Rijksinstituut voor Volksgezondheid
en Milieu
*Ministerie van Volksgezondheid,
Welzijn en Sport*

RIKILT
WAGENINGEN UR

FRONT OFFICE FOOD AND PRODUCT SAFETY

Assessment of furazolidone in animal feed: transfer to the fattening pig

Risk assessment requested by:	NVWA-BuRO
Risk assessment drafted by:	RIVM and RIKILT
Date of request:	14-07-2014
Date of risk assessment:	24-07-2014
Project number:	V/090130

Subject

Transfer of furazolidone in animal feed to the fattening pig.

Questions

1. What is the transfer of furazolidone (20 and 500 µg/kg feed, i.e. 20 and 500 ppb) in animal feed to the metabolite 3-amino-2-oxazolidinone (AOZ) in the tissue (liver, muscles, kidneys) of fattening pigs (20–110 kg), over an exposure period of no more than 12 weeks?
2 Under such circumstances, would a washout period of 6 weeks suffice to bring the AOZ content below the minimum AOZ quantification limit of 1 µg/kg tissue?
3. Does the predicted AOZ content in tissue pose a health risk?

Conclusions

1) At an exposure level of 20 µg/kg feed during 12 weeks, AOZ concentrations in muscle tissue remain below 1 µg/kg tissue. In the liver and kidneys, this content may increase up to a respective 3.8 and 17.8 µg/kg tissue. For the liver (but not for kidneys), a washout period of 6 weeks will suffice, to return the content to below 1 µg/kg tissue.
2) At an exposure level of 500 µg/kg feed for 12 weeks, the AOZ content of muscles, liver and kidneys may increase to a respective 3.1, 98 and 447 µg/kg tissue. In the liver and kidneys, this content will still be well over 1 µg/kg tissue after a washout period of 6 weeks.
3) AOZ concentrations in the liver, kidneys and muscles, following a maximum transference from feed containing 500 µg furazolidone/kg, pose a negligible risk of cancer under normal consumption levels, even if the predicted content is well over the minimal AOZ quantification limit of 1 µg/kg tissue.

Introduction

Furazolidone is a substance, the use of which is prohibited in food-producing animals. Despite this ban, the substance has been found in animal feed. The case at hand concerns the occurrence of furazolidone in pig feed (20 or (maximally) 500 µg/kg feed) and the corresponding transfer of furazolidone to its metabolite 3-amino-2-oxazolidinone (AOZ) in muscles, liver and kidneys of fattening pigs.

The furazolidone concentration levels in feed as mentioned above are representative of the content levels recently found in pig feed by the Netherlands Food and Consumer Product Safety Authority (NVWA). In actual practice, the presence of furazolidone in the feed has led to an exposure time for the animals of (maximally) three months, followed by a washout period of (currently) 1.5 weeks. Because these animals cannot be brought to slaughter, the washout period will increase further. For this assessment, it was assumed that this period will not be more than 6 weeks in total.

The fate of furazolidone in pigs

Following uptake, the furazolidone in pigs is rapidly excreted from their body. A single amount of 600 mg furazolidone (equivalent to an intake through feed with a content of 400 mg furazolidone/kg) could no longer be found in liver, kidney and muscle tissue 12 hours after exposure (McCracken et al., 1995). Exposure of pigs to furazolidone leads to the forming of protein-bound metabolites, particularly furazolidone metabolites containing the 3-amino-2-oxazolidinine (AOZ) side chain. Such metabolites are of toxicological importance because of the reasons below (Hoogenboom *et al.,* 1992):

- Under mildly acidic circumstances (stomach), the AOZ side chain may separate from the protein-bound metabolites. Through this mechanism, the consumption of products that originated from animals that were exposed to furazolidone (and therefore contain both protein-bound and unbound AOZ) may lead to AOZ exposure in humans.
- Metabolism may turn AOZ into β-hydroxy-ethylhydrazine. This last compound is both mutagenic and carcinogenic.
- AOZ gives a positive response in bone marrow micronucleus tests (Hoogenboom *et al.*, 2002).
- The half-life of protein-bound metabolites is much longer than that of the parent compound furazolidone. Thus, AOZ is found in the body for weeks (Hoogenboom et al., 1992, McCracken et al., 1997; Cooper et al., 2005; Liu et al., 2010). This effect is likely to be caused by the protein turnover in the tissue.

