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Residues of nitrofurantoin antibiotic parent compounds and metabolites in eyes of broiler chickens

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3 **Residues of nitrofurantoin antibiotic parent compounds and**
4 **metabolites in eyes of broiler chickens**
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Abstract

Nitrofurantoin antibiotic residues in food continue to be of international concern. The finding of sources of semicarbazide (SEM), other than through the misuse of nitrofurazone, present a challenge to the use of SEM as a definitive marker residue for this drug. Detection of intact (parent) nitrofurazone would avoid confusion over the source of SEM residues. Broiler chickens were fed sub-therapeutic nitrofurantoin-containing diets and tissues analysed for parent compounds and metabolites by LC-MS/MS. Depletion half-lives in muscle were longer for tissue-bound metabolite residues, 3.4 days (AOZ, AMOZ) to 4.5 days (SEM), than total metabolite residues, 2.0 days (AOZ) to 3.2 days (SEM). Metabolite concentrations were higher in eyes than muscle. Metabolite half-lives in eyes ranged from 8.5 days (AHD) to 20.3 days (SEM). Nitrofurantoin parent compounds were also detected in eyes.. Furaltidone was detected in single eyes after 21 days withdrawal of a 6 mg kg⁻¹ furaltidone diet. When 50 eyes from broilers containing metabolites in muscle close to the 1 µg kg⁻¹ MRPL were pooled into single samples, 1.2 ng furazolidone and 31.1 ng of furaltidone were detected, but nitrofurazone was not detected due to the long depletion half-life of SEM in muscle. Further studies are required to improve LC-MS/MS nitrofurazone sensitivity and refine the sample size necessary to use nitrofurazone detection in pooled eyes as a complement to SEM detection in muscle.

Keywords: *nitrofurans, broiler chickens, eyes, metabolites, depletion, semicarbazide*

Introduction

The monitoring of residues of nitrofurans metabolites in the food chain has been a major focus in the international control of veterinary drug residues for several years. Nitrofurans antibiotics were banned within the European Union (EU) (Commission Regulation 1995) due to concerns over their carcinogenicity and mutagenicity (Van Koten-Vermeulen et al. 1993) and should not be used in food-producing animals or be present in foods produced in, or imported into, the EU. However, improved liquid chromatography-tandem mass spectrometry (LC-MS/MS) confirmatory analytical methods developed under the EU funded research project FoodBRAND (<http://www.afsni.ac.uk/foodbrand>; Cooper et al. 2005b) were instrumental in uncovering the widespread abuse of nitrofurans in various world-wide food industries during 2001-02. These included primarily the poultry and aquaculture industries in S.E. Asia and S. America, in addition to isolated findings of nitrofurans in European pork (O'Keeffe et al. 2004) and poultry (Food Standards Agency 2003), and egg powders and honey from various countries (European Commission RASFF). These findings disrupted trade relations between the EU and some exporting countries. The measures taken included the adoption of Decisions that required the mandatory pre-import testing of aquaculture products from Vietnam (Commission Decision 2002a), poultry and aquaculture products from Thailand (Commission Decision 2002b) and poultry products from Brazil (Commission Decision 2002c). In the wake of improved analytical techniques and subsequent changes to national legislation and monitoring programmes instigated by some exporting countries, the above decisions were revoked and the frequency of EU Rapid Alerts for nitrofurans gradually fell. The 18-month period from January 2005 produced 73 Rapid Alerts for nitrofurans metabolites (European Commission RASFF). Shrimps from S.E. Asia and India continue to be the

