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Determination of the furazolidone metabolite, 3-amino-2-oxazolidinone, in porcine tissues using liquid chromatography–thermospray mass spectrometry and the occurrence of residues in pigs produced in Northern Ireland

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Abstract

A method is presented for the detection of the furazolidone metabolite, 3-amino-2-oxazolidinone (AOZ), in porcine tissue. Bound and extractable residues are detected following methanol–water homogenisation, repeated solvent washing and derivatisation with 2-nitrobenzaldehyde. Samples are analysed by using thermospray mass spectrometry–liquid chromatography, monitoring the positive ion m/z 253 with filament-assisted ionisation. There is no interference from tissue matrices or excess 2-nitrobenzaldehyde reagent. The limit of determination for liver and muscle is 10 ng/g. Recoveries are greater than 80%. The assay was used to investigate the occurrence of furazolidone residues in pigs from Northern Ireland. One hundred samples were analysed. Seventeen of these contained bound AOZ residues. The stability of tissue samples *post mortem* was investigated in order to achieve optimum storage conditions for samples. When liver was stored at 4°C, the concentration of bound AOZ decreased by 22% within 48 h. However, there were no significant changes in AOZ concentrations in liver that was stored at −20°C for six months.

Keywords: Furazolidone; 2-Amino-2-oxazolidinone

1. Introduction

The nitrofuran, furazolidone ([N-(5-nitro-2-furfurylidene-3-amino)-2-oxazolidinone]) is a widely used antibacterial veterinary drug (Fig. 1), effective against *Salmonella* spp. and *Eschericha coli* and is also of value as an antiprotozoan, especially in poultry. It is commonly used as a feed additive to prevent bacterial enteritis as well as for maintaining adequate growth rate and feed conversion. The occurrence of furazolidone residues in edible tissue has been of major concern. In June 1995, the European Union failed to establish a maximum residue limit (MRL) for furazolidone [1]. This followed the joint Food and Agriculture Organisation–World Health Organisation expert committee report on food additives [2], which failed to establish an acceptable daily intake (ADI) for furazolidone, based on evidence of genotoxic and carcinogenic effects. The committee also concluded that there was insufficient information regarding a suitable marker residue for furazolidone and little information con-

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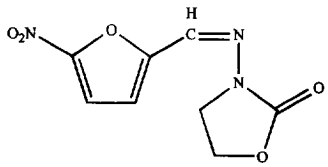
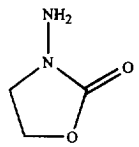
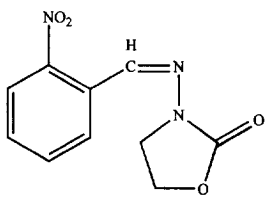


furazolidone



3-amino-2-oxazolidinone (AOZ)



3[[(2-nitrophenyl)methylene]-amino]-2-oxazolidinone (NPAOZ)

Fig. 1. The structures of furazolidone, 3-amino-2-oxazolidinone (AOZ) and 3[[(2-nitrophenyl)methylene]-amino-]-2-oxazolidinone (NPAOZ).

cerning the nature and quantity of the total residues present.

Evidence for the instability of residues of the parent compound has been reported previously [3,4]. The concentration of furazolidone found in liver and kidney of pigs, treated with furazolidone at the therapeutic level, fell below 5 ng/g within 6 and 4 h, respectively. The elimination half-lives of furazolidone in diaphragm and *longissimus dorsi* were both 1 h, falling below the 5 ng/g level within 12 h of administration of the drug [3]. Such information indicates that analysis of intact furazolidone in tissue is both difficult and of limited value.

