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Risk evaluation of nitrofurans in animal food products

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Abstract

Residues from non-allowed pharmacologically active substances are sometimes found in food products of animal origin in EU border controls. Nitrofurans are one such class of substances, to which nitrofurazone, nitrofurantoin, furazolidone and furaltadone belong. Nitrofurans have been used in both human and veterinary medicine for their antibacterial and antiprotozoal activities. In the 1990s, they were completely banned from use in food producing animals within the EU due to their genotoxicity. The only nitrofuran still in use is nitrofurantoin, which is utilised in human medicine to treat urinary tract infections. The aim of this thesis was to review the research on nitrofurans and to determine if any levels can be allowed in food products of animal origin. All substances, except for furaltadone, have shown to be genotoxic and mutagenic *in vitro*. No clear conclusions regarding the genotoxicity *in vivo* could be drawn due to contradicting results. Concerning the reproductive toxicity of these compounds only nitrofurazone and nitrofurantoin have clearly shown that they are toxic to the reproductive system of animals. The lowest daily dose which caused adverse effects on the reproduction was 10 mg nitrofurazone per kg BW. In carcinogenicity tests the most commonly observed effect was an increase in mammary tumours. Nitrofurazone and furazolidone was shown to be carcinogenic, while nitrofurantoin may be carcinogenic. The lowest dose that caused this effect was 0.16 mg nitrofurazone per kg BW.

The margin of exposure (MoE) approach was used in order to determine the risk for the Swedish population from nitrofurans in food products of animal origin. Several benchmark dose lower confidence limits (BMDLs) were derived from carcinogenicity studies and the lowest BMDL was chosen for the MoE. The exposure to nitrofurans for adults and children were estimated from intake data in Riksmaten, a Swedish national dietary survey, and the level in food was assumed to be 1 µg/kg. The MoE for adults only exposed to nitrofurans via medical products was calculated. It is considered to be a risk when the MoE value is lower than 10000. The MoEs for adults treated with nitrofurantoin for urinary tract infections were below 10000 indicating that there may be a risk for those on a course of treatment with nitrofurantoin. Hence, the safety of nitrofurantoin as a drug used in human medicine should be reevaluated. The MoEs indicated that there is a negligible risk to the health of the Swedish population from nitrofurans in food at the level of 1 µg/kg. If all food products of animal origin consumed in one day contain 8 µg/kg there may be a risk to human health. Therefore, it is advised that the current reference point of action (RPA) of 1 µg/kg should be

retained. Food products of animal origin containing nitrofurans over this level should not be allowed to enter the market, thus protecting the health of the Swedish population.

1 Introduction

Residues from pharmacologically active substances can be present in food from animals treated with veterinary medicinal products (VMPs) prior to slaughter, sampling of milk, eggs and honey. In Regulation (EEC) No 2377/90, later repealed by Regulation (EC) 470/2009, the European Council stated that in order to protect consumers from the potentially harmful effects of these residues maximum residue limits (MRLs) in food of animal origin should be established for all pharmacologically active substances used in VMPs to treat food producing animals. The substances could fall into four annexes. Annex I contained a list of the substances for which MRLs had been established. Annex II contained a list of the substances for which no MRLs were necessary because they pose no risk to the public health, although with some exceptions. Annex III contained a list of the substances for which provisional MRLs had been established. Annex IV contained a list of the substances for which MRLs could not be established because they pose a risk to the public health at any concentration. The allowed substances and their MRLs are now presented in the first table in Commission Regulation (EU) No 37/2010. The prohibited substances from annex IV are found in the second table of the same regulation.

In Council Regulation (EEC) No 2309/93, later repealed by Regulation (EC) No 726/2004, it is stated that the applicant shall submit an application for a VMP to the European Medicines Agency (EMA) who will form opinions on MRLs. The risk assessments and opinions of the EMA on MRLs are formulated by the Committee for Medicinal Products for Veterinary Use (CVMP), established by Council Directive (EEC) No 81/851, after reviewing the data provided by the applicants concerning the toxicological and pharmacological effects, pharmacokinetics and pharmacodynamics, and also physicochemical properties of the substances as well as validated analytical methods (Council Directive 81/851/EEC, Council Regulation (EEC) No 2309/93). The provided opinion consists of a scientific risk assessment and risk management recommendations (Regulation (EC) No 470/2009).

In order to establish MRLs the CVMP first reviews the toxicological, pharmacological, pharmacokinetic, microbiological and other studies submitted (Figure 1) (European Commission 2005). The results from acute and repeated dose toxicity studies,

mutagenicity, carcinogenicity and reproductive toxicity studies, and sometimes other studies, are needed to form a MRL. From the toxicological studies the no observed adverse effect (NOAEL), with respect to the most sensitive parameter in the most sensitive appropriate test species, is selected. An acceptable daily intake (ADI) is calculated by dividing the NOAEL with uncertainty factors. Uncertainty factors are mainly used to account for inter- and intraspecies differences, normally 100, but in certain cases additional uncertainty factors must be applied to account for data limitations or certain unwanted effects. To relate the ADI to bodyweight the ADI is multiplied by 60, an arbitrary defined average human bodyweight (kg). In order to establish MRLs from the ADI the levels of consumption of foods from animal origin have to be considered. Therefore it is assumed that an average person, anywhere in the world, each day consumes; 500 g of meat (made up of 50 g of fat, 50 g of kidney, 100 g of liver and 300 g of muscle), 100 g of eggs or egg products, 1.5 L of milk and 20 g honey. The total amount of residues in the daily food basket is not allowed to exceed the ADI. MRLs are then distributed to the individual food commodities according to distribution in the animal body (European Commission 2005).

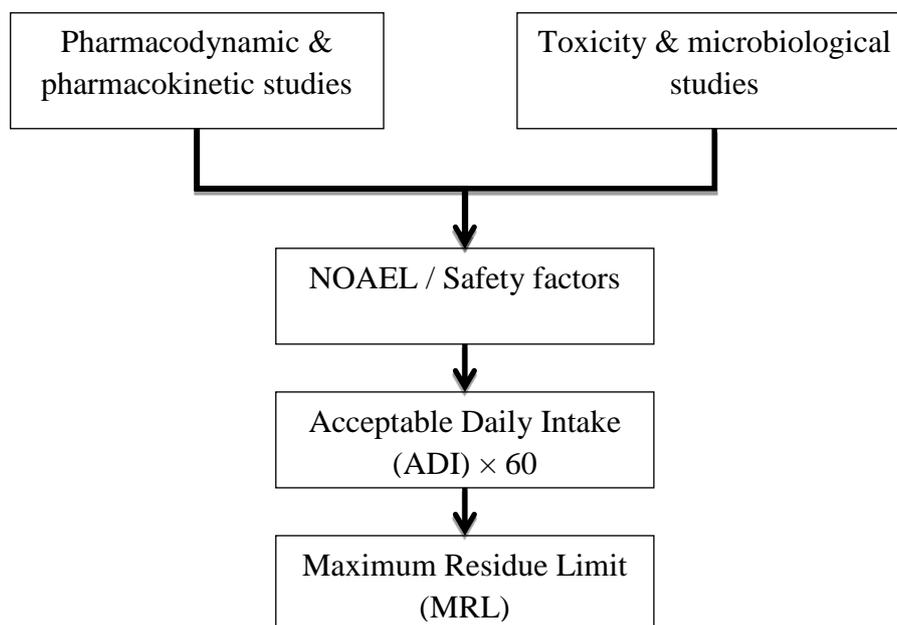


Figure 1. The road to establish a maximum residue limit (MRL)

The Codex Alimentarius Commission (CAC) is an organisation formed by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) in the early 1960s (National Food Agency 2013a). CAC has the responsibility to create international standards, codes of practice and guidelines regarding food safety, quality and equality within the international food trade (Codex

Alimentarius 2013). This includes the establishment of MRLs for VMPs by CAC after scientific evaluations by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and discussions in the codex committee for residues of veterinary drugs in food (CCRVDF) (Codex Alimentarius 2014, National Food Agency 2013b).

To ensure that the MRLs are not exceeded, withdrawal periods should be determined for the concerned pharmaceuticals and species (Directive 2001/82/EC). The withdrawal period is the time after the last administration of a VMP during which the animal is not allowed to be slaughtered or during which eggs or milk is not allowed to be taken for human consumption. The withdrawal periods are determined by the CVMP or the Member States for each species of food-producing animal and their edible products (National Food Agency 2013c). To determine the withdrawal periods marker residue depletion studies are performed on individual animal species, e.g. swine, poultry, cattle, horses and sheep (European Medicines Agency 2011). During the study the highest treatment dose is used for the maximum intended duration. The studies show how long it takes for the marker residue to be depleted down to the MRL after the treatment has ceased (European Medicines Agency 2011).

In the European Council directive 96/23/EC it is stated that Member States should perform controls to determine residue levels in animal products and to make certain that regulations are followed. The National Food Agency (NFA) in Sweden analyses around 5000 samples of food products (milk, fish, eggs, honey and meat during slaughter) each year (Nordlander *et al.* 2013). Before this directive the analysis of non-allowed residues of certain substances in food products from animals differed in limits of detection between, and even in, Member States. The differences lead to differential treatment of food producers supplying different countries in the European Union (EU). As the methods of analysis became more sophisticated the limits of detection decreased which lead to more non-allowed substances being detected. To ensure the same level of consumer protection throughout the EU and to harmonize the treatment of imported food products it was decided that minimum required performance limits (MRPLs) of analytical methods, to be used for substances for which no MRLs have been established, should be implemented (Commission Decision 2002/657/EC). The MRPLs are not health based and should not be interpreted as it is safe to consume food products containing levels below this limit. The MRPLs only establish the lowest levels that Member States must be able to analyse residues in food stuff. The substances that have

specified MRPLs are; chloramphenicol, malachite green, medroxyprogesterone, and nitrofurans metabolites (Commission Decision 2002/657/EC as amended by Commission Decision 2003/181/EC and Commission Decision 2004/25/EC).

