using the developed method.

1. Introduction

The nitrofuran group of compounds are antibacterial agents which have been used in animal feed as growth promoters and as both prophylactic and therapeutic treatments for gastrointestinal infections caused by *Escherichia coli* and *Salmonella* spp. in livestock including cattle, pigs and poultry. They have also been used for the treatment of bacterial and protozoan infections in the aquaculture and apiculture industries [1,2]. They are still being used in both veterinary and human medicine, variously, as topical applications for skin burns and infections (NFZ), and for the treatments of cholera (FZD) and urinary infections (NFA) [3–5].

The use of FZD and, subsequently, all nitrofuran antibiotics in animals producing food for human consumption has been banned within the European Union (EU) since 1993 [6] and 1995 [7] respectively, as evidence was produced to show that they have potential risks for causing both mutagenic and carcinogenic effects in humans [8,9]. From 2002 to date, import restrictions have been imposed on a number of countries following the detection of nitrofuran residues in food exports bound for the EU market [10,11]. Since 2003 there have been more than 450 Rapid Alerts issued by EU Member States in relation to the finding of nitrofuran residues in food originating from more than 30 countries.

The nitrofuran parent compounds (Fig. 1), containing a characteristic 5-nitrofuran ring structure, are unstable *in vivo* and are rapidly metabolised following ingestion into their respective tissue bound side-chain metabolites [12,13]. This inherent instability in animal tissues has made the monitoring of nitrofuran abuse through the targeting of the parent compounds problematic. Their metabolites, however, have been shown to persist in animal tissues for an extended period [14–16] and it has been suggested that the metabolic mechanism for this occurs via cleavage of the nitrofuran ring leaving the specific tail group covalently bound to tissue proteins. These can then be subsequently released by the action of mild acids, at concentrations similar to those found in the human stomach. The residues so released are, in turn, bioavailable to a second species eating contaminated meat [17]. The prolonged persistence of residues in tissues makes the nitrofuran metabolites the most suitable marker residues for regulatory monitoring programmes.

The most commonly detected nitrofuran metabolite has been the controversial marker residue semicarbazide (SEM), which is the metabolite of NFZ. Any confirmed detection of this metabolite following analysis was originally taken as proof of the illegal use of the parent compound. The reliability of this method of testing for nitrofuran abuse was later questioned, however, as SEM contamination of foodstuffs was shown to have been caused by exposure to

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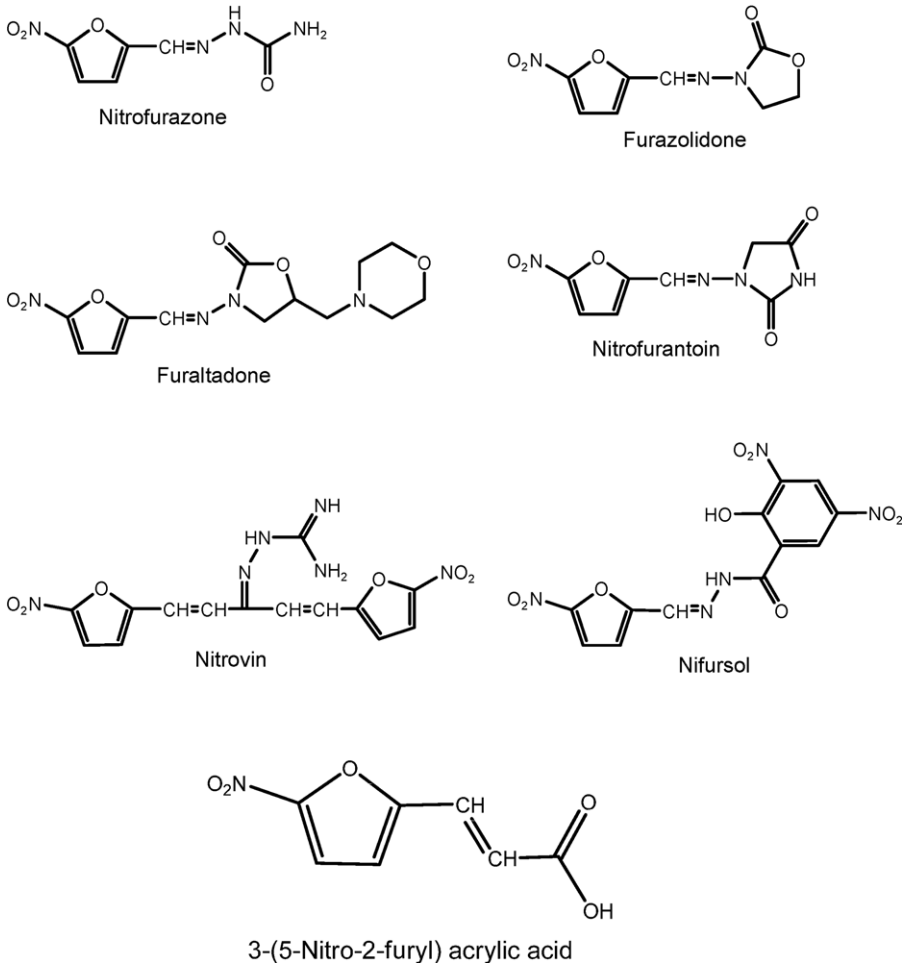