It has been shown, using radiolabelled furazolidone [5], that administration of the drug to animals can result in the occurrence of protein bound metabolites that are not readily extractable. Some of these bound residues contain furazolidone metabolites that contain a 3-amino-2-oxazolidinone (AOZ, Fig. 1) side chain [6]. The AOZ side chain can be released from the bound residues under mildly acidic conditions and detected by reaction with nitrobenzaldehyde to produce a 3[[(2-nitrophenyl)methylene]amino]-2-oxazolidinone derivative (NPAOZ, Fig. 1), which may be measured using HPLC with diode array detection. Using this procedure, it was demonstrated [7] that hepatic bound metabolites containing the AOZ side chain had a much longer half-life than that of the parent compound. Also, in so far as these bound metabolites did contain an intact AOZ side chain, these residues did, in part, resemble the parent compound. Furthermore, it is conceivable that furazolidone residues, which are considered to be extractable, may also contain an AOZ analogue. It may therefore be prudent to measure AOZ in both extractable and protein bound residues if AOZ is to be considered as a suitable marker residue for furazolidone.

The assay was used in a survey of the occurrence of furazolidone residues in pigs within the Northern Ireland pig industry. The stability of AOZ in tissue samples in vitro was also investigated in order to evaluate the most appropriate storage conditions for samples.

2. Experimental

2.1. Chemicals and solvents

All chemicals and solvents used were of analytical-reagent grade and HPLC grade, respectively. 3-Amino-2-oxazolidinone was a gift from Orphahell (Mijdrecht, Netherlands). A stock standard solution (100 μg/ml) was prepared in methanol and working standards of 1.0 and 100 ng/ml were prepared by serial dilution of the stock.

NPAOZ was synthesised according to the method described previously [7]. The purity of the NPAOZ was checked using LC–MS. In a full scan mass spectrum, no significant peaks, other than at m/z 253, were observed. A stock standard of 1.0 mg/ml was prepared in acetonitrile and working solutions of 1.0 μg/ml and 100 ng/ml were prepared in acetonitrile–water (1:1, v/v).

2.2. LC–MS system

The LC system comprised a Hewlett-Packard (Cheadle, UK) series 1050 pump, a series 1050 automatic sampler and a Merck-Lichrocart (E. Merck, Darmstadt, Germany) reversed-phase column, 125×4 mm I.D., containing LiChrospher RP18 (end-capped; 5 μm particles) and fitted with an RP18 4-4 guard column. The column was interfaced to a Hewlett-Packard HP5989A Engine mass spectrometer fitted with a thermospray source. Instrument control was via a HP Chem Station.

2.3. Sample preparation

Frozen tissue was pulverised to a fine powder in a domestic food blender. A 1.0-g portion was placed in a 30-ml centrifuge tube and homogenised for 1 min with 1.0 ml of water and 8 ml of methanol. The homogenate was centrifuged at 1500 *g* for 10 min at 4°C. Hereafter, the bound fraction (pellet) and the extractable fraction (supernatant) were treated differently.

2.3.1. Bound residues

The pellet was washed three times with 4 ml of ice-cold methanol, twice with 4 ml of ethanol and twice with 4 ml of diethyl ether. All washings were collected and added to the extractable fraction. The remaining pellet was dried gently under a stream of nitrogen and resuspended in 4 ml of water. Following the addition of 0.5 ml of 1 *M* hydrochloric acid and 150 μl of 2-nitrobenzaldehyde (50 m*M* in dimethylsulphoxide), the tube was stoppered and incubated in a water bath at 37°C for 18 h. Fortified samples were prepared by adding an appropriate volume of AOZ working standard to washed blank tissue immediately prior to the addition of hydrochloric acid and 2-nitrobenzaldehyde.

2.3.2. Extractable residues

Pooled solvent supernatants were evaporated to dryness under nitrogen and the residue was resuspended in 4 ml of water. Hydrochloric acid and 2-nitrobenzaldehyde were added as before and the stoppered tube was incubated at 37°C for 18 h. Recoveries were prepared for the extractable fractions by addition of AOZ standard to the pooled supernatants prior to evaporation.