Nitrofurans are a class of drugs that have been used for both veterinary and human medicine due to their antibacterial and antiprotozoal properties (Reynolds 1982, Sweetman 2002). To this class belong the substances nitrofurazone, nitrofurantoin, furazolidone and furaltadone. Of these compounds only nitrofurantoin is still in use in Sweden, whereas the others were withdrawn from the market in the 50s, 60s and 70s (FASS 2014a, 2014b, 2014c, 2014d). European legislation prohibited all nitrofurans, except furazolidone, from use in food-producing animals in 1993 due to their genotoxicity (Council Regulation (EEC) No 2901/93). Furazolidone was also prohibited two years later (Commission Regulation (EC) No 1442/95). During controls of food products the presence of marker residues for these nitrofurans are investigated. For nitrofurazone, nitrofurantoin, furazolidone and furaltadone the marker residues are: semicarbazide (SEM), 1-aminohydantoin (AHD), 3-amino-2-oxazolidone (AOZ) and 5-morpholinomethyl-3-amino-2-oxazolidone (AMOZ), respectively (National Food Agency 2012).

Residues of non-allowed and prohibited substances have been found in food of animal origin in the controls both in Sweden and other European countries (Gustavsson *et al.* 2012, European Commission 2013, Nordlander *et al.* 2013). In 2011 three samples of shrimp from China tested positive for nitrofurans metabolites in Swedish controls (Gustavsson *et al.* 2012). Between 2005 and March 2014 there have been 365 notifications on nitrofurans metabolites in food across Europe via the Rapid Alert System for Food and Feed (RASFF) (RASFF Portal 2014). The number of RASFF notifications was around 50 per year between 2005 and 2008. In 2009 that number increased to 94, but then dropped the following years to around 20 notifications per year (Figure 2). Since genotoxic substances are considered to exert their effects at any concentration no MRLs for such substances can be determined and consequently no withdrawal period can be established (Falk-Filipsson *et al.* 2007). Pharmacologically active substances not mentioned in Table 1 of Commission Regulation (EU) No 37/2010 are not allowed to be used in food-producing animals (Regulation (EC) No 470/2009). When such substances are found in residue surveillance above their

reference point of action (RPA), then that country's responsible agency is required to take action (European Commission 2013). However, these actions can differ between member countries, leading to a continued differential treatment of imported food products within the EU. When levels lower than the RPA, or in the case of nitrofurans the MRPL, are found it is up to that country if actions will be taken (European Commission 2013). For nitrofurans metabolites the RPA is the MRPL (1 µg/kg) (EFSA Panel on Contaminants in the Food Chain 2013). Sometimes during controls these metabolites are found in low concentrations, below the MRPL, and it can be difficult to know exactly what risk they pose to the consumers and what, and even if, actions should be taken. The objective of this master's thesis is to determine if any levels of nitrofurans can be allowed in animal food products. Is there a point where the levels in food are so low that the risk to the human population is negligible? What actions should be taken when nitrofurans are detected above and below the RPA?

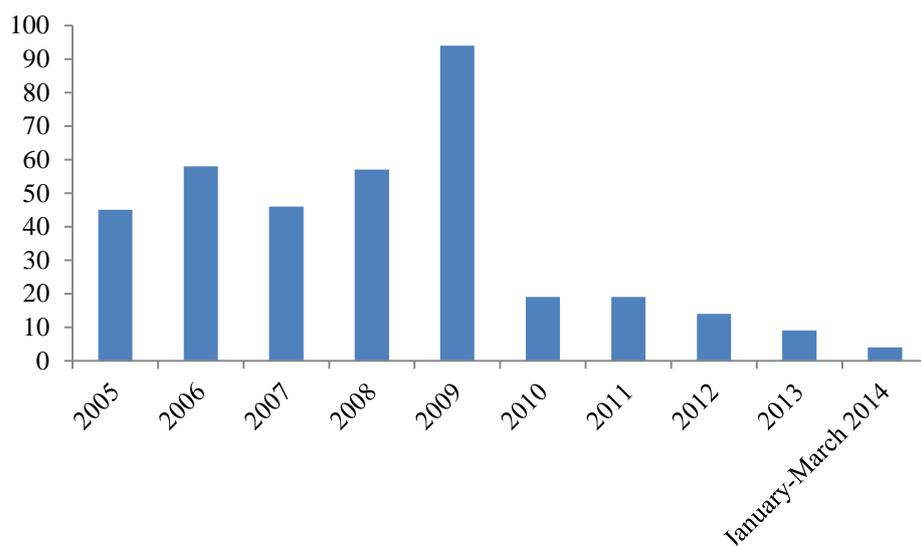


Figure 2. The number of RASFF notifications from European border controls regarding nitrofurans in food products between 2005 and 2014

2 Nitrofurazone (nitrofur)

Nitrofurazone is a broad spectrum antibiotic effective against both Gram-positive and Gram-negative bacteria (Reynolds 1982, Sweetman 2002). In human medicine it was used in the treatment of burns, ulcers, wounds and skin infections (Reynolds 1982, Sweetman 2002). In veterinary medicine it was used to treat necrotic enteritis in pigs and coccidiosis (a parasite) in farm animals and poultry (Reynolds 1982).

2.1 Physicochemical properties

International Non-proprietary Name (INN)

Nitrofurazone (nitrofurazone)

Chemical Abstract Service (CAS) name

5-Nitro-2-furaldehyde semicarbazone

CAS number: 59-87-0

Structural formula

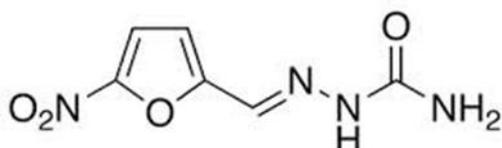


Figure 3. Structural formula of nitrofurazone

Molecular formula

C₈H₆N₄O₄

Molecular weight

198.14

Table 1. Physicochemical properties (Debnath *et al.* 1991, FAO 1993a)

Melting point	236-240 °C
Solubility in water (pH 6.0-6.5)	Very slightly soluble (1:4200)
Solubility in ethanol	Slightly soluble (1:590)
Solubility in benzene	Not soluble
Octanol/Water Partition Coefficient	Log K _{ow} = 0.23

2.2 Pharmacodynamics

Ali *et al.* (1988) orally administered nitrofurazone (7.5, 15 or 30 mg/kg BW) to male turkeys for two weeks and then measured the levels of luteinizing hormone (LH) and prolactin (PRL). It was shown that nitrofurazone treatment (30 mg/kg BW) significantly increased the levels of PRL and significantly decreased the levels of LH.

Depending on the concentration, nitrofurazone is either bacteriostatic (low concentration) or bactericidal (high concentration) (Dodd & Stillman 1944, Cramer & Dodd 1945).

2.3 Pharmacokinetics

2.3.1 *In vitro* studies

Nitrofurazone was incubated with 8500g supernatant of liver homogenate prepared from male rats aged 6-10 weeks (Akao *et al.* 1971). After 60 minutes of incubation under

aerobic conditions there was a change in optical absorbance corresponding to a loss of a nitro group. This was also the case under anaerobic conditions, but there was also an increase in absorbance at the maximum absorption wave length of 5-amino-2-furaldehyde semicarbazone. It was concluded that 5-amino-2-furaldehyde semicarbazone is a metabolite of nitrofurazone.

Human liver microsomes were incubated with 50 μ L nitrofurazone or 250 μ L 14 C-nitrofurazone for two hours at 37 °C (Wang *et al.* 2010). Using liquid chromatography (LC)-radiometric and liquid chromatography-tandem mass spectrometry (LC-MS/MS) a metabolite was detected and identified as a cyano metabolite.

2.3.2 In vivo studies

2.3.2.1 In pig

Pigs (n=18) were fed feed containing nitrofurazone (400 mg/kg feed) *ad libitum* for ten days, corresponding to around 24 mg/kg BW per day, followed by a withdrawal period for 6 weeks (Cooper *et al.* 2005). Three pigs were sacrificed each week of the withdrawal period and samples of muscle, liver and kidney were taken and analysed for nitrofurazone and semicarbazide (SEM) using LC-MS/MS and high performance liquid chromatography-UV (HPLC-UV). Nitrofurazone was detected in all muscle samples (4-21.9 μ g/kg) at week 0 of the withdrawal period. SEM was detected in all samples and the levels found at week 6 of the withdrawal period were around 50 μ g/kg in kidney and liver and 250 μ g/kg in muscle. The depletion half-lives of SEM in muscle, liver and kidney were 15.5 \pm 3.1 days, 7.3 \pm 0.6 days and 7.0 \pm 0.9 days respectively

2.3.2.2 In bovine

A cow was administered a capsule containing; 0.88 mg/kg BW furazolidone, nitrofurazone and furaltadone and 4.4 mg/kg BW nitrofurantoin (Chu & Lopez 2007). Milk samples were then collected for two weeks at intervals of 12 hours. Milk from non-treated cows was used as control. The levels of nitrofurazone side-chain residues in the milk were determined using LC-MS/MS. The level of the side-chain of nitrofurazone, SEM, in milk was highest 12 hours after dosing (~32 μ g/kg) and decreased rapidly. Seventy-two hours after dosing the level of SEM was below the detection limit (0.2 μ g/kg).

Three cows were treated with ¹⁴C-nitrofurazone to investigate its distribution after intramammary (cow 1), intrauterine (cow 2) or topical ocular (cow 3) administration (Smith *et al.* 1998). Cow 1 was injected with nitrofurazone (~0.1 mg/kg BW) into the udder, cow 2 was injected with nitrofurazone (~0.2 mg/kg BW) into the uterus, and 2.1 mg nitrofurazone per day was applied to the surface of the eye of cow 3 for four days, corresponding to roughly 3 µg/kg BW per day. Blood samples were collected at different time points and urine and faeces was collected during the entire experiment. Milk was sampled at 12 hour intervals. The cows were killed 72 hours after the administration for cows 1 and 2, and 144 hours after the first treatment for cow 3. The tissue and fluid samples were analysed for nitrofurazone.

The highest levels of nitrofurazone in blood were reached one hour after intrauterine (cow 2) and topical ocular (cow 3) administration and three hours after intramammary (cow 1) administration (Smith *et al.* 1998). After this the levels in blood decreased. Overall about 1 % of the administered dose was excreted via the milk by cow 1. Cows 2 and 3 excreted 0.5 % of the administered dose via milk. The major excretion pathway for nitrofurazone residues was via the urine except for cow 3, treated by ocular administration. The major excretion pathway was for that cow via the faeces indicating that ocular administration resulted in less absorption than the other routes. Cows 1, 2 and 3 excreted 62.9, 43.7 and 17.5 % of the administered dose via the urine, respectively. The excreted nitrofurazone residues in faeces constituted 20.2, 18.5 and 28.5 % of the total dose for cows 1, 2 and 3, respectively. After intramammary treatment the highest levels of nitrofurazone residues were found in the stomach complex, blood and skin. The highest levels after intrauterine administration were seen in liver, stomach complex and skin. After topical ocular administration the highest levels were found in head, skin and stomach complex (Smith *et al.* 1998).