Fig. 1. Molecular structures of six nitrofuran drugs and the nitrofuran mimic.

azodicarbonamide, which is used both as a flour treatment agent in bread baking [18,19] and as a blowing agent in the manufacture of lid gaskets used in the production of, for example, jars of baby food [20–22].

It has been known for some time that the retinal pigment melanin can bind drug residues [23,24] and many EU laboratories use this matrix routinely as part of their statutory monitoring programmes for members of the β-agonist family of drugs. Previous work has shown that, unlike in tissues, parent nitrofurans may also persist in the retina of the eye for an extended period of time through a similar mechanism [25,26]. It was therefore determined that it would be highly desirable to develop a quick and reliable screening test for the parent compounds in avian eyes.

The minimum required performance limit (MRPL) currently set by the European Commission for the nitrofuran metabolites in all edible tissues of animal origin is 1 $\mu g\,kg^{-1}$ [27]. While no MRPLs have been established for the parent compounds, there is however a policy of "zero tolerance" towards the abuse of these banned compounds, which are listed in Table 2 of Commission Regulation (EU) No. 37/2010 [28]. Any analytical test aimed at both their detection and the provision of conclusive evidence of treatment, leaving no ambiguity as to the possibility of contamination by any means other than exposure to the parent nitrofurans themselves, should therefore be as sensitive as possible.

Methods of analysis have been reported for a large variety of matrices and until now have been based around such physicochemical procedures as HPLC and LC–MS/MS [29–32] usually following derivatisation of the nitrofuran metabolite using *ortho*-nitrobenzaldehyde (*o*-NBA) [33] and with limits of detection generally in the low parts per billion (typically <0.5 $\mu g\,kg^{-1}$). These procedures, while being reliable and sensitive, do have the disadvantage of being expensive and time-consuming when applied to the analysis of a large number of samples. In contrast, biosensor technology [34,35] offers the advantages of allowing rapid and simple sample preparation, as well as being reliable and cost effective and has been established as a screening tool for drug residue testing for many years. Indeed it has now been incorporated into both developmental work and the statutory national testing schemes of many laboratories worldwide [36–39].

This current study describes a novel approach to nitrofuran screening by analysing whole avian eyes for detection of the parent nitrofuran compounds utilising biosensor technology. A small experimental trial was also undertaken to ascertain if the developed procedure could be successfully applied to naturally incurred material. Biosensor results for parent NFZ in incurred avian eyes, along with the LC–MS/MS confirmatory results of the metabolite concentration in the corresponding liver samples, are presented.

2. Materials and methods

2.1. Instrumentation

An optical biosensor (BIACORE®Q) was obtained from GE Healthcare (Uppsala, Sweden). Instrument operation and data handling were performed using BIACORE®Q Control Software (Version 3.0.1).

Table 1
Cross-reactivity profile and IC_{50} values of the rabbit polyclonal antibody.