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2
3 most commonly violative foodstuff (58 Alerts), the remaining food sources being fish
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5 (5), honey (4), poultry meat (3), bovine (1), porcine (1) and baby food (1). The
6
7 majority of cases concern findings of semicarbazide (SEM, 48 Alerts), the remaining
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9 being 3-amino-2-oxazolidinone (AOZ, 22) and 3-amino-5-morpholinomethyl-2-
10
11 oxazolidone (AMOZ, 3).
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17 The nitrofurantoin antibiotics are unstable *in vivo*, breaking down in edible tissues within
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19 hours (McCracken et al. 1995). However, they give rise to stable tissue-bound
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21 metabolites, which persist in muscle and liver for many weeks (Hoogenboom et al.
22
23 1991; Cooper et al. 2005b). These metabolites, for example SEM which is the
24
25 metabolite of nitrofurazone (NFZ), are the marker residues used to indicate the use of,
26
27 or contamination with, their parent compounds in animal tissues. However, the validity
28
29 of SEM as a unique marker residue for NFZ has been called into question following
30
31 recent evidence that SEM in food may arise from sources other than from this illegal
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33 antibiotic. In 2003 SEM was detected in baby food (EFSA, 2003). Subsequent
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35 investigations (Stadler et al., 2004) demonstrated that the SEM was a breakdown
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37 product of azodicarbonamide; the blowing agent used in the manufacture of plastic
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39 gaskets inside the jar lids. Azodicarbonamide is also a flour treatment agent used in the
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41 bread making process, which may account for SEM found in breaded chicken but not in
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43 the chicken meat (Kennedy et al., 2004; Pereira et al., 2004). Carrageenan, a widely
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45 used thickening agent derived from red seaweed, has also been implicated in the
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47 production of SEM under certain conditions (Hoenicke et al., 2004). Low levels of
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49 SEM, possibly of natural origin or a by-product of boiling, have also been observed in
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51 cooked crayfish samples (Saari and Peltonen 2004). Consequently, the presence of
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53 SEM can no longer be used as definitive proof of administration of NFZ to an animal
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3 and some national authorities have become reluctant to take enforcement action on the
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5 basis of SEM detection.
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10 One approach to overcoming this uncertainty surrounding SEM would be to find a
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12 depot of intact NFZ in tissues of NFZ-treated animals. This laboratory has
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14 demonstrated that the four major nitrofurans furazolidone (FZD), furaltadone (FTD),
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16 nitrofurantoin (NFT) and NFZ all accumulate in chicken eggs and are not broken down
17
18 rapidly as in muscle and liver (McCracken and Kennedy 2006). Our previous finding
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20 of nitrofurans metabolites, including SEM, at high concentrations (mg kg^{-1}) in the
21
22 retina/choroid layer in eyes of pigs suggested that at least some of these residues may
23
24 be present in the form of their parent nitrofurans drugs (Cooper and Kennedy 2005). We
25
26 subsequently demonstrated that NFZ does indeed accumulate in whole eyes of laying
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28 hens (Cooper et al. 2005a). In birds fed only 0.3 mg NFZ per kg of feed
29
30 (approximately 0.1% of a therapeutic dose; National Office Of Animal Health Limited,
31
32 1992) the mean NFZ detectable by LC-MS/MS in a single eye was 0.5 ng eye^{-1} . The
33
34 mean tissue-bound SEM concentration in muscle of these chickens was $3.3 \mu\text{g kg}^{-1}$.
35
36 This suggested that, if multiple eyes could be pooled from chickens of a single flock, a
37
38 test for parent NFZ could be developed with sensitivity comparable to that of tissue-
39
40 bound SEM in muscle. The current methodology for statutory monitoring of NFZ
41
42 abuse in animal tissues is detection of tissue-bound SEM in muscle or liver and
43
44 regulatory laboratories are required by EU legislation to set in place confirmatory
45
46 methods which can quantify SEM at $1.0 \mu\text{g kg}^{-1}$ or less (the Minimum Required
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48 Performance Limit, MRPL, set down in Commission Decision 2003/181).
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The current study aimed to assess the suitability of testing for nitrofurantoin parent compounds in eyes of broiler chickens by addressing the following objectives:

1. To demonstrate the depletion profile of nitrofurantoin metabolite residues in broiler eyes, muscle and liver tissues following drug withdrawal.
2. To demonstrate the presence of nitrofurantoin parent compounds in eyes of nitrofurantoin-medicated broiler chickens.
3. To demonstrate the depletion profile of the nitrofurantoin parent compounds in broiler eyes following drug withdrawal.
4. To determine if monitoring nitrofurantoin parent compounds in pooled broiler eyes is comparable to monitoring their tissue-bound metabolites in muscle tissue.

Materials and methods

Materials and instrumentation

Internal standards D₄-3-amino-2-oxazolidinone, D₅-3-amino-5-morpholinomethyl-2-oxazolidinone, ¹³C₃-1-aminohydantoin and ¹³C¹⁵N₂-semicarbazide were supplied by Witega Laboratorien Berlin-Adlershof (Berlin, Germany). 3-Amino-2-oxazolidinone (AOZ), semicarbazide (SEM), furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFT) and nitrofurazone (NFZ) were supplied by Sigma-Aldrich (Poole, UK). 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), and the nitrofurantoin parent compound internal standards D₄-furazolidone, D₅-furaltadone and ¹³C¹⁵N₂-nitrofurazone were supplied by Chemical Synthesis Services (Belfast, UK). 1-Aminohydantoin (AHD) was a gift from Proctor and Gamble Pharmaceuticals USA.

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3 Unless stated, all other chemicals and solvents were obtained from Sigma-Aldrich and
4
5 were analytical and HPLC grade, respectively.
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10 An Agilent 1100 Series HPLC system (Agilent Technologies, USA) coupled to a
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12 Quattro Ultima® Platinum tandem mass detector (Micromass/Waters, Manchester,
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14 UK), both operating under MassLynx® software, were used for tissue analyses. The
15
16 mass spectrometer operated in electrospray positive and negative modes and data
17
18 acquisition was in multiple reaction monitoring mode (MRM). The HPLC system was
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20 equipped with a Columbus C18(2) 3 µm, 2.0 x 150 mm column (Phenomenex, UK). A
21
22 binary gradient mobile phase was used at a flow rate of 0.2 ml/min, solvent A being 0.5
23
24 mM ammonium acetate and methanol (80:20 v/v mix), solvent B being 100%
25
26 methanol. Commercial crumbed feed for broiler chickens was supplied by McLarnons
27
28 Ltd. (Randalstown, UK). Muscle and liver were minced in a Mini Prep Plus domestic
29
30 food processor (Waring, Torrington, CT, USA) then were homogenised in a SL2
31
32 laboratory homogeniser (Silverson Machines Ltd, Chesham, England). Whole chicken
33
34 eyeballs were homogenised in self-contained, disposable FOODcon7 homogenisation
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36 units using a FASTH21 high throughput, automated tissue homogenisation system
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38 (Consular SA, Switzerland).
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48 *Production of incurred broiler tissues*