2.3.3. Extraction of NPAOZ

Following incubation, the pH of both tubes was adjusted to approximately 7.4 by the addition of 5 ml of 0.1 *M* dipotassium hydrogen orthophosphate and 0.5 ml of 0.8 *M* sodium hydroxide. The contents of both tubes were extracted twice with 5 ml of ethyl acetate and the solvent was removed under nitrogen at 50°C. To the tube containing the bound residue fractions, 200 μl of acetonitrile–water (1:1, v/v) were added, the tube was vortex-mixed and the solution was transferred to an auto-sampler vial for LC–MS analysis. To the tube containing the extractable residues, 500 μl of acetonitrile–water (1:1, v/v) were added, the tube was vortex-mixed and then centrifuged at 1500 *g* for 10 min. An aliquot of the supernatant was transferred to an auto-sampler vial for LC–MS analysis.

2.4. LC–MS analysis

The HP Engine was calibrated using a solution of poly(propylene glycol) (PPG) 425 and PPG 725 in methanol–water (25%, v/v) containing 0.1 *M* ammonium acetate. The mobile phase consisted of 0.1 *M* ammonium acetate–acetonitrile (65:35, v/v). For sample analysis, the instrument was operated in the positive ion mode with filament-assisted ionisation. The MS source was maintained at a temperature of 200°C, with a typical thermospray stem temperature of 125°C. The electron multiplier and high energy dynode voltages were typically 2000 and 8000 V, respectively. Full mass spectra were collected in the scan mode, but for maximum sensitivity, samples were analysed using selected-ion monitoring (SIM) of the ion *m/z* 253 with a dwell time of 500 ms. The flow-rate of the mobile phase was 1 ml/min and the sample injection volume was 50 μl. Results were quantified by comparing peak areas of samples with those of standards.

2.5. Validation of the assay

Validation of this assay differed from the normal protocol used in this laboratory, since it is impossible to fortify known negative samples with bound AOZ residues. Instead, we fractionated known negative tissue samples into bound and extractable fractions. At that point, they were each fortified with AOZ at a concentration equivalent to either 10 or 20 ng/g.

This was carried out on six replicates on each of three days for each tissue, at each concentration. The linearity of the assay was established using NPAOZ standards, equivalent to an AOZ concentration of between 0 and 4260 ng/g.

2.6. Stability of AOZ residues in porcine liver

A portion of liver, removed from an animal that had been treated with furazolidone at the therapeutic level for five days, was placed in a polythene bag and stored in a refrigerator at 4°C. At intervals from 0 to 48 h, a 2-g portion of the liver was taken, cut into small pieces, placed in a cryogenic vial and immersed in liquid nitrogen. Samples were subsequently stored at −20°C until analyses for bound and extractable residues of AOZ.

2.7. Ad-hoc survey of AOZ residues in Northern Ireland pigs

One hundred porcine kidneys were randomly selected for analysis from the samples regularly submitted to this laboratory from Northern Ireland meat plants, under a number of residue testing schemes. Kidneys had been removed from the animals at the time of slaughter by Department of Agriculture inspectors, based at each meat plant. Samples had been frozen upon collection, were delivered to the laboratory in sealed boxes and were stored at −20°C prior to analysis. The samples were derived from five meat plants and 70 different pig producers. All samples were received between February and July 1995.

3. Results and discussion

3.1. AOZ assay

A full scan LC–MS spectrum of a 100 μg/ml solution of AOZ is shown in Fig. 2. The M_r of the NPAOZ derivative is 235, however the base peak observed is the M+18 $[M+NH_4]^+$ ion at m/z 253. A small peak at m/z 236, (M+1) $[M+H]^+$ is also observed, however, there are no other significant fragment ions present. Single ion chromatograms for a NPAOZ standard, the bound fraction of a blank