Analyte	Buffer % cross reactivity	Buffer IC_{50} (ng mL^{-1})	Avian eye % cross reactivity	Avian eye IC_{50} (ng eye^{-1})
Nitrofurazone	100.0	4.23	100.0	2.61
Furaltadone	627.6	0.94	302.5	0.32
Furazolidone	584.3	0.98	279.2	0.40
Nitrofurantoin	367.7	0.70	589.3	0.42
Nifursol	143.0	1.89	85.4	3.73
Nitrovin	0.0	N/A	0.0	N/A

2.2. Reagents and chemicals

CM5 sensor chips (certified grade) and an amine coupling kit were obtained from GE Healthcare (Little Chalfont, England). Reference standards for NFZ, FTD, FZD, NFS, NFA and nitrovin (NTV) along with N-ethyl-N′-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS), jeffamine (2,2′-(ethylenedioxy) bis-ethylamine), 2-(N-morpholino) ethanesulfonic acid (MES) and dimethylformamide (DMF) were supplied by Sigma–Aldrich Chemical Company Ltd. (Poole, Dorset, U.K.). The nitrofuran mimic 3-(5-nitro-2-furyl) acrylic acid was obtained from TCI Europe NV (Zwijndrecht, Belgium). Strata X solid phase extraction columns were obtained from Phenomenex U.K. Ltd. (Macclesfield, England, U.K.) and Anotop 10 filter units were supplied by Whatman International Ltd. (Maidstone, England, U.K.).

Biosensor buffer was prepared in-house and contained HEPES pH 7.4 (0.01 M), sodium chloride (0.15 M), EDTA (3 mM) and Tween 20 (10%). The solution was degassed and filtered prior to use.

All other chemicals were HPLC grade and were supplied by BDH (Poole, Dorset, U.K.).

2.3. Biosensor assay development

2.3.1. Immobilisation of the nitrofuran mimic

A CM5 sensor chip was immobilised with the nitrofuran mimic (Fig. 1). Briefly, the carboxymethyl dextran surface was activated by contact with a 1/1 mixture of 0.2 M EDC/0.05 M NHS (50 μL) for 20 min. Reactants were removed and 1 M jeffamine (50 μL) was added and allowed to remain in contact with the chip surface for 1 h. Any unreacted sites were deactivated by incubating with 1 M ethanolamine pH 8.5 (50 μL) for 20 min. The nitrofuran mimic (5 mg) was dissolved in dimethylformamide (250 μL) and combined with a solution of EDC (5 mg) and NHS (3 mg) in sodium acetate (10 mM, pH 4.5, 250 μL) and immediately applied to the prepared amine surface (50 μL). This was allowed to react overnight at room temperature. The chip surface was then washed with deionised water and dried with nitrogen gas. The immobilised sensor chip was stored refrigerated in the presence of a desiccant when not in use.

2.3.2. Immunogen and antibody production

A nitrofuran mimic – ovalbumin immunogen was prepared and anti-nitrofuran polyclonal antiserum produced in a rabbit. Conjugation was via the carbodiimide reaction [40]. Briefly, the nitrofuran mimic (16 mg) was dissolved in DMF (1.6 mL). EDC (30 mg) and NHS (15 mg) were dissolved in MES pH 5.0 (300 μL) and slowly added, while stirring for 10 min, to activate the drug solution. Ovalbumin (16 mg) was dissolved in MES pH 5.0 (2 mL). The activated hapten solution (600 μL) was slowly added to the ovalbumin solution and reacted for 4 h at room temperature with constant stirring. The immunogen was purified overnight by dialysis against saline solution.

The immunisation protocol was as described in a previously published paper [41].