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53 Two-week old broiler chickens, purchased from a local supplier, were housed in deep
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55 litter pens in 4 groups of 43 birds. Birds were allowed *ad libitum* access to fresh water
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57 and feed at all times. Commercial broiler chick crumb feed was used to prepare four
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59 medicated diets containing NFT at 50 mg kg⁻¹ feed, FZD at 10 mg kg⁻¹ feed, FTD at 6
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3 mg kg⁻¹ feed and NFZ at 1 mg kg⁻¹ feed. These concentrations were designed to
4
5 produce tissue-bound nitrofurans metabolite residues in muscle close to the EU MRPL
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7 of 1 µg kg⁻¹ after a drug withdrawal period of between 1 to 3 weeks. Approximate
8
9 calculations were based on the limited literature data available for dietary nitrofurans
10
11 levels and muscle residues in chickens (Zuidema et al. 2004; McCracken et al. 2005;
12
13 Cooper et al. 2005a) and pigs (Cooper et al. 2005b). The four groups were fed a
14
15 medicated diet for 7 days after which the medicated diets were withdrawn and replaced
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17 with conventional unmedicated broiler grower crumb feed for a further 21 days'
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19 withdrawal period. Birds (3 per group) were euthanised by cervical dislocation at
20
21 intervals during the withdrawal period. Breast muscle and liver were sampled and
22
23 stored at -20°C. Whole, intact eyeballs were removed and stored at -80°C. During the
24
25 withdrawal period, muscle samples were analysed immediately for total nitrofurans
26
27 metabolite concentration as described below. McCracken et al. (1997) demonstrated
28
29 that approximately 60% of total AOZ residues in kidney of FZD treated pigs was
30
31 present in the form of tissue-bound residues. Unpublished data from this laboratory
32
33 demonstrates that approximately 80% of total SEM residues in muscle of NFZ treated
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35 chickens is present in the form of tissue-bound residues. Therefore, when total muscle
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37 metabolite concentrations had fallen to approximately 1 to 3 µg kg⁻¹ in the present
38
39 study, 25 birds from each group were euthanised and muscle, liver and eyes were
40
41 sampled. These groups of 25 birds provided 50 eyes that could be pooled for analysis
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43 of nitrofurans parent compounds. The remaining birds continued on the withdrawal
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45 rations and were euthanised (3 per group) and tissues sampled every 3 to 4 days.
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58 *Tissue analysis by LC-MS/MS*
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3 Tissue-bound nitrofurans metabolites were measured in 1 g samples of chicken muscle
4 and liver essentially as described by Cooper et al. (2005b). Briefly, samples were
5 homogenised and extractable residues were removed by repeated washing in methanol,
6 ethanol and diethyl ether. Tissue-bound metabolites were hydrolysed in mild acid and
7 derivatised with 2-nitrobenzaldehyde before extraction into ethyl acetate. Sample
8 preparation and LC-MS/MS analysis were as described by Cooper et al. (2005b) with
9 the exceptions that a Columbus C18 HPLC column and an additional $^{13}\text{C}_3$ -AHD
10 internal standard were used. Highly concentrated samples were diluted as required for
11 analysis.
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27 Total nitrofurans metabolites were measured in 1 g samples of chicken muscle as
28 described by Cooper and Kennedy (2006). This method omits the sample solvent pre-
29 washing steps and employs minor differences in reagent volumes compared to the
30 tissue-bound metabolite method.
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39 Total nitrofurans metabolites were measured in single whole chicken eyes as described
40 by Cooper et al. (2005a). Eyes were cut into four pieces and placed in FOODcon7
41 homogenisation units while still frozen in order to retain the vitreous humour. If
42 allowed to defrost, the retina, which contains the vast majority of the ocular nitrofurans
43 residues, partially disintegrates and is dispersed with the humour when the sclera is cut
44 open.
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55 Nitrofurans parent compounds were extracted from single whole chicken eyes
56 essentially as described by Cooper et al. (2005a). Sliced eyes were homogenised in
57 FOODcon7 units with 5 ml water fortified with 10 ng per eye mixed internal standard
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3 and extracted into ethyl acetate, reconstituted in acetonitrile and washed with hexane.
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5 Dried extract was finally reconstituted in 100 µl of methanol : water (50 : 50 v/v) and
6
7 centrifuged at 13 000 rpm. LC-MS/MS conditions for the analysis of the four
8
9 nitrofurantoin parent compounds were as described by McCracken and Kennedy (2006).
10
11 Nitrofurantoin parent compounds were also analysed in pooled samples comprising 50
12
13 pooled eyes, homogenising 5 eyes in 6 ml water in each of 10 FOODcon7 units,
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17 fortifying with 1 ng per unit mixed internal standard. After transferring the contents to
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19 glass tubes and rinsing the units with 2 ml water, samples were extracted into ethyl
20
21 acetate (9 + 7 ml) and reconstituted in acetonitrile (3 ml). The samples were washed
22
23 with hexane (3 + 3 ml) before combining the ten acetonitrile extracts, evaporating to
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25 dryness and reconstituting the single combined extract (50 eyes) in 100 µl of methanol :
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27 water (50 : 50 v/v) and centrifuging at 13 000 rpm.
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Results and discussion