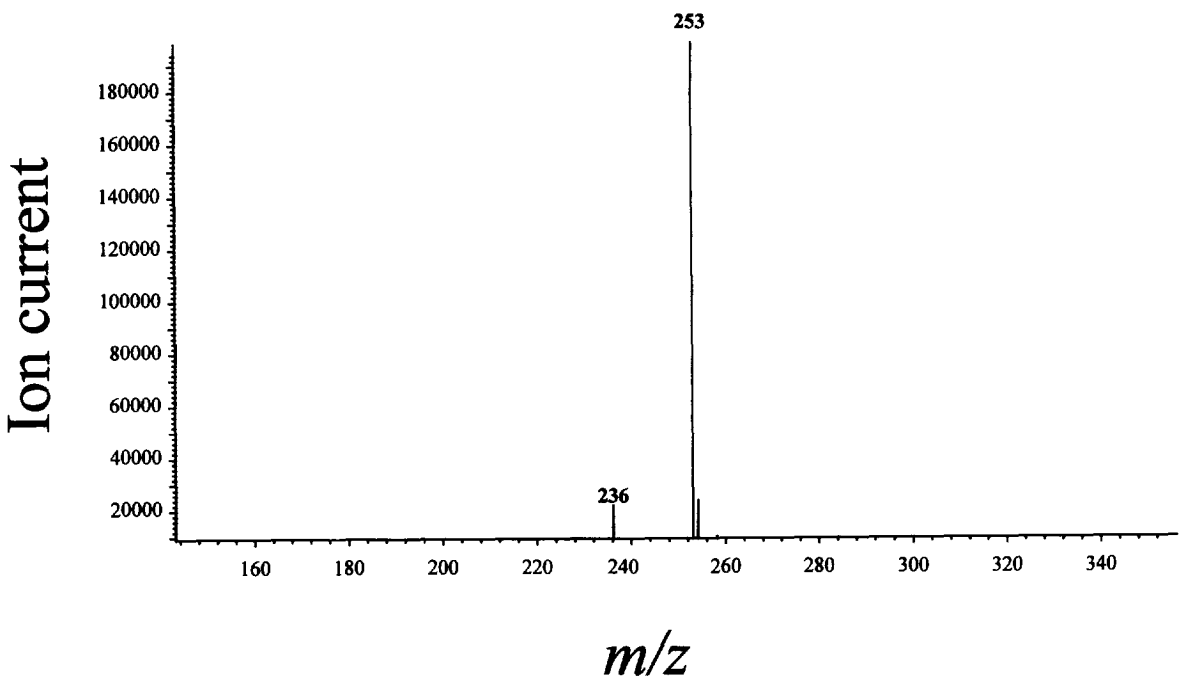


Fig. 2. The positive ion, filament-assisted ionisation, LC–MS spectrum of NPAOZ.

liver, the bound fraction of a blank liver fortified with AOZ (10 ng/g) and the extractable fraction of a blank liver fortified with AOZ (10 ng/g) are shown in Fig. 3. The NPAOZ derivative elutes at 3.2 min and there is no interference from co-eluting compounds in either the extractable or bound sample fractions. Similar chromatograms for an incurred muscle sample are shown in Fig. 4.

The linearity of the assay was checked by running a series of dilute NPAOZ standards, equivalent to AOZ concentrations of 0, 4, 8, 42.5, 85, 425, 850, 1700 and 4260 ng/g. The equation of the best-fit line was $y=412x+43$ ($r=0.9999$).