2.3.3. Sample preparation and extraction procedure for biosensor analysis

Avian eyes were trimmed of excess muscle and fat and stored frozen prior to analysis. While still frozen, all negative and sample eyes were cut in half. Both halves of the eyeball including the aqueous humour, vitreous humour and lens were transferred to glass universal bottles (both eyes from each animal were included per analysis). Calibrants (containing 0 and 40 ng mL^{-1} NFZ) were prepared by adding working standards (50 μL) to two known negative eyeballs to produce calibration points. Control samples were prepared in the same way using the appropriate working controls. All calibrants, controls and samples were allowed to stand for 10 min at room temperature and were then treated identically. Hydrochloric acid (0.1 M, 10 mL) was added to each universal and then vortexed vigorously for 10 s followed by mixing end over end for 1 h. All universals were centrifuged at 3700 rpm for 10 min at 10 °C. Solid phase extraction columns (Strata-X) were activated and equilibrated by the addition of methanol (3 mL) and deionised water (3 mL) respectively. Extracts (2 × 4 mL aliquots) were applied to their corresponding columns and allowed to pass through under gravity. Columns were washed with methanol:deionised water (10/90, v/v, 3 mL) prior to elution with ethyl acetate (3 mL). Eluents were collected in glass test tubes and evaporated to dryness using a TurboVap® LV sample concentrator at 50 °C under a stream of nitrogen gas. The dried residue was reconstituted immediately in biosensor buffer (300 μL), vortexed vigorously for 1 min, transferred to micro-centrifuge tubes and centrifuged at 13,000 rpm for 10 min. The resulting extract was passed through Anotop 10 filter units (0.20 μm, 10 mm) prior to analysis.

2.3.4. Production of nitrofuran incurred material

Seven chickens, known to be free from exposure to nitrofurans, were purchased from a local supplier and randomly divided into two groups; a control group of two birds and a medicated group of five birds. The control group received non-medicated feed throughout the trial. The medicated group received NFZ medicated feed at a target therapeutic dose of 300 mg kg^{-1} bodyweight for a period of seven days. At the end of this time both groups were immediately sacrificed and experimental samples were taken. These were stored frozen until analysed.

3. Results and discussion

3.1. Antibody characterisation

The rabbit polyclonal antibody produced was assessed for cross-reactivity, in assay buffer and matrix, with a range of the major parent nitrofuran drugs. Significant cross-reactivity was found with five of the six parent compounds tested (Table 1).

3.2. Sensor chip preparation

Immobilisation of the nitrofuran mimic on the biosensor chip surface proved critical to the development of the assay. After previous attempts utilising ethylenediamine to create the amine surface had proven unsuccessful, production of a stable sensor chip surface