Nitrofurantoin metabolites in edible tissues

Table I presents the concentrations of total nitrofurantoin metabolites in broiler breast muscle during the first week of drug withdrawal. Depletion half-lives, calculated on the basis of linear regression of metabolite concentrations over this 7 day period, ranged from 2.0 days (AOZ) to 3.2 days (SEM). This is the first publication of total metabolite half-lives for all four of the nitrofurantoin drugs in broiler chickens. Zuidema et al. (2004) published total AOZ and AMOZ depletion data in broilers fed therapeutic doses. These graphs suggested AOZ and AMOZ half-lives were in the region of 4 days. It was on the basis of estimates made from the measurements of the total

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3 metabolite concentrations in the present study, that broilers were euthanised on days 8,
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5 9 and 10 in order to produce muscle samples with tissue-bound nitrofurans metabolite
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7 concentrations close to the MRPL of $1 \mu\text{g kg}^{-1}$. Groups of birds were euthanised on
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9 different days due to the varying concentrations of dietary nitrofurans employed and the
10
11 variable metabolite half-lives observed.
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15 [Insert TABLE I about here]
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18 Whilst European legislation does not specify whether nitrofurans metabolites should be
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20 monitored in the form of tissue-bound or total fractions, analysis of tissue-bound
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22 metabolites became the norm in this laboratory, initially because of the cleaner extracts
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24 obtained following solvent washing. This approach was reinforced by AFSSA, the
25
26 Community Reference Laboratory for nitrofurans drugs, which, following the discovery
27
28 that SEM could arise from degradation of azodicarbonamide, used as a flour treatment
29
30 agent, issued a statement in 2003 that *“when testing composite food, only analyse the*
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32 *part of the product which is of animal origin, for example, only the meat part of*
33
34 *breaded products...In the case of a non-compliant sample for total SEM, a sample must*
35
36 *be reanalysed for the bound residues of SEM only”* (Sanders 2003). Given that chicken
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38 muscle is the world’s most popular meat, and that nitrofurans residues have been an
39
40 issue of concern in the global poultry industry, there is a need for data on the depletion
41
42 half-lives of the tissue-bound nitrofurans metabolites in edible broiler tissues. Figures 1
43
44 and 2 illustrate the depletion profiles and half-lives of the four major tissue-bound
45
46 nitrofurans metabolites in muscle and liver respectively. (Note: the nitrofurans drugs
47
48 were fed at different dietary concentrations, so absolute metabolite concentrations
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50 cannot be compared meaningfully across drug groups). Depletion half-lives for all
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52 metabolites were longer in muscle (ranging 3.4 to 4.5 days) than in liver (ranging 2.2 to
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54 3.1 days), possibly reflecting the lower metabolic rate and slower cellular turnover of
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3 muscle tissue. This tissue difference was also evident in our previous studies of
4 nitrofurans depletion in porcine tissues (Cooper et al. 2005). SEM exhibited the longest
5 half-life in broiler muscle (4.5 days) and was detectable after 3 weeks withdrawal of a
6 low contamination level diet (1 mg kg⁻¹, normal therapeutic dose being 200-300 mg kg⁻¹).
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[Insert FIGURE 1 and FIGURE 2 about here]

Nitrofurans metabolites in broiler eyes

The recent controversy over sources of SEM in foods, other than through abuse of NFZ, led this laboratory to investigate eyes as a potential depot of nitrofurans residues. We demonstrated that total nitrofurans metabolites were detectable at parts per million concentrations in retina of pigs fed therapeutic nitrofurans doses (Cooper and Kennedy 2005). We then proceeded to show that NFZ-treated laying hens had similarly high concentrations of total SEM in whole eyeballs (Cooper et al. 2005a) and that SEM was even detectable in a single eye when feeding a diet containing NFZ at only 0.03 mg kg⁻¹. Figure 3 shows that AOZ, AMOZ, AHD and SEM residues can be detected by LC-MS/MS in single eyes of broiler chickens fed sub-therapeutic nitrofurans doses. The depletion half-lives of total metabolites in eyes are considerably longer (ranging 8.5 to 20.3 days) than the depletion half-lives for tissue-bound metabolites in muscle and liver (Figures 1 and 2). As is the case with some other xenobiotic residues such as the β -agonist clenbuterol (Malucelli et al. 1994), ocular tissue, specifically the retina, appears to be a metabolic “dead end” tissue where nitrofurans metabolites accumulate to

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3 high concentrations and where they are not subject to the higher cellular turn-over and
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5 clearance rates of other tissues such as muscle and liver. The pigment melanin has been
6
7 identified as the site of binding of various drugs in pigmented ocular tissues (Ings
8
9 1984).

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11
12 [Insert FIGURE 3 about here]

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15 The concentrations of nitrofurans metabolites are also significantly greater in eyes
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17 (measured as ng per eye; each eye weighing approximately 1 g) than in muscle and
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19 liver (measured as ng per g tissue). The MRPL for the nitrofurans is $1.0 \mu\text{g kg}^{-1}$ in
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21 poultry meat and aquaculture products (Commission Decision 2003). Commission
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23 Decision 2005/34/EC has recently harmonised the standards applied for monitoring
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25 banned substances, including the nitrofurans, in foods. The MRPL is now to be
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27 considered a “reference point(s) for action irrespective of the matrix tested.”
28
29 Furthermore, “where the results of analytical tests on products are below the MRPL...
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31 the products will not be prohibited from entering the food chain.” Where recurrent
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33 sub-MRPL findings are observed, “the Commission shall bring the matter to the
34
35 attention of the competent authority in the country of origin and shall make appropriate
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37 proposals” (Commission Decision 2005). Given the higher concentrations and longer
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39 half-lives of nitrofurans metabolites in eyes compared to muscle and liver, and the
40
41 possibility of applying the MRPL as an reference point for action irrespective of the
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43 matrix tested, it is now possible, in principle at least, that a chicken may be condemned
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45 as unfit for human consumption on the basis of metabolites detected in its eyes long
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47 after the metabolite concentration in muscle or liver has fallen below the MRPL, further
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49 diminishing the temptation to misuse these drugs.
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Nitrofurantoin parent compounds in broiler eyes