Furaltadone and nitrofurazone (14.0 mg/kg BW) suspended in milk were given orally to five preruminant MRV male calves (Nouws *et al.* 1987). Furaltadone was given three days before nitrofurazone. Blood samples were taken at different time points after each administration and urine was collected from three calves and analysed for the nitrofurans. The maximum concentration of nitrofurazone in plasma (3.5 µg/mL) was achieved three hours after administration and the half-life of nitrofurazone was calculated to be around five hours.

2.3.2.3 In fish

The depletion of nitrofurans and their tissue-bound residues in channel catfish (*Ictalurus punctatus*) was investigated by Chu *et al.* (2008). Fish (n=55) were orally administered furazolidone, nitrofurantoin, nitrofurazone and furaltadone (1 mg/kg BW) at the same time. After 2, 4, 8 and 12 hours, and 1, 4, 7, 10, 14, 28 and 56 days, five fish were killed and muscle samples collected for analysis of parent nitrofurans and their tissue-bound residues. The highest concentration of nitrofurazone in muscle (104 µg/kg) was reached 12 hours after administration. Nitrofurazone could no longer be detected 96 hours after administration. The level of SEM was highest (31.1 µg/kg) 24 hours after administration. The elimination of all tissue-bound residues was biphasic and could still be detected 56 days after administration. The half-life for SEM was calculated to be 63 days.

Chu *et al.* (2008) also examined the levels of nitrofurans and tissue-bound metabolites in muscle of fish after waterborne exposure to nitrofurans. Fish (5 per treatment) were exposed to nitrofurantoin, nitrofurazone, furazolidone or furaltadone (10 mg/L) for 8 hours. After this time the fish were killed and their muscle tissue was analysed for parent nitrofuran and tissue-bound metabolites. The concentrations of nitrofurazone and SEM were around 61 and 18 µg/kg, respectively, at 8 hours.

¹⁴C-nitrofurazone (1 mg/kg BW) was given orally to channel catfish for metabolic profiling and sampled after 18 hours (Wang *et al.* 2010). The major metabolite found was a cyano metabolite containing the SEM side-chain and the nitroreduced ring portion of nitrofurazone.

Channel catfish (n=45) were orally administered nitrofurazone (10 mg/kg BW) and after 2, 4, 8, 12, 96, 168, 192, 240 and 336 hours five fish at each time point were killed, muscle samples taken and analysed for nitrofurazone and the cyano metabolite. The level of nitrofurazone was highest eight hours after administration and could be detected in muscle up to 48 hours. The half-life was calculated to be 6.3 hours. For the cyano metabolite the level was highest after 10 hours and could be measured even two weeks after the administration. The half-life in the terminal phase of elimination was calculated to be around 81 hours.

2.3.2.4 In poultry

McCracken *et al.* (2005a) fed six broiler hens and one cockerel 120-140 g of feed containing nitrofurazone (400 mg/kg feed), corresponding to 24-28 mg/kg BW per day.

Eggs were collected and analysed for SEM and when it was clear that nitrofurazone residues had transferred to eggs, eggs laid after this were collected and allowed to hatch. After hatching four chicks were sacrificed at determined intervals and muscle and liver samples were analysed for SEM. SEM could be detected up to slaughter age of 42 days. The levels of SEM in liver and muscle in one day old chicks were approximately 30 µg/kg. In 42 days old chicks the levels of SEM in liver and muscle were around 0.2 µg/kg.

Twenty-four laying hens were fed feed containing 300 mg furaltadone, nitrofurazone, nitrofurantoin or furazolidone per kg feed for one week, corresponding to about 15 mg/kg BW per day (McCracken & Kennedy 2007). Eggs were then collected for two days and immediately analysed for nitrofurazone parent compound and their bound residues in albumen, yolk and shell using LC-MS/MS. The levels of nitrofurazone in yolk, albumen and shell were 0.828, 0.258 and 0.0476 mg/kg, respectively. The levels of SEM in yolk, albumen and shell were 1.14, 0.634 and 1.82 mg/kg, respectively.

Broiler hens (n=30) were fed feed containing nitrofurazone (0.03, 0.3, 3, 30 or 300 mg/kg feed), corresponding to around 6 µg/kg BW to 60 mg/kg BW, for 16 days (Cooper *et al.* 2008). The experiment was then terminated except for the hens treated with 300 mg/kg feed who were kept on a control diet for an additional 16 days. Eggs were collected during the entire experiment and analysed for nitrofurazone and SEM. The levels of nitrofurazone and SEM quickly increased in eggs during the exposure and reached a steady-state around day 4 for all doses except for 0.03 mg/kg feed. It was calculated that during the steady-state 28 % of detected SEM was in the form of nitrofurazone. The half-life of nitrofurazone and SEM in eggs was calculated to be 1.1 and 2.4 days, respectively. SEM could still be detected in eggs after the 16 days long withdrawal period, but nitrofurazone could not be detected after eight days. In eggs from the hens treated with 3, 30 and 300 mg/kg feed around 60 % of the nitrofurazone was found in the yolk and 40 % in the albumen. Around 75 % of SEM was found in yolk and 25 % in albumen.

2.3.2.5 In rat

Samsonova *et al.* (2008) investigated which proteins would bind to nitrofurazone metabolites containing the SEM side-chain. A Sprague-Dawley rat was fed a total of

315 mg nitrofurazone in the feed over a period of seven days, corresponding to 225 mg/kg BW per day. After this time the rat was sacrificed and proteins were extracted from the liver. Proteins binding to metabolites were identified as albumin, liver regeneration-related protein LRRG03 and glutathione S-transferase.

Paul *et al.* (1960) administered nitrofurazone to rats and examined the urine for metabolites. They tentatively identified a urinary metabolite of nitrofurazone as hydroxylaminofuraldehyde semicarbazone or aminofuraldehyde semicarbazone.

Yeung *et al.* (1983) administered nitrofurazone (0.13 mg/kg) to male germfree (n=4) and conventional (n=3) Sprague-Dawley rats by gavage. The authors investigated if any metabolites of nitrofurazone could be found in the urine of treated rats. They found one reduced metabolite, 4-cyano-2-oxobutyraldehyde semicarbazone, in the urine of both rat types. The amount of 4-cyano-2-oxobutyraldehyde semicarbazone found in the urine of conventional rats was almost the double of that found in the urine of germfree rats, 68 and 37 nmoles respectively.

2.3.3 Conclusions on pharmacokinetics of nitrofurazone and its metabolites

The pharmacokinetic studies that have been performed showed that nitrofurazone is rapidly absorbed, distributed throughout the body and excreted. After oral administration of nitrofurazone to calves the half-life in blood was calculated to be around five hours. In cows administered nitrofurazone via intramammary and intrauterine injection the major excretion pathway was via urine (43-63 % of administered dose). Around 20 % of the administered dose was excreted via the faeces. It was also shown that nitrofurazone can be transferred to milk of treated cows and to eggs of treated poultry. In eggs, the half-life of nitrofurazone was approximately 24 hours. The half-life of nitrofurazone in fish muscle after oral administration was around six hours.

The metabolites of nitrofurazone can be seen in Table 2. The most commonly found metabolite of nitrofurazone is the side-chain SEM which is the marker residue for nitrofurazone. Quite recent studies have revealed some pharmacokinetic properties of SEM. SEM has been shown to be transferred to milk in cows and to eggs in poultry. In eggs, the half-life of SEM was about two days. The half-lives have been shown to be longer in other animals. In pigs fed nitrofurazone SEM could be detected in muscle,

kidney and liver with the highest levels found in muscle. The calculated half-lives were 15 days in muscle and 7 days in kidney and liver. In fish muscle SEM had a half-life of 63 days.

Table 2. Metabolites of nitrofurazone in different species found in the literature

Metabolite	Species/cells	Reference
4-cyano-2-oxobutylaldehyde semicarbazone	Rat	Yeung <i>et al.</i> (1983)
Semicarbazide (SEM)	Broiler, pig, cow, fish	McCracken <i>et al.</i> (2005a), Cooper <i>et al.</i> (2005), Chu & Lopez (2007 & 2008)
Cyano metabolite	Human liver microsomes, fish	Wang <i>et al.</i> (2010)
Hydroxylaminofuraldehyde semicarbazone	Rat	Paul <i>et al.</i> (1960)
5-amino-2-furaldehyde semicarbazone	Rat	Akao <i>et al.</i> (1971), Paul <i>et al.</i> (1960)

2.4 Toxicology of nitrofurazone

2.4.1 Acute toxicity

Acute toxicity tests with nitrofurazone have been performed on rats and mice (JECFA 1993a). The lethal dose for 50 % of the test animals (LD₅₀) after oral administration was determined to be between 590 and 800 mg/kg BW in rats, and between 380 and 590 mg/kg BW in mice. In one study mice were administered nitrofurazone intraperitoneally and the LD₅₀ was determined to be 300 mg/kg BW (JECFA 1993a).

2.4.2 Conclusions on acute toxicity of nitrofurazone

Based on the results from the different studies on the acute toxicity of nitrofurazone described in JECFA (1993a) nitrofurazone is slightly toxic to rats and mice. However, the studies are quite old and it is not clear whether they follow guidelines or good laboratory practice (GLP).

2.4.3 Chronic toxicity

No chronic toxicity studies were found.

2.4.4 Reproductive toxicity including teratogenicity

2.4.4.1 Mice

In a teratogenicity study by Nomura *et al.* (1984), summarized in JECFA (1993a), 16 pregnant ICR/Jcl mice were administered nitrofurazone (100 mg/kg BW) via subcutaneous injection on gestation days 9-11. Another group of six pregnant mice were administered 300 mg/kg BW via subcutaneous injection, only on gestation day 10. No

increase in foetus malformations was seen in the 100 mg/kg BW dose group. The group dosed with 300 mg/kg BW did have an increased incidence of malformations, particularly tail anomalies, leg defects and oligodactyly in their offspring. The overall incidence of malformations was 0.3 % in controls and 21 % in the 300 mg/kg BW dose group.