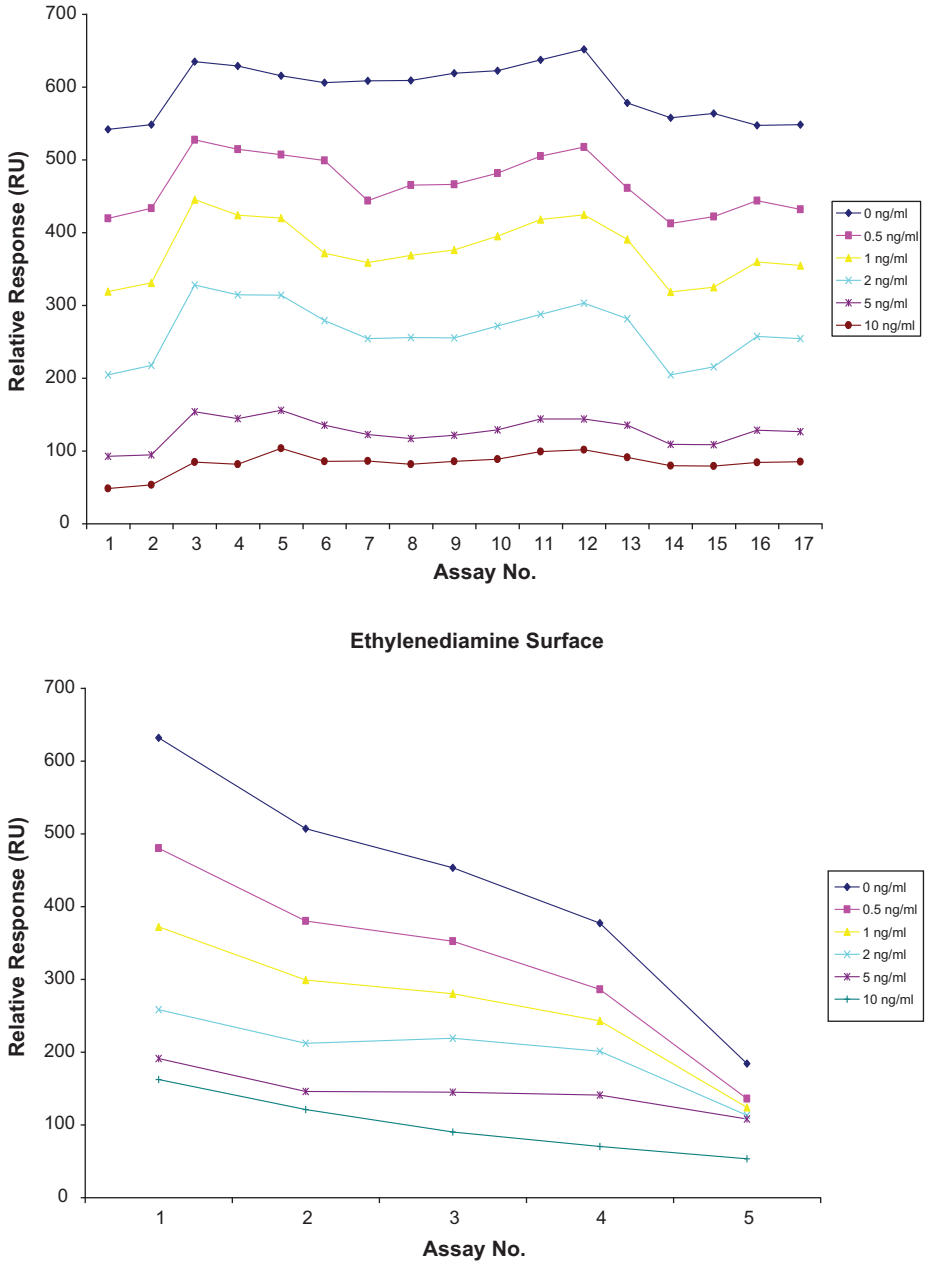

Fig. 2. Stability graphs charting sensor chip performance over time showing buffer calibrant (ng/mL) responses (single data points represented).

was achieved through immobilisation of the nitrofuran mimic via an amine surface prepared using jeffamine (Fig. 2).

3.3. Sample preparation

It was determined during the course of this study that the amount of retina that can be recovered from an avian eye was minimal, ranging from 0.10 g to 0.14 g ($n = 20$). When attempting to recover the retina, an amount of retinal material is inevitably lost when the lens and the aqueous and vitreous humours are removed from the whole eyeball. Furthermore the process of attempting to remove the retina from avian eyes was found to be extremely time consuming and labour intensive. For these reasons it was decided to proceed with the study based on an extraction from the whole eye. These were simply cut into two halves while frozen and then homogenised thus ensuring no loss of retina during sample preparation. As a result of the adoption of this procedure, all results obtained were therefore expressed in terms of ng eye^{-1} (or μg eye^{-1}) rather than ng g^{-1} (or μg kg^{-1}).

By employing the current procedure, 24 samples could be extracted and analysed within 12 h.

3.4. Biosensor assay validation

The developed immunobiosensor assay was validated in accordance with Commission Decision 2002/657/EC [42]. Nitrofuran free avian eye samples were extracted and analysed, as has been previously detailed, both blank ($n = 21$) and fortified with NFZ at a concentration of 1.0 ng eye^{-1} ($n = 21$). Seven blank and seven fortified samples were extracted and analysed in a single run and this was repeated on three successive days to determine the detection capability (CCβ) of the method for NFZ. To achieve the required sensitivity it was necessary to use two eyeballs as a test portion. To ensure homogeneity of the sample pool a single eyeball

from one animal was randomly paired with a single eyeball from a second animal. This combined pair was analysed as a negative sample. The remaining eyeballs from each of these animals were then paired together and analysed as the corresponding fortified sample. Results obtained showed that the method had a CCβ of <1.0 ng eye^{-1} for NFZ in avian eyes.