The major attraction of testing chicken eyes is their ability to retain the intact nitrofurantoin parent compounds in contrast to edible tissues in which the parent compounds are broken down inside a matter of hours (McCracken et al. 1995, Cooper et al. 2005b). We previously demonstrated that NFZ was detectable in eyes of laying hens (Cooper et al. 2005a). Table II now demonstrates that FZD, FTD and NFT are also detectable in single broiler chicken eyes when no withdrawal period is observed. Whilst direct comparisons cannot be made between the absolute concentrations of the four nitrofurantoin parent compounds in eyes because of the differing dietary concentrations administered, we have shown that accumulation of nitrofurantoin parent compounds in broiler eyes is not restricted to NFZ. The failure to detect NFZ in single eyes in this study (Table II) may have been due to the fact that SEM has a long depletion half-life in muscle and therefore it was necessary to feed NFZ at a very low dietary level (1 mg kg^{-1}) to achieve SEM muscle concentrations close to the MRPL within the confines of a short term study. NFZ was also the least sensitive of the nitrofurantoin parent compounds under LC-MS/MS ionisation. However, monitoring compliance with the prohibition on the use of NFZ in food-producing animals may still be feasible by measuring NFZ in the retina of larger food-producing animals (e.g. cattle and pigs).

[Insert TABLE II about here]

NFT was still detectable in a single eye after 3 d withdrawal of a 50 mg kg^{-1} NFT diet. FTD, by contrast, when fed at only 6 mg kg^{-1} feed, was still detectable (0.08 ng eye^{-1}) in a single eye after 21 days withdrawal (Table II; depletion half-life 3.4 days). The ability to detect FTD over such a period is due partly to FTD being the most sensitive of the nitrofurantoin parent compounds under the LC-MS/MS method described above.

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2
3 However, FTD does appear to be the most abundant nitrofuran parent compound in
4 ocular tissues, given that in pigs fed 400 mg kg⁻¹ FTD, the parent compound was
5 detected in isolated retina at mg kg⁻¹ concentrations, much higher than the µg kg⁻¹
6 concentrations found in FZD treated pigs (Cooper et al. 2005a).
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15 Twenty-five broilers from each group were euthanised after 8, 9 or 10 days withdrawal
16 when it was estimated (based on real-time analysis of total metabolites) that tissue-
17 bound metabolite concentrations in muscle would be close to the 1 µg kg⁻¹ MRPL.
18 Table III shows that two groups were below (AHD 0.8 µg kg⁻¹, SEM 0.4 µg kg⁻¹) and
19 two groups above the MRPL (AOZ 1.4 µg kg⁻¹, AMOZ 1.6 µg kg⁻¹) on these dates.
20 Processing and pooling 50 whole eyes into a single extract reconstituted in 100 µl of
21 solvent proved to be easier than expected. Eyes were split across 10 groups of 5 eyes,
22 each independently extracted and washed with hexane to remove the considerable
23 orange oily fraction present in intact eyeballs, before combining to a single extract
24 which could be dissolved in 100 µl of 50% methanol. Ultra-centrifugation further
25 clarified the samples such that extracts of 50 eyes were of a similar clarity to extracts of
26 a single eye and suitable for injection onto a HPLC column. However, greater
27 ionisation suppression was evident during tandem MS analysis of extracts of 50 eyes
28 compared to single eye samples (peak areas reduced by a factor of approximately 4).
29 Comprehensive validation of the LC-MS/MS method is hindered by the enormous
30 number of eyes required for the sample compositing process, however, the authors
31 estimate that in a fortified sample of 50 broiler eyes, nitrofuran parent compounds can
32 be detected down to approximately 4 ng NFZ, 2 ng NFT, 1 ng FZD and 0.2 ng FTD per
33 pooled sample.
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[Insert TABLE III about here]