Transfer model: base definition

The fact that furazolidone is discharged from the body much faster than its metabolite AOZ means that AOZ concentrations in organs can be directly related to the intake of furazolidone in feed (for details, see Annex 1). The connected transfer model states that the rate at which the amount of AOZ in the tissue changes ($\frac{dA_{AOZ,tissue}}{dt}$) results from the increase rate which equals a fraction (α_{tissue}) of the daily dose of furazolidone (D) and the decrease rate which equals the amount of AOZ in tissue ($A_{AOZ,tissue}$) and an elimination rate constant of (k_A). In mathematical terms, this translates to the following differential equation:

$$\frac{dA_{AOZ,tissue}}{dt} = \alpha_{tissue} \cdot D - k_A \cdot A_{AOZ,tissue}$$

In which:

$\frac{dA_{AOZ,tissue}}{dt}$	is the rate at which the amount of AOZ changes in the tissue of animals exposed to furazolidone (mg/day)
α_{tissue}	is the constant in the transfer of furazolidone from feed to AOZ in certain tissue types (dimensionless)
D	is the furazolidone intake (mg/day)
k_A	is the AOZ elimination rate constant (day^{-1})
A_{AOZ}	is the amount of AOZ in tissue (mg)

The model contains two parameter values, α_{tissue} and k_A, which can be estimated from experimental observations. For the conversion of AOZ amounts into AOZ concentrations, the tissue weight must also be known (see Annex 2).

Transfer model: calibration

In McCracken et al. (1997), pigs (with a body weight of 20 kg) were exposed for five days to feed with a furazolidone content of 400 mg/kg (feed intake: 1 kg/day). This was followed by a period of 42 days in which the animals were fed on furazolidone-free feed. During this washout period, the half-life of AOZ was estimated in kidneys (5.9 days), muscles (9.1 days) and liver (5.8 days) (note that the half-life with respect to one-compartment kinetics can be converted to the required parameter k_A).

In a second experiment by McCracken et al. (1997), pigs (with a body weight of 20 kg) were exposed for 5 days to feed with a furazolidone content of 0, 0.5, 2.3, 7.9 or 18.4 mg/kg feed (feed intake: 1 kg/day). At the end of this exposure period, AOZ concentrations in kidneys, muscles and liver were estimated[1] (see Annex 3). Given the abovementioned elimination rate constants, for each of the examined doses in this experiment, the transfer constant α_{tissue} from feed to tissue was determined (see Table

[1] digitalised from the publication, using DigitizeIt software

1). Table 1 shows that, in this study, this constant did not depend on either feed concentration or tissue type.

Table 1 Transfer constant for furazolidone from feed to AOZ in the liver, muscles or kidneys of pigs (McCracken et al., 1997).

Feed concentration (mg/kg)	Transfer constant (α_{tissue})		
	Liver	Muscles	Kidneys
0.5	0.0026	n/a	0.0033
2.3	0.0027	0.0026	0.0020
7.9	0.0020	0.0020	0.0018
18.4	0.0038	0.0034	0.0029

n/a: no data available

In Liu et al. (2010), pigs (with a body weight of 15–18 kg) were exposed for 7 days to feed with a furazolidone content of 400 mg/kg (feed intake: 0.8 kg/day). This was followed by a period of 63 days during which the animals were fed on furazolidone-free feed. By the end of the exposure period, AOZ concentrations were found in plasma (1969 µg/l, muscles (691 µg/kg), liver (2170 µg/kg) and kidneys (1012 µg/kg), while the washout period was used for determining the half-life of AOZ in the various tissue types (plasma: 13.7 days, liver: 13.6 days, kidneys: 13.6 days and muscles: 15.0 days). This shows that, in this study, the half-life did not depend on the type of tissue. In comparison, Cooper et al. (2005) reported an AOZ half-life of 11.5 days in muscles, 7.3 days in liver and 6.9 days in kidneys. Table 2 provides the transfer constant α_{tissue}, based on that determined in the Liu study.

Table 2 Transfer constant for furazolidone from feed to AOZ in the liver, muscles or kidneys of pigs (Liu et al., 2010).

Feed concentration (mg/kg feed)	Transfer constant (α_{tissue})		
	Liver	Muscles	Kidneys
400	0.00071	0.0030	0.000055

In Hoogenboom et al. (1992), pigs (aged 12 weeks; body weight 30–40 kg) were exposed for 7 days to feed with a furazolidone content of 300 mg/kg (feed intake: 1.2 kg/day). This was followed by a period of 28 days in which the animals were fed on furazolidone-free feed. By the end of the exposure period, AOZ concentration levels were measured in muscles (144 µg/kg), liver (1070 µg/kg) and kidneys (765 µg/kg). Hoogenboom et al. (1992) reports no organ-specific half-life. Therefore, to calculate the transfer constant α_{tissue} for this study (see Table 3), for each organ, the shortest known half-life was used, i.e. the half-life from the McCracken study. This is a 'worst-case' approach, with respect to the estimation of the transfer constant.

Table 3 Transfer constant for furazolidone from feed to AOZ in the liver, muscles or kidneys of pigs (Hoogenboom et al., 1992).

Feed concentration (mg/kg feed)	Transfer constant (α_{tissue})		
	Liver	Muscles	Kidneys
300	0.00075	0.0017	0.000090

Transfer model: simulations

The model calibrations in the studies named above show mutual differences in transfers constants and the AOZ half-life in tissue types. For example, the study with a relatively low exposure to furazolidone (McCracken study) gives a relatively high transfer constant for liver and kidneys, compared to the two studies with a relatively high exposure to furazolidone (Liu and Hoogenboom studies). All three studies, however, give a comparable transfer constant for muscle tissue.