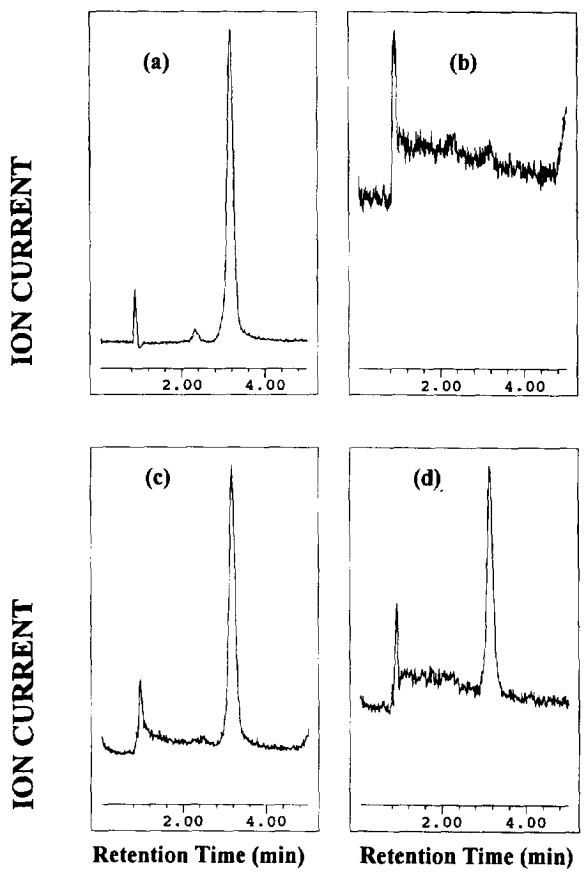


Fig. 3. Single ion chromatograms (normalised) at m/z 253 of 50-μl injections of: (a) NPAOZ standard (equivalent to 20 and 50 ng/g, respectively, in bound and extractable sample fractions); (b) blank liver (bound fraction); (c) blank liver fortified with 10 ng/g AOZ (bound fraction); (d) blank liver fortified with 10 ng/g AOZ (extractable fraction).

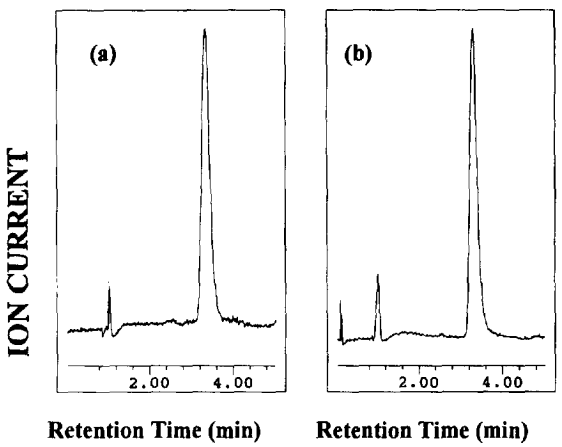


Fig. 4. Single ion chromatograms (normalised) at m/z 253 of 50-μl injections of: (a) bound fraction of an incurred muscle sample containing 10 ng/g AOZ; (b) extractable fraction of an incurred muscle sample containing 16 ng/g AOZ.

The assay was validated by measuring the recovery of AOZ from known negative samples of liver and muscle that had been fortified with AOZ at concentrations of 10 and 20 ng/g on three separate occasions (Table 1). Recoveries were measured in both bound and extractable fractions of the tissue samples. The limit of determination, defined as the smallest analyte concentration for which the method has been validated with specified accuracy and precision [8], for liver and muscle was 10 ng/g. Overall, recoveries in liver and muscle were greater than 80%, with good precision at the concentrations stated. However, recoveries measured in the bound sample fractions do not reflect the extent of AOZ release from bound residues and therefore only serve to indicate the efficiency of the NPAOZ derivatisation procedure in a tissue matrix. The limit of detection of the assay, based on a signal-to-noise ratio of 3:1, was approximately 0.2–0.5 ng/g. The detection limit cannot be defined exactly because of differences in probes, etc.

Extraction of NPAOZ from incubated samples was, at times, hampered by protein gels having formed during the incubation. However, the addition of di-potassium hydrogen orthophosphate and sodium hydroxide and adjusting the pH to approximately 7.4 improved the extraction of NPAOZ into ethyl acetate.