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3 FZD was detected at 1.2 ng in 50 pooled eyes (Table III) some 10 days after
4 withdrawal of a diet containing 10 mg kg⁻¹ FZD (5% of a therapeutic dose). This
5 equates to 0.02 ng eye⁻¹, which would be undetectable in a single broiler eye using
6 current LC-MS/MS techniques. In contrast, the stability of FTD was reinforced by the
7 detection of 31.1 ng in 50 pooled eyes 10 days after withdrawal of a diet containing
8 only 6 mg kg⁻¹ FTD. This equates to 0.62 ng eye⁻¹, which is consistent with the
9 detection of 0.6 ng FTD in single eyes at 7 days withdrawal (Table II). Pooled eyes
10 were unavailable for testing for NFT. However, on the basis of the concentrations of
11 NFT in single eyes (Table II), it can be estimated that NFT would have been detected in
12 50 pooled eyes at approximately 10 ng. Possibly the most significant result was the
13 inability to detect NFZ in 50 pooled eyes after 8 days withdrawal of a 1 mg kg⁻¹ NFZ
14 diet. This can again be attributed primarily to the long depletion half-life of SEM in
15 muscle (4.5 days) that results in levels of SEM close to the MRPL being detected in
16 muscle even following withdrawal of a low contamination level diet. This was
17 compounded by the poor sensitivity of NFZ under the applied LC-MS/MS conditions.
18 It may be possible that, if MS/MS sensitivity can be improved and samples analysed
19 when SEM in muscle is closer to the 1 µg kg⁻¹ MRPL rather than the 0.4 µg kg⁻¹ in the
20 current test samples, NFZ may be detectable in a 50 eyes pooled sample. However, a
21 larger sample size may also be considered to improve test sensitivity. Given the scale
22 of commercial broiler production facilities, obtaining 50 or 100 eyes from a chicken
23 house (in which all birds receive the same feed and are therefore suitable for pooling
24 into a single sample) would present little difficulty. However, it must be recognised
25 that the testing of eyes for nitrofurans parent compounds is unlikely to replace the
26 testing of edible tissues for nitrofurans metabolites as the routine method of nitrofurans
27 residue monitoring. Imported poultry meat is rarely accompanied by the eyes from the

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3 source bird. Nevertheless, the testing of nitrofurantoin parent compounds in pooled eyes
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5 from a poultry flock would be a valuable tool within a monitoring programme. It
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7 would remove any doubt over the source of nitrofurantoin metabolites and enable poultry
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9 producers to police their own supply chains and competent national authorities to carry
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11 out comprehensive investigative work within their domestic primary production
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13 facilities. Ultimately it would assist EU Member States to enforce fully the ban on the
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15 use of nitrofurantoin drugs in primary production. Furthermore, the higher concentrations
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17 of nitrofurantoin metabolites detected in eyes may permit laboratories lacking LC-MS/MS
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19 technologies to monitor nitrofurantoin abuse in their primary production using HPLC-UV
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21 detection as an alternative to monitoring tissue-bound metabolites in edible tissues,
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23 albeit at a level which would not meet EU confirmatory criteria.
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32 This study has provided useful data on the depletion profiles and half-lives of the
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34 nitrofurantoin metabolites in broiler chicken muscle and liver. It has also demonstrated that
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36 higher concentrations of metabolites with longer half-lives are detectable through the
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38 analysis of whole eyes of broilers. This study, in conjunction with our previous work
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40 (Cooper et al., 2005a), has now shown that all four major nitrofurantoin parent antibiotics,
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42 as opposed to their metabolites, are detectable in broiler eyes, with FTD, in particular,
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44 being detectable even after 3 weeks withdrawal of a contamination level diet. This was
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46 the first attempt to measure nitrofurantoin parent compounds in pooled samples of eyes
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48 from broilers whose muscles contained nitrofurantoin metabolites close to the EU MRPL.
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50 Measuring FZD in 50 pooled eyes was shown to be an alternative to measuring tissue-
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52 bound AOZ in single muscle samples. FTD was detectable in single eyes of broilers
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54 with MRPL concentrations of AMOZ in their muscle. However, NFZ could not be
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56 detected in 50 pooled eyes of broilers with 0.4 $\mu\text{g kg}^{-1}$ SEM in their muscle. Further
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3 study is required to improve NFZ sensitivity under LC-MS/MS and to refine the sample
4 size necessary to enable NFZ detection in pooled eyes to be applied as an alternative to
5 SEM detection in muscle. Such a test would circumvent the problem of alternative
6 sources of SEM in the food chain and would help protect both the consumer from
7 eating meat of birds that have received a banned substance, and of poultry producers
8 from being wrongly accused of administering nitrofurazone on the basis of
9 semicarbazide detection in muscle.
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TABLE AND FIGURE CAPTIONS:

Figure 1. Depletion, during 3 weeks withdrawal, of tissue-bound nitrofuran metabolites, AOZ, AMOZ, AHD and SEM from breast muscle of broiler chickens fed, FZD (10 mg kg⁻¹), FTD (6 mg kg⁻¹), NFT (50 mg kg⁻¹ feed) and NFZ (1 mg kg⁻¹) respectively for 7d. Mean ± SE (n=3).

Figure 2. Depletion, during 3 weeks withdrawal, of tissue-bound nitrofuran metabolites AOZ, AMOZ, AHD and SEM from liver of broiler chickens fed FZD (10 mg kg⁻¹), FTD (6 mg kg⁻¹), NFT (50 mg kg⁻¹ feed) and NFZ (1 mg kg⁻¹) respectively for 7d. Mean ± SE (n=3).

Figure 3. Depletion, during 3 weeks withdrawal, of total nitrofuran metabolites AOZ, AMOZ, AHD and SEM from single whole eyes of broiler chickens fed FZD (10 mg kg⁻¹), FTD (6 mg kg⁻¹), NFT (50 mg kg⁻¹ feed) and NFZ (1 mg kg⁻¹) respectively for 7d. Mean ± SE (n=3).