With respect to AOZ half-life, Liu et al. (2010) states around two weeks, while, in contrast, McCracken et al. (1997) provide a half-life that varies from 4.4 to 9.1 days. In addition, Cooper et al. (2005) reports an AOZ half-life of 11.5 days for muscles, 7.3 days for liver and 6.9 days for kidneys (in 8-week old pigs that were exposed for 10 days to 400 mg furazolidone/kg feed, followed by a washout period of 6 weeks). Furthermore, the studies by McCracken, Hoogenboom and Liu are limited to animals with a more or less constant body weight, varying from 15 to 40 kg. In practice, however, exposure to furazolidone occurs mainly in growing animals.

In order for optimally fitting practical simulations to model calibration results, the calibrated transfer model was expanded with growth data on Dutch fattening pigs in 2014 and the connected feed intake (for details, see Annex 2). Moreover, the practical simulations distinguished between a ‘maximum case’ scenario (highest transfer constant and longest AOZ half-life) and a ‘minimum case’ scenario (lowest transfer constant and shortest half-life). In this way, uncertainties in the experimentally determined parameters for the transfer of furazolidone to AOZ and the AOZ half-life were also taken into account.

Simulations were conducted at furazolidone levels of 20 and 500 µg/kg feed. These levels are representative of that in the contaminated pig feed found by the Netherlands Food and Consumer Product Safety Authority. Furthermore, a continuous exposure of 12 weeks was assumed. This period is comparable with the maximum possible exposure period at pig farms. And, lastly, the simulations began with exposure to polluted feed of pigs aged 8 weeks (body weight: 20 kg), because the model calibration was conducted with pigs from this body weight onwards. The simulations thus assume that animals up to the age of 8 weeks have not been exposed to furazolidone. Therefore, should there have been any such exposure in actual practice, then the presented simulations would underestimate the AOZ accumulation in the animals’ organs. An overview of simulation specifications is provided in Box 1.

The simulations show that, at a feed concentration of 20 µg/kg, for the 'minimum case' scenario, after an exposure of 12 weeks, AOZ concentrations in all three tissue types remained far below the minimum AOZ quantification level of 1 µg/kg (Figure 1). For the 'maximum case' scenario, AOZ concentrations exceeded the limit of 1 µg/kg in liver and kidneys, but not in muscle tissue (Figure 2). For liver tissue, a washout period of 6 weeks was sufficient for levels to return below 1 µg/kg; for kidneys, however, this period was found to be insufficient. From the simulation may be concluded that an exposure period of 12 weeks to 20 µg/kg furazolidone in feed may lead to exceedance of the minimum AOZ quantification level in the liver and kidneys of pigs. For this level of exposure, a washout period of 6 weeks is not long enough to cause the content in the kidneys to return to the AOZ quantification level of 1 µg/kg.

At feed concentrations of 500 µg/kg, AOZ concentrations even below the level of the 'minimum case' scenario (Figure 1), exceeded the minimum quantification level of 1 µg/kg in all three tissue types. Here, a washout period of 6 weeks was found to suffice for lowering the level to below 1 ppb, for all three tissue types. Under the 'maximum case' scenario (Figure 2), AOZ concentrations were found to far have exceeded the limit of 1 µg/kg, in all three tissue types. For muscle tissue, a washout period of 6 weeks would suffice to bring the level at the end of the exposure period back to below 1 µg/kg. For liver and kidneys, this period of 6 weeks would not suffice. From these simulations, it may be concluded that exposure to 500 µg/kg furazolidone in feed for a period of 12 weeks would lead to exceedances of the minimum AOZ quantification level in the liver, kidneys and muscles of pigs. In this case, a washout period of 6 weeks would not be sufficient for the content in liver and kidneys to return to this minimum level.

Transfer model: health risks of simulated tissue concentrations

As described, the furazolidone in pig feed, in actual practice, led to an exposure period of maximally three months; followed by a washout period of 1.5 weeks. Thus, this washout period of 1.5 weeks reflects the highest projected AOZ tissue concentration that could be found in these animals, at that time. Assuming 500 µg furazolidone/kg feed ('maximum case'), the simulated AOZ concentration in kidneys would be 300 µg/kg. In the liver this would be around 60 µg/kg, and in muscle tissue around 2 µg/kg. To obtain a sense of the possible risks to consumers in relation to such content levels, a margin of exposure (MOE) may be calculated on the basis of predicted daily intake levels that may be expected to cause cancer. This intake level was set at the so-called BenchMarkDose10 (BMD10) – in other words, the daily life-long intake for which the chance of developing cancer is 0.10. For AOZ may be calculated that the BMD10 range runs from 11 to 2000 mg/kg (Front Office Food and Product Safety, 2014). Table 4 summarises the MOEs, for the three tissue types and under certain assumed consumption levels, using a lower bound (LB)/upper bound (UB). These reflect the uncertainties in the predicted BMD10 for AOZ carcinogenicity.