To demonstrate the ability of the test to detect residues of the other nitrofuran parent drugs, a reduced number of samples ($n = 5$) were fortified at various concentrations and evaluated against 0 and 1.0 ng eye^{-1} NFZ extracted calibration standards. From the results obtained it was shown that the method will also detect the parent compounds FZD, FTD and NFA at <1.0 ng eye^{-1} and NFS at <5.0 ng eye^{-1}. The assay was further validated by determining the inter-assay ($n = 3$) and intra-assay ($n = 10$) coefficients of variation following fortification of whole avian eyeballs with NFZ. Intra-assay variations were 12.9% and 10.1% at concentrations of 1 ng eye^{-1} and 2 ng eye^{-1} NFZ respectively, while inter-assay variations were 10.8% and 4.7% at the same NFZ concentrations respectively.

The assay is qualitative as it cannot distinguish between the various nitrofurans but as a result of the specificity of the antibody, it does indicate that one or more is present. Any nitrofuran detected following biosensor analysis of samples will represent the total concentration of NFZ (immunoreactivity) equivalents in the sample.

3.5. Incurred samples

The incurred avian eye samples were analysed by the developed biosensor based procedure and the corresponding liver samples by LC–MS/MS [43]. Of the five animals that were fed medicated meal during the trial, all gave screening results significantly above 1 ng eye^{-1}. All corresponding liver samples gave LC–MS/MS confirmatory results of >5 ng g^{-1} for the semicarbazide metabolite. The unmedicated birds tested compliant using both methods.

4. Conclusions

A stable sensor chip surface, used in combination with a polyclonal antibody, has resulted in the successful development of a simple, reliable and novel screening procedure based on optical biosensor technology, which has been shown to be capable of detecting low concentrations of a range of parent nitrofurans in avian eyes.

Both eyes from sample animals were used in an attempt to maximise the sensitivity of the test and it is perfectly feasible that larger numbers of eyes could be pooled from a single flock to further enhance sensitivity. This type of "bulk screening" of samples from a single flock is a common practise in many instances when analysing samples from poultry farms [16]. The value of CCβ obtained in this study demonstrated that the procedure is suitable for the analysis of the parent nitrofurans in the concentration range from <1.0 to 5.0 ng eye^{-1}.

There is an ongoing need for a rapid, generic screening test capable of detecting nitrofuran contaminants present in a range of food products and animal feedstuffs. Such an assay would be welcomed by food producers, countries exporting foods to the EU and the regulatory authorities of European Member States alike. In particular, there is a need for a screening test which can detect parent nitrofurazone as opposed to its marker residue, the controversial metabolite semicarbazide. Member States have been hindered in the enforcement of a ban on nitrofurazone use, as a number of natural sources of semicarbazide in animal tissues have recently been identified. The ability to categorically link parent levels of the nitrofurans in eyes to metabolite levels in muscle and liver samples would be an invaluable tool for EU Member States in the enforcement of the ban on these drugs.

When the developed method was further applied to a small number of NFZ incurred samples, it was shown to be capable of detecting the parent drug in the eyes of the treated broilers. Confirmatory analysis of the corresponding liver samples by LC–MS/MS produced similarly high levels of the metabolite semicarbazide.

As was previously determined by the work of Cooper et al. [16] into the accumulation of NFZ in the eyes of medicated broilers, the levels of the parent NFZ remained detectable (mean = 4.8 ng eye^{-1}) up to 16 days following withdrawal of medicated feed from the diet after the birds had been fed at a therapeutic dose (300 mg kg^{-1}) for 16 days. Their results would appear to suggest that levels of nitrofurans in the eyes of animals fed therapeutic doses of the compounds may persist in sufficient concentrations and for a sufficiently extended period of time post withdrawal to allow parent NFZ in eyes to be used as a marker of nitrofuran abuse.

To determine if the procedure is suitable for the long term detection of nitrofuran abuse in broilers following drug withdrawal from the diet, it is proposed that a much more extensive depletion study will be undertaken. This study will attempt to ascertain if a relationship can be established between accumulation of parent drug in the eye and that of the marker compound in the liver and muscle. Persistence within the eye would need to be of sufficient duration to allow detection of the parent by biosensor analysis until such time as concentrations of the marker residue in liver and muscle has fallen to at least the MRPL (1 μg kg^{-1}) established for the marker compounds.

Although work to date has focused solely on the application of the method to avian eyes, it would seem likely that the assay could be equally well applied to other species. Future work will investigate this possibility including the requirement for "pooling" of eyeballs which, in larger species, may be unnecessary.

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