Table I. Depletion, during first week of withdrawal, of total nitrofuran metabolites AOZ, AMOZ, AHD and SEM from breast muscle of broiler chickens fed FZD (10 mg kg⁻¹), FTD (6 mg kg⁻¹), NFT (50 mg kg⁻¹ feed) and NFZ (1 mg kg⁻¹) respectively for 7d. Mean (n=3) ± SE.

1
2
3 Table II. Detection of nitrofurantoin parent compounds in single whole eyes of broiler
4 chickens fed FZD (10 mg kg⁻¹), FTD (6 mg kg⁻¹), NFT (50 mg kg⁻¹ feed) and NFZ (1
5 mg kg⁻¹) for 7d. ND = not detected. Mean (n=3) ± SE. Depletion half-life of FTD was
6
7 3.4 d.
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14 Table III. Detection of nitrofurantoin parent compounds in 50 pooled eyes of medicated
15 broiler chickens when tissue-bound nitrofurantoin metabolites in breast muscle (n=5) were
16 close to the MRPL of 1 µg kg⁻¹.
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Figure 1

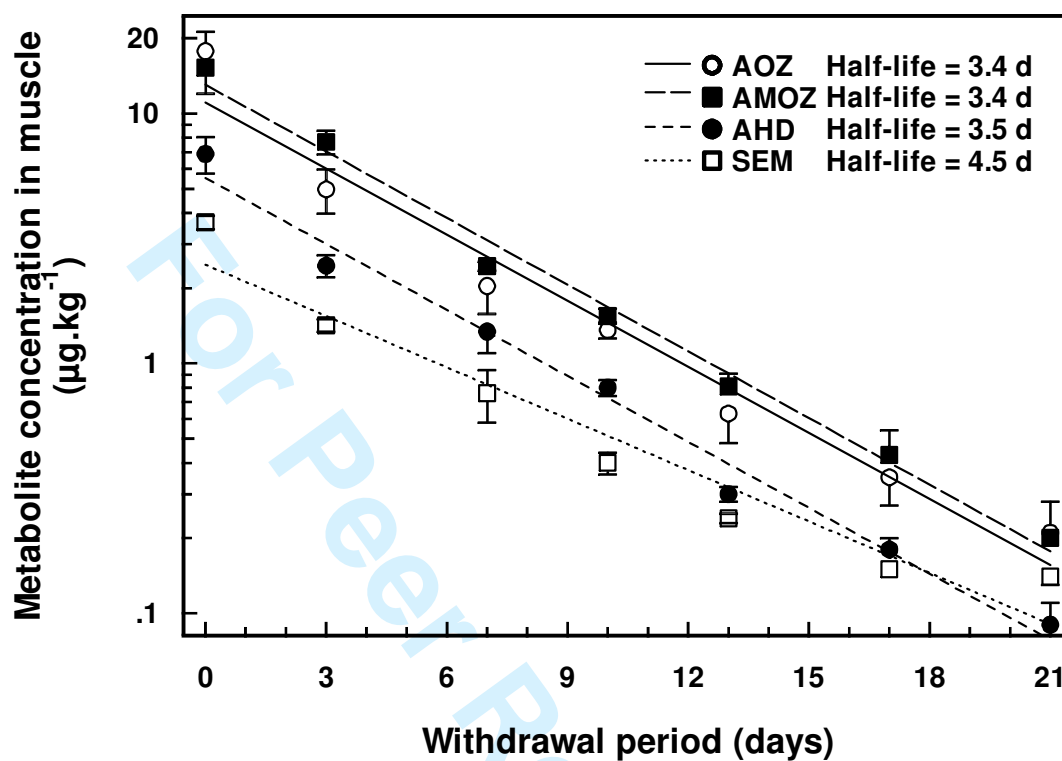