Table 4 Margin of Exposure (MOE) under a certain consumption level of kidney, liver and muscle tissue and a 'maximum case' AOZ concentration per tissue type, assuming a BMD10 of 11 (LB) and 2000 (UB) mg/kg body weight/day.

Tissue type	'Maximum case' concentration (µg/kg)	Consumption (g)	Intake (µg)[2]	Intake (µg/kg bw)[3]	MOE (LB)	MOE (UB)
Kidneys	300[1]	165	50	1	1.1×10^4	2×10^6
Liver	60	330	20	0.4	2.8×10^4	5×10^6
Muscles	2	500	1	0.02	5.5×10^5	100×10^6

[1] Sum of unbound and protein-bound AOZ
[2] Assuming 100% absorption of the AOZ evident in tissue. For AOZ evident in tissue, Gottschall and Wang (1995) report a bioavailability in rats of maximally 40%.
[3] Illustrative body weight of 50 kg.

The European Food Safety Authority (EFSA) uses a content level for carcinogenic substances of 10,000 as the margin of exposure (MOE), as the level at which there is no reason for concern. As the table shows, all calculated MOEs are above this level. Moreover, this MOE of 10,000 is assumed to apply to life-long, daily exposure. In this case, however, in all probability, it only concerns single exposures for individual consumers. Therefore, the predicted AOZ content in pigs, based on the maximum level of furazolidone in pig feed of 500 µg/kg, may be considered to pose a negligible risk of cancer, even though the content is well over the minimum AOZ quantification limit of 1 µg/kg tissue.

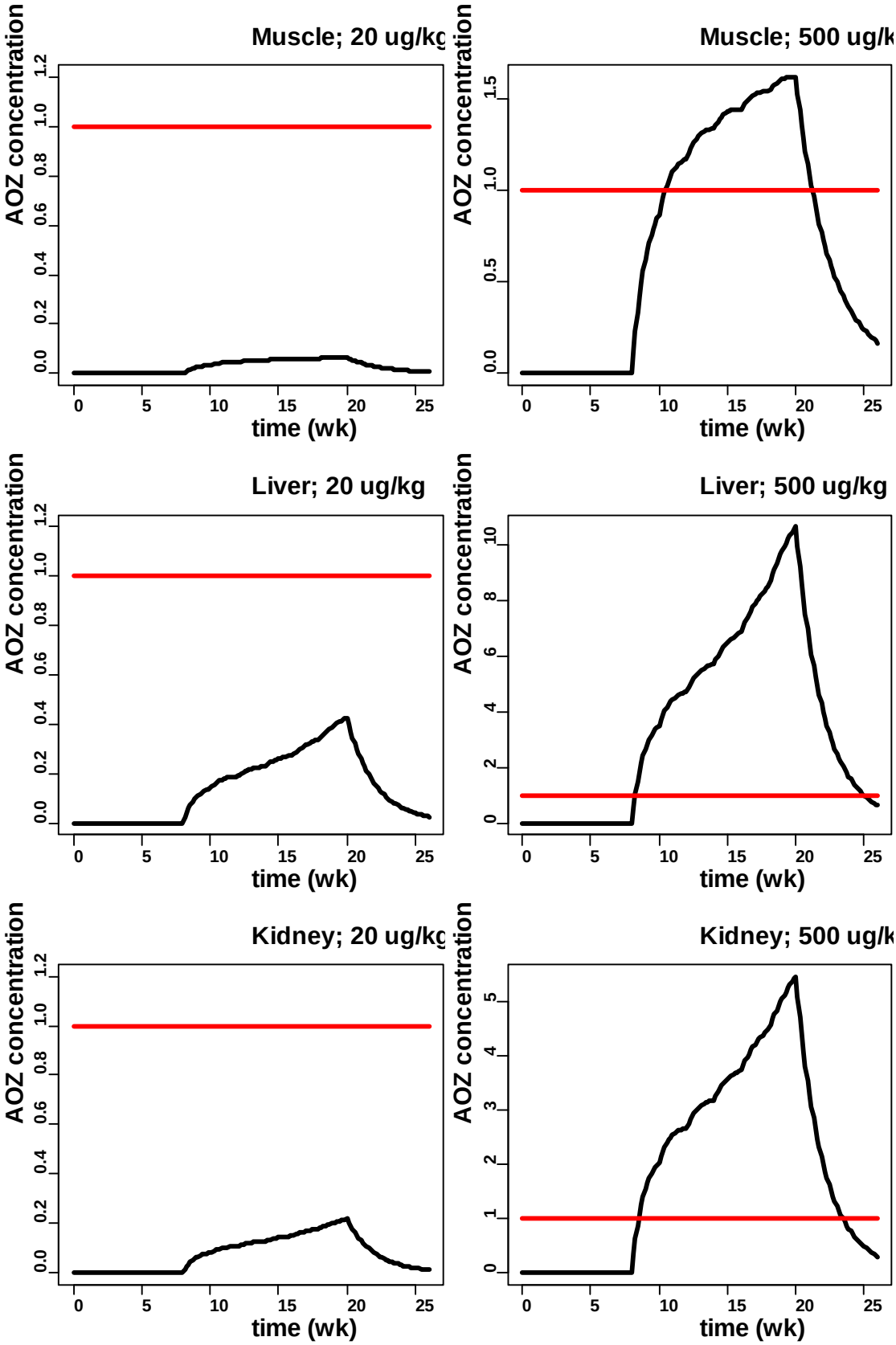


Figure 1 'Minimum case' scenario simulations of the accumulation of AOZ in the liver, muscles and kidneys of fattening pigs exposed to 20 or 500 µg/kg furazolidone in their feed. The exposure ran from week 8 to 20.