The study does not follow today's study guidelines and probably does not follow GLP. It was only seen as a summary in JECFA (1993a). There is no mention of maternal toxicity but a single dose of 300 mg/kg BW at gestation day 10 caused malformations in the foetus.¹

A reproductive toxicity study by Hardin *et al.* (1987) is presented in JECFA (1993a). On gestation days 6-13 female CD-1 mice were administered nitrofurazone (100 mg/kg BW per day) via gavage. After treatment with nitrofurazone a decrease in the number of viable litters was seen. Also the birth weights were slightly reduced. The authors therefore concluded that nitrofurazone was embryotoxic.

The study was only accessed as a summary in JECFA (1993a). The study does not meet today's standard and most likely does not follow GLP. It does not report if there was any maternal toxicity but results do indicate that nitrofurazone is embryotoxic at the only dose tested, 100 mg/kg BW.

Nitrofurazone was studied in a two generation reproduction study (George *et al.* 1996). CD-1 Swiss mice, 100 of each sex, were continually fed nitrofurazone (0, 14, 56 or 102 mg/kg BW per day). The F₀ generation were treated for a total of 29 weeks. For 14 weeks animals were housed in breeding pair within each dose group. The litters were evaluated and immediately euthanized on post natal day (PND) 0. The fertility was significantly reduced in the highest dose group (102 mg/kg BW per day). Only 17 % of the breeding pairs produced a first litter, as compared to 98 % in the control. One pair produced a second litter and all pairs were infertile for the rest of the cohabitation. In the middle dose group (56 mg/kg BW per day) all pairs produced their first litter but their fertility decreased with each litter. By the second litter 79 % were fertile and by the fifth litter only 47 % were fertile, as compared to 88 % in the control. No effect on fertility was seen in the lowest dose group (14 mg/kg BW per day).

¹ The texts written in cursive are comments to the individual studies.

Due to the observed decrease in fertility a crossover mating trial was carried out using F₀ control and high dose animals (George *et al.* 1996). At week 23 the animals were cohabited for maximum one week and fed control feed. They were then separated and the females were allowed to deliver the litters. The pups from the litters were evaluated and euthanized on PND 0 and the F₀ generation were killed and necropsied at week 29. Treated males mated with control females produced no live pups and control males and treated females had a significant decrease in the number of live pups per litter. The necropsies and histological examinations of the F₀ generation revealed that liver and kidney weights were significantly increased in middle- and high-dose males and testis weight was significantly decreased in high-dose males. At all dose levels the incidence of seminiferous tubule degeneration and atrophy was increased. At the low- and middle-dose groups there was a significant increase in the percentage of aberrant sperm. In high-dose males and females hepatic hypertrophy was observed. Compared to the control, females in the highest dose group had an altered oestrus cycle and at all doses the relative ovary plus oviduct weight was decreased (George *et al.* 1996).

The last litter produced by the F₀ generation was saved and used for F₁ fertility assessment (George *et al.* 1996). Pups from each dose group (0, 14 or 56 mg/kg BW per day) were randomly selected on PND 21 and fed nitrofurazone in the same doses as the parents. At 74 days of age breeding pairs were housed together for a maximum of one week and then separated. The litters were euthanized on PND 0 and the F₁ generation were killed at 119 days of age. The fertility of the 56 mg/kg BW per day breeding pairs was significantly decreased as well as the number of live pups per litter. The necropsies and histology showed that males (56 mg/kg BW per day) had decreased testis weight and epididymal sperm concentration. They also had an increased percentage of aberrant sperm. Hepatic hypertrophy was also seen in these males. Females had altered oestrus cycles at all dose levels and females in the middle-dose group had reduced liver and ovary weights.

The study does not follow European guidelines but does follow GLP. The results show that nitrofurazone reduced fertility in mice at 56 and 102 mg/kg BW per day. At the lowest dose tested, 14 mg/kg BW per day, the oestrus cycle was altered in F₁ females and there was an increase in abnormal sperm in F₀ males. Therefore, no NOAEL can be set.

2.4.4.2 Rats

In a study by Ito *et al.* (2000) SPF Sprague-Dawley rats (n=40) were administered nitrofurazone, 50 mg/kg BW, via stomach tube for two or four weeks, or 100 mg/kg BW for two weeks. Control animals were given the vehicle (0.5 % methylcellulose) for four weeks. After the medication period the animals were sacrificed, visceral organs were examined macroscopically, and testis and epididymis were examined histologically. Rats treated with 100 mg/kg BW showed decreased spontaneous locomotive activity and salivation. Three of these rats died during the medication period. No symptoms were observed in the 50 mg/kg BW dose groups. All rats administered nitrofurazone had decreased body, testis and epididymis weights compared to controls. The decrease in epididymis weight was significant in all treatment groups while the decrease in testis weight was significant in the rats treated with 100 mg/kg BW for two weeks and 50 mg/kg BW for four weeks. Atrophy of the seminiferous tubules and reduced number of spermatozoa was seen in all treated animals.

The study demonstrates that nitrofurazone induce testicular toxicity in rats. The study does not follow guidelines or GLP.

2.4.4.3 Conclusion on reproductive toxicity including teratogenicity

The results from the reproductive toxicity tests are presented in Table 3 and show that nitrofurazone is reproductively toxic. Exposure to at least 14 mg/kg BW over an extended period of time caused toxic effects on the reproductive system in mice. Mice exposed to 56 or 102 mg/kg BW per day had decreased fertility. No NOAEL can be set due to that the lowest dose tested (14 mg/kg BW per day) caused abnormal sperm in F₀ males.

Table 3. Summary on reproductive toxicity studies including teratogenicity of nitrofurazone

Species	Dose (mg/kg BW)	Duration	Effects	Reference
Mouse	100, 300	3 days (gestation days 9-11), 1 day (gestation day 10)	No effects ↑foetal malformations	JECFA (1993a)
Mouse	100	8 days (gestation days 6-13)	↓viable litters and birth weights	JECFA (1993a)
Mouse (F ₀)	14, 56 or 102	29 weeks	↓fertility, seminiferous tubule degeneration, ↑abnormal sperm, altered oestrus cycle, ↓testis & ovary weights	George <i>et al.</i> (1996)
Mouse (F ₁)	14 or 56	17 weeks	↓testis & ovary weights, abnormal sperm, altered oestrus cycle, ↓fertility	George <i>et al.</i> (1996)
Rat	50 or 100	2-4 weeks	↓testis & epididymis weight, ↓number of spermatozoa	Ito <i>et al.</i> (2000)

2.4.5 Mutagenicity and genotoxicity

2.4.5.1 *In vitro* studies on nitrofurazone

Anderson & Phillips (1985) investigated the genotoxicity of nitrofurazone in a chromosome aberration test. Chinese hamster ovary (CHO) cells were incubated for two hours at 37 °C with nitrofurazone (0, 25, 50, 100 or 200 µg/mL medium) in the presence or absence of S9-mix. Cyclophosphamide (CPA) (50 µg/mL medium) and ethyl methanesulphonate (EMS) (2000 µg/mL medium) were used as positive controls. At 4, 8 or 24 hours after the start of the treatment the cells were fixed and the number of chromosome aberrations, in 100 metaphase cells, was counted. A significant dose-related increase in chromosome aberrations was seen at all sampling times in the presence of S9 fraction. In the absence of S9-mix a significant increase in chromosome aberrations was found at the 8 and 24 hour time points.

The study mostly follows today's guidelines and shows that nitrofurazone is genotoxic to CHO cells in vitro.

In an *in vitro* mammalian cell gene mutation test, performed two times, CHO-K1-BH₄ cells were incubated for two hours at 37 °C with nitrofurazone (0, 25, 50, 100 or 200 µg/mL medium) in the presence or absence of S9-mix (Anderson & Phillips 1985). Cells were then cultured for seven days and on the eighth day after treatment cells were transferred to plates with hypoxanthine-free medium either containing or not containing thioguanine. The cells were incubated for an additional seven days and the number of mutant colonies was calculated. EMS and benzo[a]pyrene was used as positive controls. The frequency of mutants did not increase in a dose-related manner after exposure to nitrofurazone but a few treatments caused an increased mutant frequency (experiment 2 without S9-mix: 25 & 100 µg/mL, experiment 1 with S9-mix: 25, 50 & 100 µg/mL).

Since the increase in mutant frequencies could not be reproduced nitrofurazone should be considered non-mutagenic in this test. The study, for the most part, follows guidelines but it is not known whether GLP was followed.

A reverse mutation test was performed on *E. coli* 343/113/R-9 by Baars *et al.* (1980). Cells were exposed to nitrofurazone (0-75 µg/mL), with and without metabolic activation from *Drosophila melanogaster* microsomes, for two hours at 37 °C. Exposure to nitrofurazone, especially at the highest dose, with metabolic activation increased the number of arg⁺ and gal⁺ mutants. The number of mutants did not increase without the metabolic activation system.

The study does not follow guidelines or GLP, but does show that metabolic activation of nitrofurazone caused mutations in E. coli 343/113/R-9 in what appeared to be a dose-related manner.

In an Ames test, using the plate incorporation method, nitrofurazone (0.25-1.5 µg/plate) was incubated with *S. typhimurium* TA 98 and 100 for 48 hours (Goodman *et al.* 1977). The revertant his⁺ colonies were then counted and it was concluded that nitrofurazone was mutagenic.

The study mostly follows guidelines and shows that nitrofurazone is mutagenic.

2.4.5.2 *In vivo* studies on nitrofurazone

Nitrofurazone was tested in a mammalian bone marrow chromosome aberration test (Anderson & Phillips 1985). Male Wistar rats were given a single dose of nitrofurazone (40, 120 or 400 mg/kg BW, n=72), 200 mg EMS/kg BW (positive control, n=24) or corn oil (negative control, n=36) via gavage. The animals were killed 6, 24 or 48 hours after administration but were intraperitoneally injected with the metaphase-arresting colchicine (3 mg/kg BW) two hours before. Bone marrow cells were harvested and 50 cells in metaphase per animal were examined for chromosome aberrations and 500 cells were examined to provide a mitotic index. There was no increase in chromosome aberrations after exposure to nitrofurazone.