Figure 2

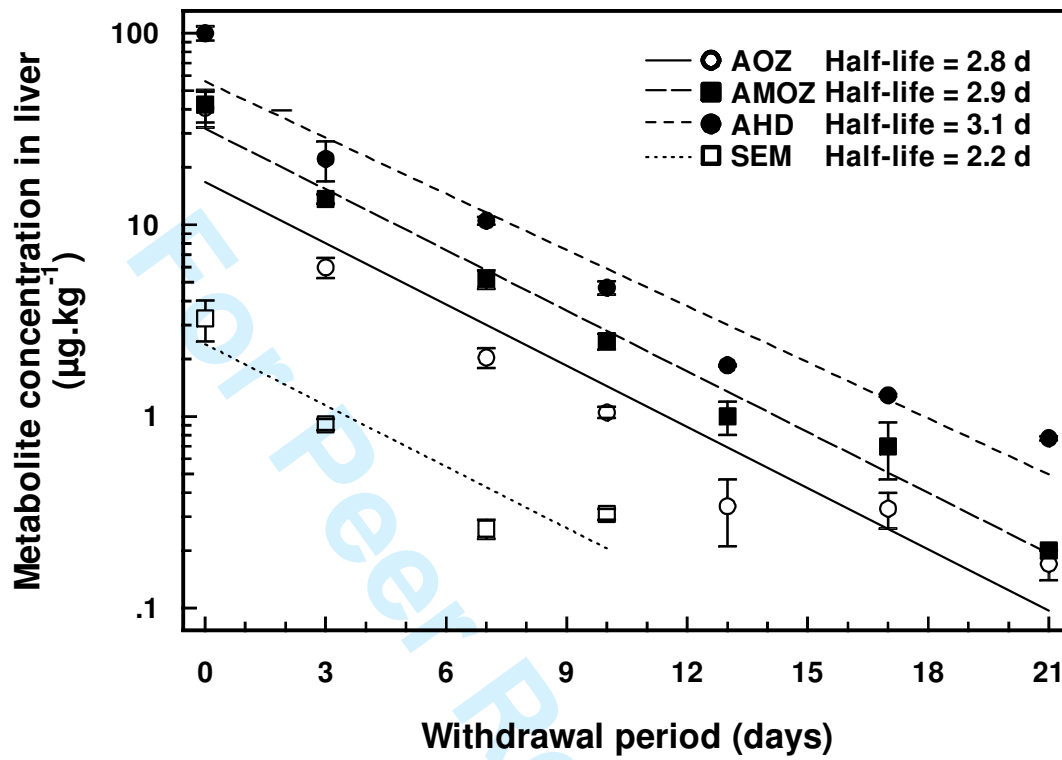


Figure 3

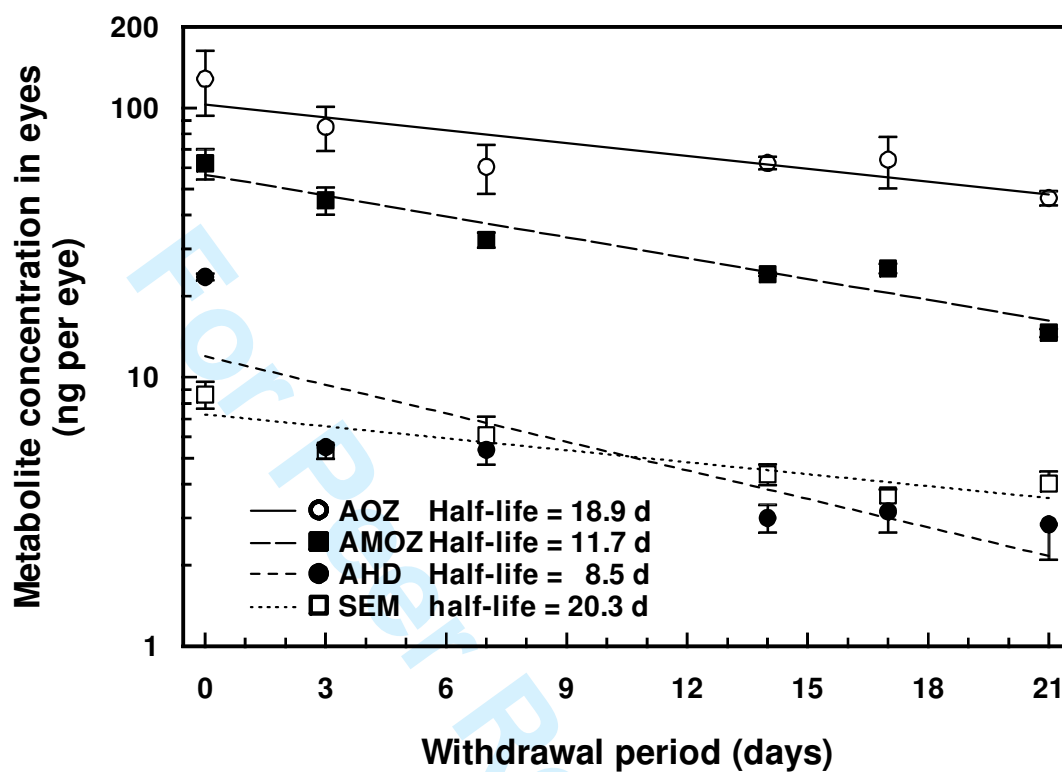


Table I

Total nitrofurans metabolites ($\mu\text{g kg}^{-1}$ muscle)				
Withdrawal (days)	AOZ	AMAZ	AHD	SEM
0	31.9 ± 6.6	19.5 ± 4.3	18.0 ± 1.6	4.8 ± 0.3
3	7.5 ± 1.0	8.9 ± 1.0	4.9 ± 0.5	1.9 ± 0.2
7	2.8 ± 0.5	3.0 ± 0.2	2.6 ± 0.5	1.0 ± 0.2
Half-life (days)	2.0	2.6	2.5	3.2

Table II

Nitrofurantoin parent compound (ng per eye)				
Withdrawal (days)	FZD	FTD	NFT	NFZ
0	8.3 ± 7.4	10.4 ± 1.7	1.8 ± 0.7	ND
3	ND	2.0 ± 0.2	0.4 ± 0.1	ND
7	ND	0.6 ± 0.2	ND	ND
14	ND	0.2 ± 0.1	ND	ND
17	ND	0.3 ± 0.2	ND	ND
21	ND	0.1 ± 0.0	ND	ND

Table III

Nitrofuran	mg kg ⁻¹ feed	Withdrawal (days)	Tissue- bound metabolite	µg kg ⁻¹ muscle ± SE	Nitrofuran parent	ng per 50 pooled eyes
NFT	50	9	AHD	0.8 ± 0.1	NFT	Not tested
FZD	10	10	AOZ	1.4 ± 0.1	FZD	1.2
FTD	6	10	AMOZ	1.6 ± 0.1	FTD	31.1
NFZ	1	8	SEM	0.4 ± 0.1	NFZ	Not detected