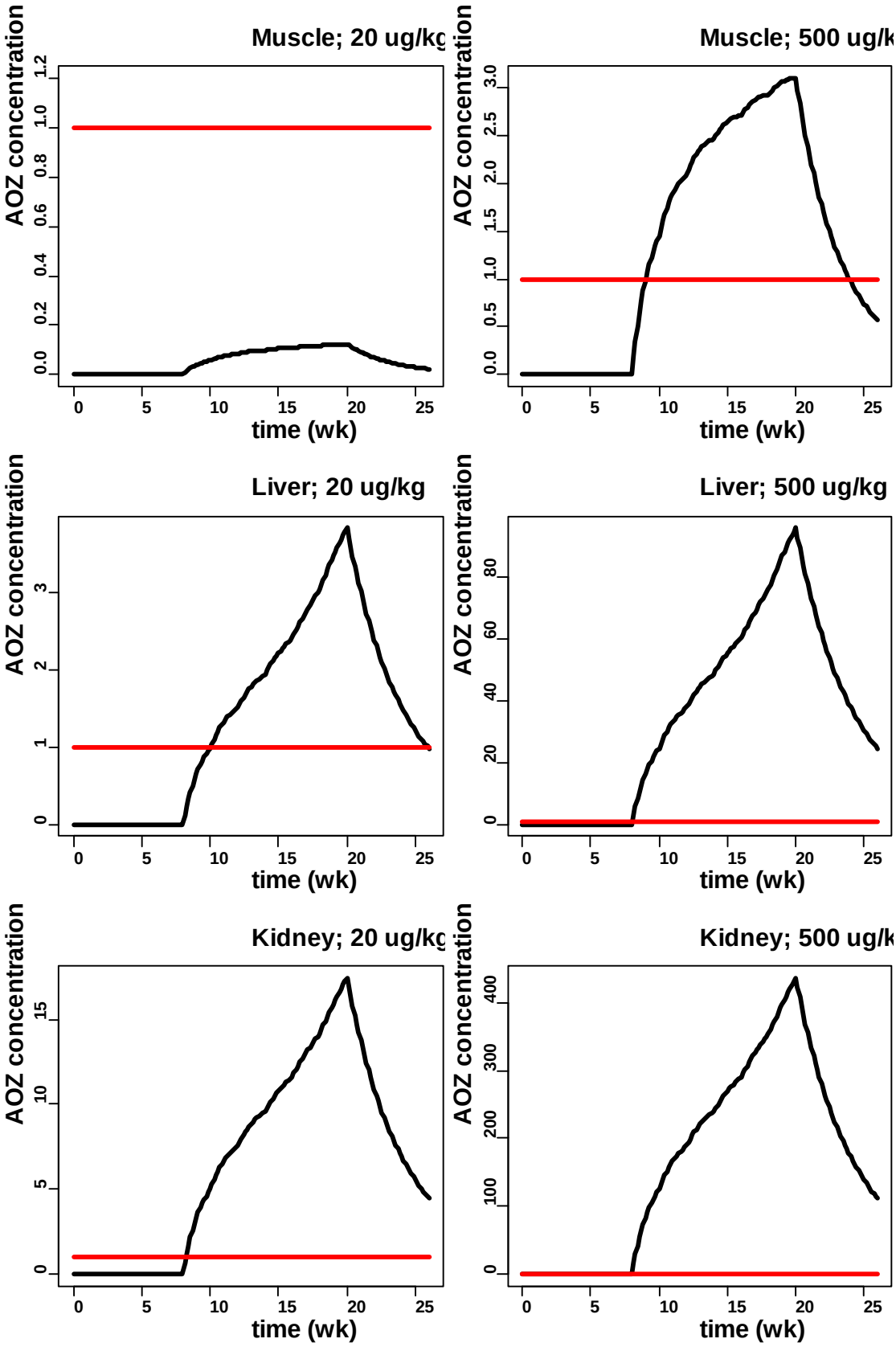


Figure 2 'Maximum case' scenario simulations of the accumulation of AOZ in the liver, muscles and kidneys of fattening pigs exposed to 20 or 500 µg/kg furazolidone in their feed. The exposure ran from week 8 to 20.