Another study was performed where rats were given daily doses of nitrofurazone (15, 45 or 150 mg/kg BW), corn oil or EMS (200 mg/kg BW) for five days. The rats were killed six hours after the last administration and bone marrow cells were harvested and examined. No increase in chromosome aberrations was observed. Colchicine (3 mg/kg BW) were intraperitoneally injected two hours before harvesting the cells.

The study does not follow guidelines but provide some indications that nitrofurazone is not genotoxic in rat in vivo.

The mutagenicity of nitrofurazone was tested in a bone marrow micronucleus test using Sprague-Dawley and Long-Evans rats (n=24) (Goodman *et al.* 1977). Rats were intraperitoneally injected with nitrofurazone (15, 30 or 60 mg/kg BW in Sprague-Dawley rats and 60 mg/kg BW in Long-Evans rats). Half of the dose was given 30 hours before sacrifice and the other half was given six hours before sacrifice. Triethylenemelamine served as positive control. The number of reticulocytes with micronuclei out of 2000 or 3000 cells per rat was then counted. Nitrofurazone did not increase the number of micronucleated reticulocytes at any dose or rat strain and was concluded to be non-mutagenic *in vivo*.

The study does not follow current guidelines. According to the authors the results show that nitrofurazone is not mutagenic under these conditions. However, the sampling only occurred six hours after the last dosing and that is not long enough for micronucleated reticulocytes to be formed. Therefore, the study is not that reliable since the results may be false negative. There is also no mention of any other signs of toxicity being observed or not observed during the study.

In a chromosome aberration test nitrofurazone (60 mg/kg BW) was injected intraperitoneally into Sprague-Dawley rats (n=5 per treatment) and samples of bone marrow were retrieved 6 and 24 hours after administration (Goodman *et al.* 1977). Triethylenemelamine served as positive control. Fifty cells in metaphase per animal were examined for chromosome aberrations. The results showed that nitrofurazone did not induce chromosome aberrations at these time points.

No mitotic index was determined, no meta-phase arresting agent was used and only 50 cells per animals were examined instead of 100. Otherwise the study follows guidelines and does not indicate that nitrofurazone is genotoxic in rat.

2.4.5.3 *In vitro* studies on the metabolite SEM

Parodi *et al.* (1981) performed an Ames test with *Salmonella typhimurium* strain TA 1535. The bacteria were exposed to SEM (67 µmol/plate) with or without S9-mix. The results showed that SEM was slightly mutagenic in the absence of S9. The mutagenicity decreased in the presence of S9-mix.

The study does not follow the guidelines or GLP. The results indicate that SEM may not be mutagenic after metabolic activation and thereby may not be mutagenic in vivo.

The mutagenicity of SEM was tested in a bacterial reverse mutation test using the plate-incorporation method with histidine-requiring *Salmonella typhimurium* mutants TA 98, TA 100, TA 1535 and TA 1537 and tryptophan-requiring *Escherichia coli* mutant WP2 *uvrA* (TNO 2004a). Bacteria were incubated with SEM at five concentrations (62-5000 µg/plate) in the presence and absence of S9-mix, transferred to minimal glucose agar plates and incubated for 48-72 hours at 37 °C. The *trp*⁺ and *his*⁺ revertant colonies were then counted. Appropriate positive controls were used. SEM was cytotoxic to *S. typhimurium* TA 100, TA 1537 and *E. coli* WP2 *uvrA* at the highest concentration in the presence of S9-mix. There was a dose-related increase in the number of revertant colonies in strain TA 1535 in the absence of S9-mix and an increase at the highest dose in the presence of S9-mix. In TA 100 the number of revertant colonies also increased in the absence of S9-mix at 1667 µg/plate and slightly at 5000 µg/plate. A slight increase in revertant colonies was seen in WP2 *uvrA* at the highest concentration in the absence of S9-mix.

The study follows GLP and the guidelines. SEM was cytotoxic to TA 100, TA 1537 and WP2 uvrA at the highest dose in the presence of S9-mix but that is not believed to have impacted the results. The results show that under these test conditions SEM is mutagenic.

In a cell gene mutation test at the *tk* locus in mouse lymphoma (L5178Y) cells, cells were exposed to 13 concentrations of SEM (0.21-10 mM) in the presence and absence of S9-mix (TNO 2004b). Methyl methanesulphonate (MMS) and 3-methylcholanthrene (MCA) were used as positive controls. Cells were cultured in wells of two 96-wells microtiter plates in TFT (4 µg/mL) containing medium. After 10-14 days of incubation the number of surviving colonies (mutants) was counted. In the presence of S9-mix the mutant frequency was only increased compared to the control at the highest concentration (10 mM). In the absence of S9 there was a dose-related increase in the mutant frequency. SEM was not cytotoxic in the presence of S9 but slightly cytotoxic in the absence of S9-mix.

The study follows GLP and guidelines. SEM is mutagenic at the TK-locus in mouse lymphoma cells.

SEM was tested in a chromosome aberration test where CHO cells were treated both with and without S9-mix (TNO 2004c). In one test, cells were treated with SEM (10-1115 µg/mL) for 4 or 18 hours and then harvested 18 hours after the start of treatment. Colcemid (0.1 µg/mL) was added two hours before harvest. Two-hundred cells in metaphase per dose were examined for chromosome aberrations. In another test cells were treated with SEM (50-900 µg/mL) for 4, 18 or 32 hours, harvested and examined 18 or 32 hours after the start of treatment. Two hours before harvest colcemid (0.1 µg/mL) was added. The results showed that SEM did not significantly increase the number of aberrant cells at any dose or time-point. However, it was shown that SEM in the presence of S9 significantly increased the number of endoreduplicated cells at the 18 hour, but not 32 hour, sampling time. It was concluded that SEM may have the potential to inhibit mitotic processes.

The results from this study indicate that SEM is not clastogenic in CHO cells but may have the potential to interfere with mitotic processes. The study followed GLP and guidelines.

In an *in vitro* sister-chromatid exchange (SCE) assay it was shown that SEM (0.5, 2.5, 5, 10 or 20 µg/mL) only caused a minor significant increase ($p=0.05$) in SCE frequency in human lymphocytes at the highest dose tested (Vlastos *et al.* 2010). SEM and MMC (positive control, 0.1 µg/mL) were incubated with the cells for 72 hours. Demolcine (0.3 µg/mL) was added two hours before harvesting the cells. Twenty-five cells in metaphase were examined per culture.

The study shows that SEM may be genotoxic in this test system at 20 µg/mL, which is stated to be 1000 times higher the worst-case intake of a 6-months old infant. The study mostly follows test guidelines but does not follow GLP.

The genotoxicity of SEM was tested in a micronucleus test in lymphocytes *in vitro* (Vlastos *et al.* 2010). Human lymphocytes were cultured for 72 hours. Cells were incubated with SEM (0, 0.5, 2.5, 5, 10 or 20 µg/mL) or mitromycin-C (MMC) (positive control, 0.5 µg/mL) starting 24 hours after the beginning of the culture period. Cytochalasin-B (6 µg/mL) was added 44 hours after culture initiation. At the end of the culture period cells were collected. The frequency of micronuclei was derived by examining 1000 binucleate cells per culture. The results showed that there was no statistical difference in the frequency of micronuclei between SEM treated cells and the control.

The study does not follow GLP but mostly follows guidelines. The results in this study indicate that SEM may not be genotoxic.

2.4.5.4 *In vivo* studies on the metabolite SEM

SEM was tested in a flow cytometry-based micronucleus assay *in vivo* (Abramsson-Zetterberg & Svensson 2005). Male Balb/C mice were intraperitoneally injected with SEM (0, 40, 80 or 120 mg/kg BW) and CBA mice were injected with 80 or 120 mg/kg BW. Three or four Balb/C mice were included in each dose group while five CBA mice per dose group were used. Colchicine (1 mg/kg BW) was used as positive control and PBS (1 mg/kg BW) was used as negative control. Blood samples were collected 42 hours after injection. Between 20000 and 100000 erythrocytes per sample were examined and the frequency of micronucleated polychromatic erythrocytes was determined. It was shown that SEM did not affect the frequency of micronucleated erythrocytes in either mouse strain at any dose.

The study mostly follows the guidelines, except for a low number of Balb/C mice, and indicates that SEM is not genotoxic in vivo.

Male Wistar rats (n=25) were orally administered SEM (0, 50, 100 or 150 mg/kg BW) or cyclophosphamide (positive control, 20 mg/kg BW) and killed 24 hours after administration (Vlastos *et al.* 2010). Bone marrow cells were collected and the frequency of micronuclei in 2000 polychromatic erythrocytes (PCEs) per animal was determined. It was shown that SEM statistically increased ($p \leq 0.05$) the frequency of micronuclei at all doses.

The study mostly follows guidelines and demonstrates a genotoxic effect of SEM in vivo but the effect was not dose-dependent. When using rat for the micronucleus assay the risk for false negatives are increased due to micronucleated erythrocytes being removed from the circulatory system by the spleen. This increases the reliability of the results in this study.

2.4.5.5 Conclusions on mutagenicity and genotoxicity studies

The results of the mutagenicity and genotoxicity studies are presented in Table 4. Nitrofurazone has been shown to be genotoxic and mutagenic in the bacterial tests and in *in vitro* mutation and chromosome aberration tests. Several other *in vitro* mutagenicity and genotoxicity studies have been performed with nitrofurazone and the results of these are presented in JECFA (1993a). Most of the results are positive for mutagenicity and genotoxicity. In the two *in vivo* studies mentioned above, nitrofurazone did not test positive in rat. However, one of these may be a false negative. From these studies it is not possible to draw any conclusions on the mutagenicity of nitrofurazone *in vivo* but nitrofurazone is mutagenic *in vitro* both with and without metabolic activation.

In the studies mentioned above the metabolite SEM has tested positive in all bacterial mutation test, but there has been both negative and positive results in the *in vitro* and *in vivo* studies. Further study is needed before any conclusions on the mutagenicity and genotoxicity of SEM can be drawn.