Table 1
Recovery of AOZ from known negative liver and muscle, fortified with AOZ at concentrations of 10 and 20 ng/g

Sample	Day 1		Day 2		Day 3		Overall	
	Bound	Extractable	Bound	Extractable	Bound	Extractable	Bound	Extractable
Liver 10 ng/g								
Mean	10.3	8.9	10.0	9.3	8.7	8.5	9.7	8.9
S.D.	0.92	0.74	1.04	1.46	0.22	0.53	1.06	1.00
C.V.	8.9	8.3	10.4	15.7	2.5	6.2	10.9	11.2
n	6	6	6	6	6	6	18	18
Liver 20 ng/g								
Mean	20.6	19.5	20.0	18.9	18.2	19.4	19.6	19.3
S.D.	1.35	1.25	2.74	0.49	1.27	1.73	2.09	1.21
C.V.	6.6	6.4	13.7	2.6	7.0	8.9	10.7	6.3
n	6	6	6	6	6	6	18	18
Muscle 10 ng/g								
Mean	8.3	9.5	8.5	8.5	7.6	10.0	8.2	9.3
S.D.	0.33	0.99	1.24	0.70	0.70	1.00	0.89	1.06
C.V.	4.0	10.4	14.6	8.2	9.2	10.0	10.9	11.4
n	6	6	6	6	6	6	18	18
Muscle 20 ng/g								
Mean	19.2	18.1	17.9	16.8	16.8	19.7	18.0	18.2
S.D.	0.90	1.72	1.70	1.85	0.85	1.56	1.52	2.00
C.V.	4.7	9.5	9.5	11.0	5.1	7.9	8.4	11.0
n	6	6	6	6	6	6	18	18

Recoveries from extractable and bound fractions are shown. S.D.: standard deviation; C.V.: coefficient of variation.

The presented method has a number of advantages over the procedure described by Hoogenboom et al. [6].

Firstly, the use of furazolidone in livestock production in the EU will be banned, with effect from 1st January 1997. Thereafter, member states will be required to have screening and confirmatory tests in place to monitor for possible furazolidone abuse. AOZ residues offer a suitable marker residue for this purpose. However, methods based on chromatographic analysis, such as the HPLC–UV method described earlier [7], without the use of molecular spectrometric detection are not suitable for use as confirmatory methods [8]. The method presented in the current study provides mass-related information and is therefore acceptable as a confirmatory technique.

Secondly, the previously reported method [7] used methanol at a concentration of 57% for the initial extraction of tissue. However, a greater degree of protein precipitation was achieved when 80% methanol was used. Comparison of the two procedures showed that the AOZ concentrations measured in incurred tissue samples following extraction with 80% methanol could be up to 50% higher than in the same samples extracted with 57% methanol (mean increase 18%, $P<0.01$, Student's t-test, $n=14$). This suggested that some of the bound residues were incompletely precipitated in 57% methanol and were consequently discarded or were considered to be extractable.

Thirdly, the previously described method is subject to interference from other matrix components that react with nitrobenzaldehyde [7]. Using that method, bound NPAOZ elutes on the leading edge of a matrix-derived peak. This interference was particularly noticeable in muscle samples. In the present study, all of the chromatograms obtained by analysing samples containing similar concentrations of incurred AOZ residues (Figs. 3 and 4) are completely free from interference.

Finally, as a result of interference from nitrobenzaldehyde, Hoogenboom et al. [6] did not observe that tissues contained substantial concentrations of extractable AOZ in addition to bound AOZ.

The AOZ side chain is known to be released from

bound metabolites under mildly acidic conditions. Dilute acid was also required for release of AOZ from residues in the extractable sample fractions. Consequently, there is a need for some clarity in deciding which residues are considered to be strictly "non-extractable" and which are "bound". In this context, the terms do not necessarily mean the same thing. Extensive solvent washing of tissue samples was necessary to ensure the removal of all extractable residues that might contain the AOZ side chain. An experiment was performed whereby individual wash fractions were collected from a liver sample with incurred concentrations of AOZ. Measurement of AOZ in these fractions showed that AOZ residues were detectable up to the penultimate ether wash.

Thermospray LC–MS was particularly useful in measuring the extractable AOZ residues, because an extensive clean-up procedure was not required. Using SIM, clean chromatograms were obtained with no interference from excess 2-nitrobenzaldehyde (NBA) reagent.