BOX 1: Overview of simulation specifications

Feed regime:

Week 0 to 8:	clean feed
Week 8 to 20:	contaminated feed (20 or 500 µg/kg, in line with the maximum exposure period as could have occured at pig farms)
Week 20 to 26:	washout period on clean feed (washout period, in practice, is currently 1.5 weeks)

During the simulation, the animals reached their slaughter weight of 110 kg after 22 weeks. Subsequently, they grew another 4 weeks, reaching their end weight of 150 kg at 26 weeks. As stated, the period between 22 and 26 weeks must be seen as extrapolation (Figures 1 and 2).
The red line in the first two figures represents the analytical minimum AOZ quantification level in tissue (1 µg/kg).

A. 'Minimum case' simulation

Parameter specifications:
Muscle transfer constant = 0.0023
AOZ half-life = 10.3 days
Liver transfer constant = 0.00073
AOZ half-life = 6.6 days
Kidney transfer constant = 0.000073
AOZ half-life = 6.4 days

B. 'Maximum case' simulation

Parameter specifications:
Muscle transfer constant = 0.0034
AOZ half-life = 15 days
Liver transfer constant = 0.0038
AOZ half-life = 13.6 days
Kidneys transfer constant = 0.0033
AOZ half-life = 13.6 days

References

Cooper, K.M., Mulder, P.P.J., van Rhijn, J.A., Kovacsics, L., McCracken, R.J., Young, P.B. and D.G Kennedy (2005). Depletion of four nitrofuran antibiotics and their tissue-bound metabolites in porcine tissues and determination using LC-MS/MS and HPLC-UV. Food Additives and Contaminants 22: 406–414.

Front Office Voedsel- en Productveiligheid [*Food and Product Safety*] (2014). Beoordeling toxicologie furazolidon and 3-amino-2-oxazolidinione (AOZ) [*Assessment toxicology furazolidone and 3-amino-2-oxazolidinione*]. G. Wolterink, S. ten Voorde and W. Slob. National Institute for Public Health and the Environment (RIVM), Bilthoven.

Gottschall, D.W and R. Wang (1995). Depletion and bioavailability of [^{14}C] furazolidone residues in swine tissues. Journal of Agricultural and Food Chemistry 43: 2520–2525.

Hoogenboom, L.A.P., Berchmans, M.C.J., Polman, T.H.G., Parker, R. and I.A. Shaw (1992). Depletion of protein bound furazolidone metabolites containing the 3-amino-2-oxazolidone side-chain from liver, kidney and muscle tissues from pigs. Food Additives and Contaminants 9: 623–630.

Hoogenboom, L.A.P., van Brichem, G.D., Sonne, K., Enninga, I.C., van Rhijn, J.A., Heskamp, H., Huveneers-Oorsprong, M.B.M., van der Hoeven, J.C.M. and H.A. Kuiper (2002). Absorption of a mutagenic metabolite released from protein-bound residues of furazolidone. Environmental Toxicology and Pharmacology 11: 273–287.

Liu, Y., Huang, L., Wang, Y., Yang, B., Ishan, A., Fang, K., Peng, D., Liu, Z., Dai, M. and Z. Yuan (2010). Tissue depletion and concentration correlations between edible tissues and biological fluids of 3-amino-2-oxazolidinonen in pigs fed with a furazolidone-mediated feed. Journal of Agricultural and Food Chemistry. 58: 6774–6779.

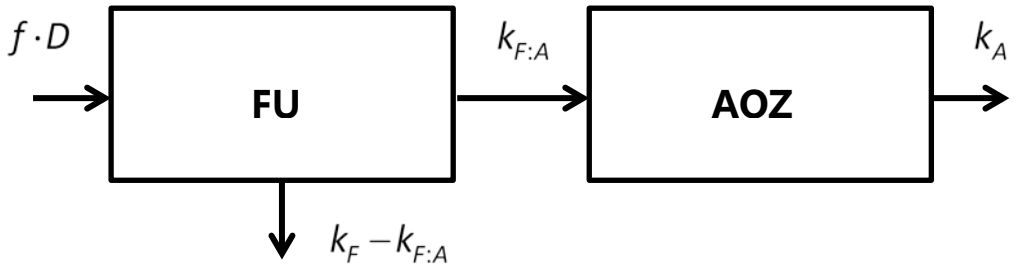
McCracken, R.J., Blanchflower, J., Rowan, C., McCoy, M.A. and D.G. Kennedy (1995). Determination of furazolidone in porcine tissue using thermospray liquid chromatography-mass spectroscopy and a study of the pharmacokinetics and stability of its residues. Analyst 120: 2347–2351.

McCracken, R.J., McCoy, M.A. and D.G. Kennedy (1997) The prevalence and possible causes of bound and extractable residues of their furazolidone metabolite 3-amino-2-oxazolidone in porcine tissues, Food Additives and Contaminants 14: 287-294.

Raamsdonk, L.W.D. van, Kan, C.A., Meijer, G.A.L. en P.A. Kemme (2007). Kengetallen van enkele landbouwhuisdieren en hun consumptiepatronen [*Index numbers on certain farm animals and their feeding patterns (in Dutch)*]. RIKILT rapport 2007.010. RIKILT-Instituut voor Voedselveiligheid [*institute of food safety*], Wageningen UR, Wageningen. Available online from: www.rikilt.wur.nl.