Table 4. Summary of mutagenicity and genotoxicity studies on nitrofurazone and its metabolite SEM

Test	Compound	Species/strain	Dose/ concentration	Result	Reference
Bacterial reverse mut. assay	Nitrofurazone	<i>E. coli</i> 343/113/R-9	0-75 µg/mL*	+	Baars <i>et al.</i> (1980)
Ames test	Nitrofurazone	<i>S. typhimurium</i> TA 98 & 100	0-1.5 µg/plate	+	Goodman <i>et al.</i> (1977)
Mammalian cell gene mut. test	Nitrofurazone	Chinese hamster ovary (CHO) cells	0-200 µg/mL*	-	Andersson Phillips (1985)
<i>In vitro</i> chrom. aberration assay	Nitrofurazone	Chinese hamster ovary (CHO) cells	0-200 µg/mL*	+	Andersson Phillips (1985)
<i>In vivo</i> chrom. aberration assay	Nitrofurazone	Wistar rats	0-400 mg/kg BW, 15-150 mg/kg BW per day for 5 days	-	Andersson Phillips (1985)
<i>In vivo</i> micro-nucleus test	Nitrofurazone	Sprague-Dawley and Long-Evans rats	0-60 mg/kg BW	- **	Goodman <i>et al.</i> (1977)
Ames test	Semicarbazide (SEM)	<i>S. typhimurium</i> TA 1535	67 µmol/plate*	+	Parodi <i>et al.</i> (1981)
Bacterial reverse mut. assay	Semicarbazide (SEM)	<i>S. typhimurium</i> TA 98, 100, 1535, 1537 & <i>E. coli</i> WP2 <i>uvrA</i>	0-5000 µg/plate*	+	TNO (2004a)
Mammalian cell gene mut. test	Semicarbazide (SEM)	Mouse lymphoma L5178Y cells	0.21-10 mM*	+	TNO (2004b)
<i>In vitro</i> sister-chromatid exchange (SCE) assay	Semicarbazide (SEM)	Human lymphocytes	0.5-20 µg/mL	+	Vlastos <i>et al.</i> (2010)
<i>In vitro</i> chrom. aberration assay	Semicarbazide (SEM)	Chinese hamster ovary (CHO) cells	10-1115 µg/mL*	-	TNO (2004c)
<i>In vitro</i> micro-nucleus test	Semicarbazide (SEM)	Human lymphocytes	0-20 µg/mL*	-	Vlastos <i>et al.</i> (2010)

Table 4 continued

<i>In vivo</i> micro- nucleus assay	Semicarbazide (SEM)	Balb/C & CBA mice	0-120 mg/kg BW	-	Abramsson -Zetterberg & Svensson (2005)
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+ positive, - negative

*With and without metabolic activation, ** May be false negative

2.4.6 Carcinogenicity and long-term toxicity

2.4.6.1 Mice

B6C3F₁ mice, 50 of each sex and dose group, were fed feed containing nitrofurazone (0, 150 or 310 mg/kg feed) for two years and were then killed and necropsied (Kari *et al.* 1989). The daily nitrofurazone dose was estimated to be 15 or 31 mg/kg BW for the low and high dose, respectively. During the first year some stimulus-sensitive seizures were observed in the high dose group and in females of the low dose group. Also there was a significant decrease in the survival of males in the high dose group. Females in the high dose group had significantly increased incidences of granulosa cell tumours. There was also a significant increase in the incidences of benign mixed ovarian tumours in the low and high-dose groups. No effects were seen in male mice.

The study mostly follows the guidelines and demonstrates that nitrofurazone can induce both benign and malignant tumours in mice.

2.4.6.2 Rats

Fisher 344 rats, 50 of each sex and dose group, were fed feed containing nitrofurazone (0, 310 or 620 mg/kg feed) for two years and were then killed and necropsied (Kari *et al.* 1989). The daily nitrofurazone dose was estimated to be 12 or 25 mg/kg BW for the low and high dose, respectively. Nitrofurazone caused abnormal posture and pelvis limb gait at all doses. There was also a significant decrease in the survival of males in the high dose group. Degeneration of joint articular cartilage was seen at all dose levels and dosed male rats had increased incidences of testicular degeneration. In female rats there was a significant increase in mammary gland fibroadenomas at both dose levels. Low-dose male rats had significantly increased incidences of carcinomas and mesothelioma in tunica vaginalis. High-dose males had significantly increased incidences of trichoepithelioma or sebaceous adenoma.

The results from the study show that nitrofurazone causes mammary gland neoplasms. The authors conclude that the results in male rats are equivocal. The study mostly follows the guidelines for carcinogenicity studies. Exposure to nitrofurazone has

previously been shown to be reproductively toxic and cause testicular degeneration in both mice and rats.

The results from a study by Siedler & Searfoss (1967) are presented in JECFA (1993a). CFE rats (20 of each sex) were fed feed containing nitrofurazone for 45 weeks. The daily intake was around 50-55 mg/kg BW per day. After this medication period the rats were fed control feed for seven weeks and then examined. The number of female rats with benign mammary tumours was significantly higher in the treated rats than the control. In male rats there was no increase in tumour incidence.

The study is quite old, does not follow GLP and does not meet today's standard of testing. Also it was only accessed as an abstract and a small number of animals were used. It does however, indicate that female CFE rats are more susceptible to the carcinogenic effects of nitrofurazone compared to males.

JECFA (1993a) summarised the results from another study by Siedler & Searfoss (1967). Female Holtzman rats (n=35) were administered nitrofurazone in the feed, corresponding to 0, 75 or 150 mg/kg BW per day, for 45 weeks and were then given control feed for eight weeks. At the end of the study there was a significant increase in the number of benign mammary tumours at all dose levels.

Only an abstract of the study was found. The study does show that nitrofurazone increases the incidences of benign mammary tumours, but due to it being old and not following guidelines it may not be very reliable.

Ertürk *et al.* (1970) administered female weanling Sprague-Dawley rats (n=60) control feed or feed containing nitrofurazone (1000 mg/kg) for 46 weeks. Over the course of the experiment this corresponded to about 50-400 mg/kg BW, depending on the weight of the rat. After the medication period the rats were fed control feed for another 20 weeks. At the end of the study there was a significant increase in the number of rats with mammary fibroadenomas in the treated group compared to the control.

The study does not follow guidelines and the survival is not stated. But it shows that nitrofurazone induced mammary fibroadenomas in female rats.

2.4.6.3 Conclusions on the carcinogenicity and long-term toxicity of nitrofurazone

The results from carcinogenicity studies are summarised in Table 5. Nitrofurazone has been shown to be carcinogenic in mice and rats. In the only mouse study found, both benign and malignant tumours were observed. In rats most tumours were benign and in female rats nitrofurazone mostly caused benign mammary tumours. In the evaluation of nitrofurazone by JECFA it is concluded that nitrofurazone is secondary carcinogen acting on endocrine-responsive organs (JECFA 1993a).

Table 5. Summary of carcinogenicity and long-term toxicity studies on nitrofurazone

Species	Dose (mg/kg BW)	Duration (medicated + non-medicated period)	Effects	Reference
B6C3F ₁ mice	15 or 31	2 years	↑granulosa cell tumours, ↑benign mixed ovarian tumours	Kari <i>et al.</i> (1989)
Fisher 344 rats	12 or 25	2 years	↑mammary gland fibroadenomas, ↑carcinomas and mesothelioma in tunica vaginalis, ↑trichoepithelioma or sebaceous adenoma and testicular degeneration	Kari <i>et al.</i> (1989)
CFE rats	50-55	1 year (45+7 weeks)	↑benign mammary tumours	JECFA (1993a)
Holtzman rats	75 or 150	53 weeks (45+8 weeks)	↑benign mammary tumours	JECFA (1993a)
Sprague-Dawley rats	50-400	56 weeks (46+20 weeks)	↑mammary fibroadenomas	Ertürk <i>et al.</i> (1970)

3 Nitrofurantoin

Nitrofurantoin is an antibacterial agent that is used to treat urinary-tract infections in humans (FASS 2010, Reynolds 1982, Sweetman 2002). It is used for prophylaxis and long-term suppression therapy (Reynolds 1982, Sweetman 2002).

3.1 Physicochemical properties

International Non-proprietary Name (INN)

Nitrofurantoin

Chemical Abstract Service (CAS) name

N-(5-Nitro-2-furfurylidine)-1-aminohydantoin

CAS number: 67-20-9

Structural formula

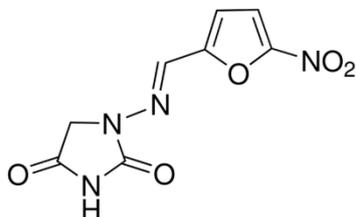


Figure 4. Structural formula of nitrofurantoin

Molecular formula

C₈H₆N₄O₅

Molecular weight

238.16

Table 6. Physicochemical properties (Debnath *et al.* 1991, Reynolds 1982, Windholz 1983)

Melting point (decomposes)	270-272 °C
Solubility in water (pH 6.0-6.5)	Very slightly soluble (1:5000)
Solubility in ethanol	Very slightly soluble (1:2000)
Solubility in dimethylformamide	Soluble (1:16)
Dissociation constant (pK_a)	7.2
Octanol/Water Partition Coefficient	Log K _{ow} = -0.47

3.2 Pharmacodynamics

Nitrofurantoin has been shown to be bacteriostatic and bactericidal, depending on the dose (Brunton *et al.* 2011).

3.3 Pharmacokinetics

3.3.1 *In vitro* studies

The metabolism of nitrofurantoin was studied by adding nitrofurantoin (~50 mg/L) to slices of rat liver, intestine, muscle or kidney and incubating them for 30 minutes Buzard *et al.* (1961). After this time the levels in all slices had dropped to 50 % of the original concentration, except in slices containing muscle. In these slices the levels had only decreased by 13 %.

Homogenates of various Sprague-Dawley rat tissues were incubated with nitrofurantoin for 45 minutes and then examined for metabolites of nitrofurantoin (Aufrère *et al.* 1978). Four metabolites were found and two could be identified. The major metabolite

was 1-[[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione and a minor metabolite was identified as aminofurantoin (1[[[(5-amino-2-furanyl)methylene]amino]-2,4-imidazolidinedione).