3.2. Stability of AOZ residues in porcine liver

Storage of *longissimus dorsi* at 4°C for 24 h causes complete degradation of incurred furazolidone residues [3]. Snap-freezing samples in liquid nitrogen and subsequent storage in liquid nitrogen failed to stabilise the compound fully. Therefore, we investigated the in vitro stability of incurred AOZ residues at +4 and −20°C. The concentrations of bound and extractable AOZ residues detected in liver, stored at +4°C, are shown in Table 2. After 48 h, AOZ concentrations in the bound and extractable sample fractions of liver had fallen by 22 and 27%, respectively. However, liver samples that had been frozen and subsequently stored at −20°C showed no significant differences in AOZ concentration after six months of storage (Table 3).

Table 2
Concentration of AOZ in liver stored at 4°C

Sampling period (h)	AOZ (bound) (ng/g)	AOZ (extractable) (ng/g)
1	1840	934
2	1801	950
4	1493	841
6	1382	830
18	1532	1001
24	1484	723
48	1435	679

Results are means of duplicate analyses.

Table 3
Concentration of AOZ in liver stored at −20°C

Sample number	$t=0$		$t=$six months	
	Bound (ng/g)	Extractable (ng/g)	Bound (ng/g)	Extractable (ng/g)
1	301	157	230	159
2	272	144	272	195
3	1540	1410	1417	1537

Results are means of duplicate analyses.

3.3. Ad-hoc survey of AOZ residues in Northern Ireland pigs

Results of the survey are shown in Fig. 5. Of the 100 kidneys analysed, seventeen had detectable concentrations of bound AOZ. Two of these also contained extractable AOZ. Seven of the samples had concentrations of between 1 and 5 ng/g, four were <1 ng/g and one sample was found to have 144 ng/g. The positive samples came from four different meat plants and from fourteen different pig producers. The rapid metabolism of furazolidone, in vivo, has in the past prevented detection of violative

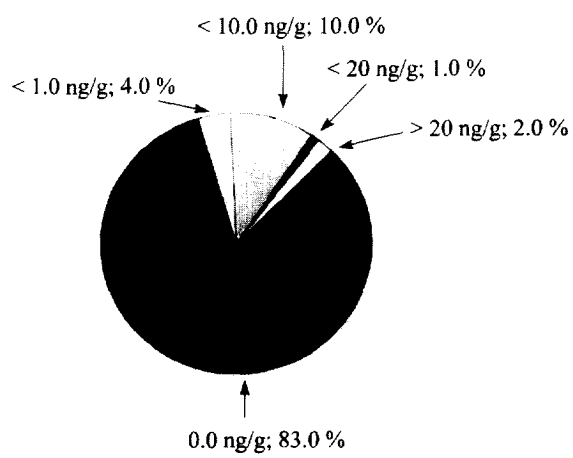


Fig. 5. Concentrations of bound AOZ residues in 100 porcine kidney samples in Northern Ireland.

levels of its residues in animals presented for slaughter. Analysis of 110 samples collected in Northern Ireland, for the UK National Surveillance Scheme, over four years, have shown no evidence of furazolidone residues. This survey indicates that it is now possible to detect residues of furazolidone, using AOZ as the marker. Other than freezing samples after collection, no special treatment is necessary. It has previously been reported that only 14% of bound furazolidone residues are releasable as AOZ [6]. Therefore, any detectable levels of AOZ may indicate that much higher residual levels of furazolidone metabolites are present in tissue. On this basis, even the relatively low concentrations of AOZ found in the positive samples may indicate the presence of potentially harmful residues.

4. Conclusions

The method presented is capable of the unequivocal detection of AOZ in extractable and bound residues in porcine tissues. The data presented on in vitro sample stability indicate that AOZ residues, either extractable or bound, are more suitable markers for furazolidone detection than the parent compound. Using the assay, we have demonstrated that furazolidone use can be detected in pigs presented for slaughter. There is evidence which suggests that furazolidone residues are bioavailable and may be released from ingested animal tissue under the mildly acidic conditions of the human stomach [9]. It is therefore essential that furazolidone residues in edible tissues are monitored to reduce the risk of potentially harmful contaminants reaching the consumer.

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