Annex 1 Kinetic model for furazolidone and its metabolite AOZ

It is assumed that furazolidone (FU) and its metabolite AOZ (AOZ) can *both* be described by 1-compartment kinetics (see Figure A1.1 below).



A fraction *f* of FU-dose *D* enters the systemic circulation. The dose applied is eliminated from the system at elimination rate k_F , part of which is metabolised to AOZ at rate $k_{F:A}$. AOZ itself is eliminated at rate k_A. The resulting concentration-time curve for AOZ can be described by:

$$A_{AOZ}(t) = \frac{k_{F:A}}{k_F} \cdot \frac{fD}{k_A} \cdot \left(1 - \frac{k_A e^{-k_F t} - k_F e^{-k_A t}}{k_A - k_F} \right)$$

This model can be further simplified by taking into account that FU-elimination kinetics is much faster than AOZ-elimination kinetics, most probably because the latter is determined by binding to plasma proteins.
Then, $k_A e^{-k_F t}$ is negligible soon after the onset of contamination relative to $k_F e^{-k_A t}$ and the amount of AOZ can be well approximated by:

$$A_{AOZ}(t) = \frac{k_{F:A}}{k_F} \cdot \frac{fD}{k_A} \cdot \left(1 - e^{-k_A t} \right)$$

However, it would be more convenient to express this in the format of a differential equation:

$$\frac{dA_{AOZ}}{dt} = \frac{k_{F:A}}{k_F} \cdot f \cdot D - k_A \cdot A_{AOZ}$$

Nevertheless, the distribution of FU and AOZ to the various tissue types may be different, and in this simple modelling approach, due to the lack of sufficient data, a specific tissue approach is required; namely one that relates the applied dose to the observed tissue concentrations:

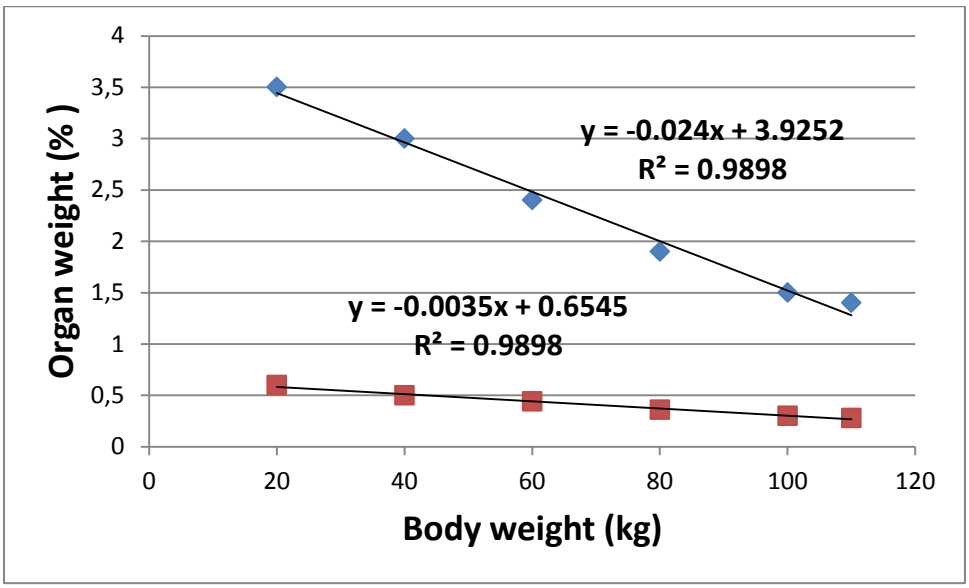
$$\frac{dA_{AOZ,tissue}}{dt} = \alpha_{tissue} \cdot D - k_A \cdot A_{AOZ,tissue}$$

where the factor α contains general kinetic ($k_{F:A} / k_F$) and absorption (*f*) information together with tissue-specific partition information.

Growing pigs are modelled (see Annex 2). Accordingly, the elimination rate parameter k_A is scaled allometrically. Note that α_{tissue} is composed of the ratio of the rate constants $k_{F:A}$ and k_F, i.e. $k_{F:A} / k_F$, and therefore is independent of body weight.