3.3.2 *In vivo* studies

3.3.2.1 In swine

Pigs (n=18) were fed feed containing nitrofurantoin (400 mg/kg feed) *ad libitum* for ten days, corresponding to around 24 mg/kg BW per day, followed by a withdrawal period for six weeks (Cooper *et al.* 2005). Three pigs were sacrificed each week of the withdrawal period and samples of muscle, liver and kidney were taken and analysed for nitrofurantoin and its marker residue 1-aminohydantoin (AHD) using LC-MS/MS and high performance liquid chromatography-UV (HPLC-UV). At week 0 of the withdrawal period nitrofurantoin was not detected in any sample while AHD was detected in all samples. The levels of AHD found at week 6 of the withdrawal period were around 4 µg/kg in kidney and liver and 8 µg/kg in muscle. The depletion half-lives of AHD in muscle, liver and kidney were 8.3±0.9 days, 5.7±0.5 days and 5.8±0.7 days respectively.

3.3.2.2 In bovine

One cow was administered one capsule containing; 0.88 mg/kg BW furazolidone, nitrofurazone and furaltadone, and 4.4 mg/kg BW nitrofurantoin (Chu & Lopez 2007). Milk samples were then collected for two weeks at intervals of 12 hours. Milk from non-treated cows was used as control. The levels of nitrofuran side-chain residues in the milk were determined using LC-MS/MS. The level of the side-chain of nitrofurantoin, AHD, in milk was highest 12 hours after dosing (~47 µg/kg), decreased rapidly and 72 hours after dosing the level of AHD was below the detection limit (0.2 µg/kg).

3.3.2.3 In fish

The depletion of nitrofurans and their tissue-bound residues in channel catfish (*Ictalurus punctatus*) was investigated by Chu *et al.* (2008). Fish (n=55) were simultaneously orally administered furazolidone, nitrofurantoin, nitrofurazone and furaltadone (1 mg/kg BW). After 2, 4, 8 and 12 hours, and 1, 4, 7, 10, 14, 28 and 56 days five fish were killed and muscle samples collected for analysis of parent nitrofurans and their tissue-bound residues. The highest concentration of nitrofurantoin in muscle (9.8 µg/kg) was reached 12 hours after administration. Nitrofurantoin could no longer be detected 96 hours after administration. The level of AHD was highest (9.1

µg/kg) 24 hours after administration. The elimination of all tissue-bound residues was biphasic and could still be detected 56 days after administration. The half-life for AHD was calculated to be 45 days.

Chu *et al.* (2008) also examined the levels of nitrofurans and tissue-bound metabolites in muscle of fish after waterborne exposure to nitrofurans. Fish (5 per treatment) were exposed to nitrofurantoin, nitrofurazone, furazolidone or furaltadone (10 mg/L) for eight hours. After this time the fish were killed and their muscle tissue was analysed for parent nitrofuran and tissue-bound metabolites. The concentrations of nitrofurantoin and AHD were around 3 and 2 µg/kg, respectively.

Channel catfish were given ¹⁴C-nitrofurantoin, 1 or 10 mg/kg BW, via intravascular administration or 1 mg/kg BW via oral administration (Stehly & Plakas 1993). The fish (n=9) which received nitrofurantoin through intravascular administration were sampled for blood over a 72 hour period. For the 16 fish treated orally with nitrofurantoin four were killed at each sampling time (4, 24, 48 and 72 hours). Bile, urine, plasma, head and trunk kidneys, muscle, liver and spleen were collected and analysed for nitrofurantoin residues. An additional five fish were given nitrofurantoin orally and their blood sampled over a 72 hour period to determine the bioavailability.

After intravascular injection the levels of nitrofurantoin in plasma quickly decreased and the half-lives were determined to be 23 and 46 minutes for the 1 and 10 mg/kg BW doses, respectively (Stehly & Plakas 1993). A two- and three-compartment pharmacokinetic model best described the plasma concentrations after intravascular administration of 1 and 10 mg/kg BW, respectively. The pharmacokinetic values indicated a limited tissue distribution and rapid clearance.

The bioavailability of nitrofurantoin was 17 % after oral administration and the highest plasma level of nitrofurantoin (0.06 µg/mL) was seen two hours after administration (Stehly & Plakas 1993). The highest concentrations of ¹⁴C-nitrofurantoin and its metabolites in tissues were observed four hours after administration. The levels were highest in plasma (1.22 mg equivalents of nitrofurantoin per kg) and liver (1.64 mg/kg) and the lowest in muscle (0.05 mg/kg). The levels in bile ranged from 9.3 to 13.8 mg/kg over the 72 hour period. The major excretion pathway for nitrofurantoin was via the urine with around 21 % of the administered dose being eliminated in the first 24 hours. The eliminated residues in urine mostly consisted of parent compound but five unidentified metabolites were found in urine.

3.3.2.4 In poultry

McCracken *et al.* (2005a) fed six broiler hens and one cockerel 120-140 g of feed containing nitrofurantoin (400 mg/kg feed), corresponding to about 96-112 mg/kg BW. Eggs were collected and analysed for AHD and when it was clear that nitrofurantoin residues had transferred to the eggs, the eggs laid after this were collected and allowed to hatch. After hatching, four chicks were sacrificed at determined intervals and muscle and liver samples were analysed for AHD. However, AHD could only be detected up to day 10. The levels of AHD in liver and muscle in one day old chicks were around 2.9 µg/kg. In five days old chicks the levels of AHD in liver and muscle were around 0.3 and 0.9 µg/kg, respectively. In 10-days-old chicks no AHD could be detected in liver and levels in muscle were approximately 0.1 µg/kg.

Twenty-four laying hens were fed feed containing 300 mg furaltadone, nitrofurazone, nitrofurantoin or furazolidone per kg feed for one week, corresponding to around 60 mg/kg BW (McCracken & Kennedy 2007). Eggs were then collected for two days and analysed immediately for nitrofurantoin parent compound and their bound residues in albumen, yolk and shell using LC-MS/MS. The levels of nitrofurantoin in yolk, albumen and shell were 66.7, 54.8 and 35.7 ng/g, respectively. The levels of AHD in yolk, albumen and shell were 169, 171, 19.6 ng/g, respectively.

3.3.2.5 In rabbit

The bioavailability of nitrofurantoin via different administration routes was investigated in rabbits (Watari *et al.* 1983). Two groups of rabbits (n=16) were given nitrofurantoin (1.25 or 10 mg/kg BW) once a week via different administration routes; intravenous infusion (i.v. inf.), intraduodenal administration (i.d.), portal vein infusion (p.v. inf.) or oral administration (p.o.). Plasma samples were taken before administration and over a 90 minute period after administration for the low dose group and over a 2.5 hour period after administration for the high dose group. Urine was collected before and eight hours after administration. The plasma concentration-time course could be described by a one-compartment model. Plasma levels were significantly lower after oral administration compared to the other administration routes. At the low dose the maximum level of nitrofurantoin was 0.26 µg/mL after oral administration and around 1.55 µg/mL after the other routes of administration. At the high dose it was 1.73 µg/mL after oral administration and approximately 14 µg/mL after the other routes of administration. In the animals given the low dose of nitrofurantoin around 16 % of the low dose was

excreted as parent compound via the urine after oral administration and 50 % after the other routes of administration. At the high dose, 20 % of the dose was excreted as parent compound via the urine, independent of the route of administration. The bioavailability was calculated to be 30 % after oral administration. The authors concluded that the reduced bioavailability of nitrofurantoin after oral administration was due to degradation of the compound in the stomach.

3.3.2.6 In rat

Female Sprague-Dawley rats with their first litter were treated via gavage with nitrofurantoin (50 mg/kg BW) on lactation day 10 (Kari *et al.* 1997). Pups were removed three hours before collection to allow milk to accumulate. Milk and plasma samples were retrieved at different times after administration. The highest levels in milk and plasma, ~100 and 20 µg/mL respectively, were found 30 and 15 minutes after administration, respectively. The levels of nitrofurantoin were higher in milk than plasma at all time-points and the milk/plasma partitioning ratio was calculated to be 23.

The absorption, metabolism and elimination of nitrofurantoin in rat were investigated by Buzard *et al.* (1961). To show at what site nitrofurantoin is absorbed after oral administration, nitrofurantoin (25 or 100 mg/kg BW) was injected into different segments of the rat gastrointestinal tract; small intestine, colon, cecum and stomach. Blood samples were then retrieved and analysed for nitrofurantoin. It was shown that nitrofurantoin was rapidly absorbed in the small intestine, somewhat absorbed in the colon and not absorbed in cecum or stomach.

Following intravenous injection of nitrofurantoin (25 mg/kg BW) in rats (n=9) the half-life was calculated to be 25 minutes Buzard *et al.* (1961). It was also shown that after bilateral nephrectomy or ligation of the ureters the half-life of nitrofurantoin increased to around 70 minutes, indicating that urinary excretion of nitrofurantoin is an important elimination pathway.

3.3.2.7 In man

Six healthy men, weighing between 62 and 80 kg, received 50 mg nitrofurantoin via intravenous infusion for 45 minutes and via oral administration, with and without food (Hoener & Patterson 1981). One week passed between each treatment. After the intravenous infusion blood and urine samples were collected over a 6 and 24 hour period. After oral administration of nitrofurantoin, blood and urine were collected over

an 8 and 24 hour period. The bioavailability of nitrofurantoin after oral administration was around 87-94 % and 34 % of the administered dose was excreted in the urine as unchanged parent compound. After intravenous infusion the fraction of unchanged parent compound was higher, i.e. 47 %. The half-life of nitrofurantoin in blood after intravenous infusion was calculated to be around 58 minutes. The metabolite aminofurantoin and possibly its acylated conjugate were found in the urine of the test subjects. It was also shown in a serum protein binding test that around 60 % of nitrofurantoin binds to plasma proteins.

Four postpartum women were given a dose of 100 mg nitrofurantoin and the levels in breast milk and plasma were examined over a period of 12 hours after the administration (Gerk *et al.* 2001). The results showed that the levels in milk were higher than those in plasma at all of the time points. The mean maximum concentration in milk was 2.71 µg/mL and was reached five hours after administration. In plasma the mean maximum concentration was 0.50 µg/mL and was reached 4.9 hours after administration. The observed milk:serum ratio (M:S) was 20 times higher than the predicted, 6.2 compared to 0.28, which indicates an active transport of nitrofurantoin into milk.