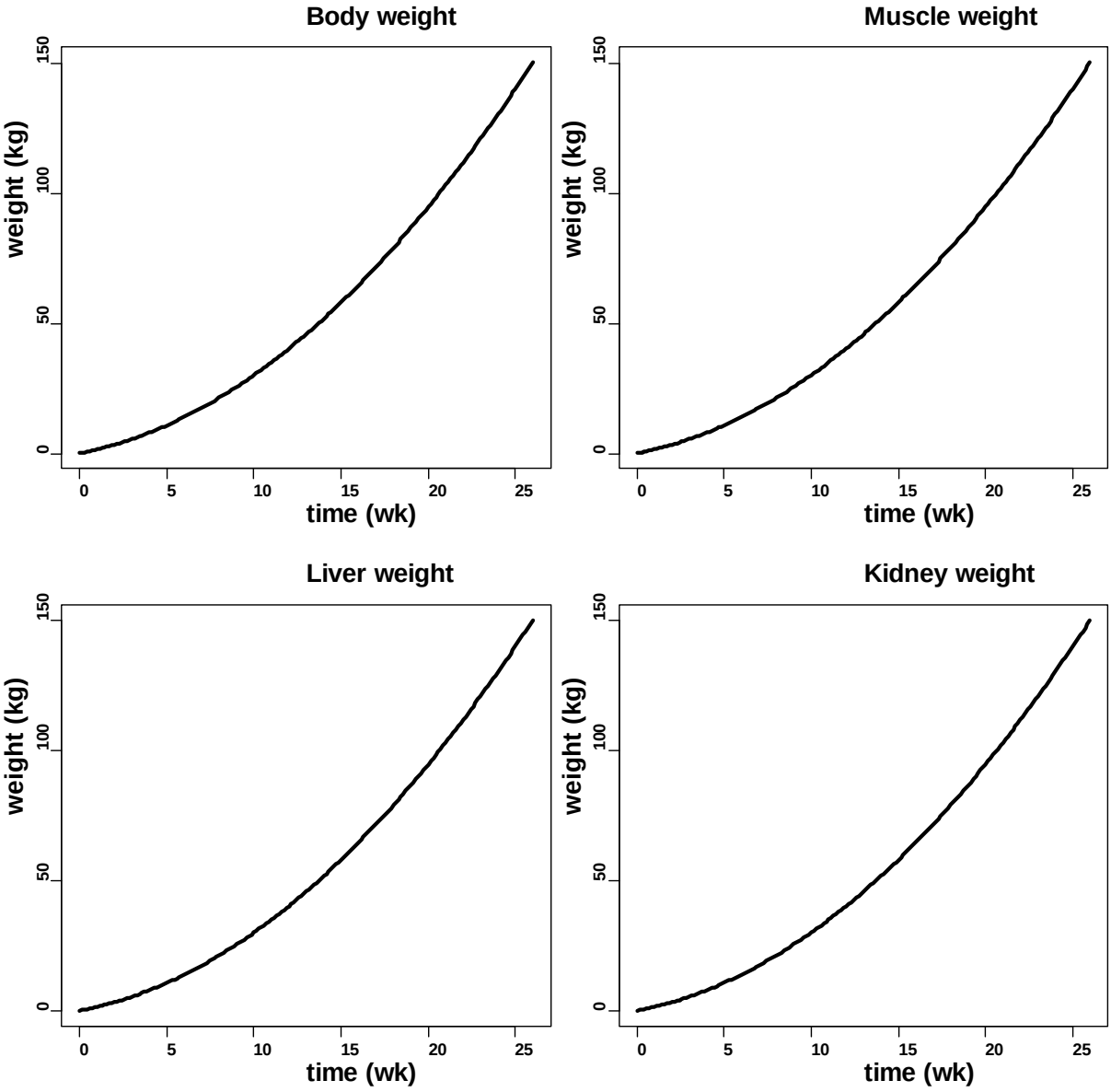
Annex 2 Body weight and tissue weight

Relative weights of liver tissue (blue diamonds) and kidney tissue (red squares), as derived from Raamsdonk et al. (2007)(see the figure A2.1 below).



Data from 2014 on the body weight and feed consumption of Dutch fattening pigs up to the age of 22 weeks were made available by the Netherlands Food and Consumer Product Safety Authority (NVWA). As indicated below, increases in body weight over time may be described according to a quadratic function.

The part of the presented connection between the ages of 22 and 26 weeks, therefore, must be considered an extrapolation. Here, it was assumed that the absolute weight of liver and kidneys would remain constant after the pigs reached their slaughter weight (see the figure A2.2 below). Increases in body weight above the slaughter weight, therefore, are due to increases in muscle and tissue types other than liver and kidneys; for example body fat.
For muscle weight, a relative body weight of 50% was assumed (for pigs in 2007, as reported by Raamsdonk et al. (2007); here between 43% and 49%).


Body weight
weight (kg)
time (wk)
Muscle weight
weight (kg)
time (wk)
Liver weight
weight (kg)
time (wk)
Kidney weight
weight (kg)
time (wk)

Annex 3 AOZ tissue concentrations as scanned from McCracken *et al.* (1997, Figure 3)

Administered furazolidone (mg/kg feed)	AOZ[1] concentration (ng/g tissue)			AOZ amount (mg)		
	liver	muscles	kidneys	liver	muscles	kidneys
0.5	7.09	n/a	5.23	0.004963	n/a	0.006276
2.3	33.83	2.51	14.64	0.023681	0.0251	0.017568
7.9	83.4	6.64	43.5	0.05838	0.0664	0.0522
18.4	381	26.06	168.4	0.2667	0.2606	0.20208

[1]AOZ as the sum of bound and unbound