3.3.2.8 In dog

Buzard *et al.* (1961) intravenously injected dogs (n=3) with nitrofurantoin (4 mg/kg BW), collected plasma samples and measured the levels of nitrofurantoin. The levels decreased quickly and the half-life was calculated to be 25 minutes. After oral (15 mg/kg BW) and intravenous (4 mg/kg BW) administration of nitrofurantoin to dogs the saliva contained nitrofurantoin. Demonstrating that nitrofurantoin is widely distributed.

Male Beagle dogs, weighing 10-16 kg, were intravenously injected with nitrofurantoin (1.5, 3, 6, 12 or 24 mg/kg BW) and samples of blood, urine and bile were collected over a period of six hours (Conklin & Wagner 1971). Thirty minutes after the dogs were administered 3 mg/kg BW, their bile to blood concentration ratio was around 200, indicating an active transport of nitrofurantoin into bile. Over a six hour time period after dosing the percentage of dose found in bile and urine were 16.5-22.6 % and 24.1-36.2 %, respectively, for the doses 1.5-6.0 mg/kg BW. For the 12 and 24 mg/kg BW doses the percentages were 15.3-18.3 % and 3.1-10.0 % in bile and urine, respectively.

The decrease in the dose recovered was concluded to be due to a saturation of the biliary and urinary excretion system. Half-lives of nitrofurantoin in blood were determined to be between 10 and 26 minutes at this dose range after intravenous injection. There was also an increase in bile flow immediately after the administration of nitrofurantoin which was proportional to the dose.

It was shown by Conklin *et al.* (1973) that nitrofurantoin is subjected to enterohepatic circulation in dogs. Donor and recipient dogs were surgically attached so that the bile from the donor dog would flow to the duodenum of the recipient dog. The donors were then administered nitrofurantoin (3 mg/kg BW) intravenously and urine, bile and blood were collected for three hours after administration. Within three hours, donors excreted around 13.9 and 21.5 % of the administered dose in bile and urine, respectively. The recipients excreted approximately 1.5 and 3.4 % of the administered donor dose. Nitrofurantoin (2-5 µg/mL) could be detected in blood of donors within 30 minutes of administration, but no nitrofurantoin was found in recipients' blood. It was estimated that around 5 % of the donors' dose is reabsorbed in the intestines.

Female Beagle dogs, weighing 10.5-12.5 kg, were administered nitrofurantoin intravenously (50 mg) or orally (50 or 100 mg) with or without food (Niazi *et al.* 1983). Blood samples were collected for four or eight hours after administration. The half-life in blood after intravenous administration was calculated to be around 31 minutes. The maximum plasma levels after oral administration of 50 mg nitrofurantoin in a solution (3.0-4.6 µg/mL) were observed 30-60 minutes after treatment, and the half-life was calculated to be around 30 minutes. The absorption of nitrofurantoin was determined to be around 94 % after oral administration of a solution. When 100 mg nitrofurantoin was given in tablet form either with or without food the elimination half-lives ranged between 39 and 63 minutes. The absorption ranged between 46 and 88 %.

3.3.3 Conclusions on pharmacokinetics of nitrofurantoin and its metabolites

Studies on the pharmacokinetics of nitrofurantoin have been performed in swine, cow, fish, poultry, rat, rabbit, dog and man. Many of these studies are quite old and may not meet today's standards in testing. In dogs the oral absorption of nitrofurantoin was 94 %, in a solution, and between 46 and 88 % in tablet form. The availability of nitrofurantoin after oral administration was 16 % in fish and 30 % in rabbit. In man it is

quickly absorbed in the gastro-intestinal tract and the bioavailability is between 87 and 94 %. It was shown that in rat most of the absorption of nitrofurantoin occurs in the small intestine. Nitrofurantoin has been found in muscle, liver, bile, plasma and milk. In both rat and man the levels of nitrofurantoin have been higher in milk than in plasma after exposure to nitrofurantoin indicating that the drug is actively transported into milk. Women who received 100 mg nitrofurantoin had a maximum concentration of 2.71 µg nitrofurantoin per mL breast milk. Nitrofurantoin is rapidly excreted with half-lives being between 30 and 60 minutes in fish, rabbit, rat, dog and man. Man had the longest reported half-life, 58 minutes. Nitrofurantoin is also subjected to enterohepatic circulation in dogs. It seems that the major excretion pathway of nitrofurantoin is via the urine. It has been reported that around 40 % of the administered dose is excreted unchanged in the urine in man (Brunton *et al.* 2011).

Metabolites that have been identified are seen in Table 7. From the studies mentioned above AHD is distributed throughout the body. In swine, after administration of 24 mg/kg BW per day for 10 days and a withdrawal period of six weeks, AHD was still found in liver and kidney (~4 µg/kg), and in muscle (~8 µg/kg). The reported half-lives of AHD are much longer than those for nitrofurantoin. The half-lives of AHD in the tissues of swine were calculated to be around six and eight days in kidney and liver, and muscle, respectively. In fish, the half-life of AHD in muscle was calculated to be 45 days. It has also been shown that AHD is transferred to milk in cows and to chicken eggs. AHD could be detected in chicks, from hens exposed to nitrofurantoin, for up to 10 days after hatching.

Table 7. Metabolites of nitrofurantoin in different species found in the literature

Metabolite	Species/cells	Reference
1-aminohydantoin (AHD)	Poultry, fish, cow, swine	McCracken & Kennedy (2007), McCracken <i>et al.</i> (2005a), Chu <i>et al.</i> (2008), Chu & Lopez (2007), Cooper <i>et al.</i> (2005)
1-[(3-cyano-1-oxopropyl)methylene]amino}-2,4-imidazolidinedione	Rat	Aufrère <i>et al.</i> (1978)
1-[(5-amino-2-furanyl)methylene]amino}-2,4-imidazolidinedione (aminofurantoin)	Rat, man	Aufrère <i>et al.</i> (1978), Hoener & Patterson (1981)

3.4 Toxicology of nitrofurantoin

3.4.1 Acute toxicity

Åkerblom & Campbell (1973) reported LD₅₀-values of 150 and 360 mg nitrofurantoin per kg BW in mice after intraperitoneal injection and by gavage, respectively. The doses tested were between 3 and 12800 mg/kg BW.

3.4.1.1 Conclusions on acute toxicity of nitrofurantoin

The results from this study indicate that nitrofurantoin is quite toxic to mice after oral and intraperitoneal administration, but the study does not follow guidelines or GLP.

3.4.2 Chronic toxicity

In a chronic toxicity study by Butler *et al.* (1990) Sprague-Dawley rats (n=240) were administered nitrofurantoin (0, 24, 48 or 96 mg/kg BW per day) for 24 months. The actual intake was 23.5, 42.8 and 81.3 mg/kg BW per day for males and 31.7, 57.3 and 116.1 mg/kg BW per day for females. Haematological and biochemical investigations were performed on a total of 25 rats per treatment. Five male and female rats were bled and necropsied at 1, 3, 6, 12 and 24 months. At the end of the study all remaining rats were killed and necropsied. Histological examinations were performed on liver, kidney, heart, gonads, thyroid, brain, uterus or prostate and pituitary. There was no effect seen on haematological and biochemical parameters. At the highest dose there was an increase in testicular degeneration in males. At all doses females had an increase in focal biliary proliferation. There was also sciatic nerve fibrosis and degeneration at the highest dose in both male and female rats.

3.4.2.1 Conclusions on chronic toxicity of nitrofurantoin

Only one study on chronic toxicity was found which mostly follows guidelines. Since focal biliary proliferation was observed at all doses in females no NOAEL can be set, but it is not clear to what extent this cell proliferation occurred and if it may be a sign of hepatotoxicity.

3.4.3 Reproductive toxicity including teratogenicity

For one month, male rats (36 per treatment) were orally administered nitrofurantoin (10 or 85 mg/kg BW per day) (Yunda *et al.* 1974). The control group consisted of 18 animals. Treatment with both doses of nitrofurantoin detrimentally affected spermatogenesis. At 10 mg/kg BW per day the concentration of spermatozoa was reduced. This was also seen in the high-dose group along with other effects. This effect was somewhat reversed 48 days after cessation of treatment. Cysteine was shown to

prevent the gonadotoxic effect of nitrofurantoin when administered at the same time as nitrofurantoin.

The study does not meet today's standard of testing, but demonstrates a gonadotoxic effect of nitrofurantoin in rat.

IARC (1990) summarized the results of a study by Prytherch *et al.* (1984). They studied the teratogenicity in rats and effects on fertility, and peri- and postnatal effects in rat and rabbit after exposure to nitrofurantoin. When doses of 10, 20 and 30 mg nitrofurantoin per kg BW were orally administered no adverse effects could be observed. No adverse effects on testicular histology or fertility in males, which were only given 10 mg/kg BW, were seen.

The results from this study could only be obtained as an abstract in IARC (1990). It is not known whether or not the study follows guidelines or GLP. It is also not known how long the exposure was. The results suggest that nitrofurantoin is not reproductively toxic.

Nomura *et al.* (1984) performed a study in mice which is reported in IARC (1990). ICR/Jcl mice were subcutaneously injected with nitrofurantoin (100 or 250 mg/kg BW) on gestation days 9-11. There was no observed increase in embryo- or fetomortality. In the high-dose group there was a decrease in birth-weight and a significant increase in the incidence of cleft palate and syndactyly.

Only a summary of the study was available. The study does not meet today's standard in testing, but demonstrates developmental toxicity of nitrofurantoin in mice.

In a study by NTP (1989) male and female F344/N rats were orally administered nitrofurantoin (~50-850 mg/kg BW per day) via the feed for 13 weeks. B6C3F₁ mice were treated with nitrofurantoin (~96-1600 mg/kg BW) per day for the same length of time. Male and female F344/N rats (100 and 60 mg/kg BW per day respectively) showed testicular and ovarian degeneration. Male mice (285 mg/kg BW per day) also showed testicular degeneration at the end of the experiment.

The study demonstrates that nitrofurantoin may be toxic to the reproductive system.

3.4.3.1 Conclusions on reproductive toxicity including teratogenicity

The results from reproductive toxicity tests are presented in Table 8. The studies presented above show that nitrofurantoin is reproductively toxic. The lowest dose that was seen to cause reproductive effects was 10 mg/kg BW per day in male rats after one month of treatment. But in another study (length of study is not known) male rats treated with the same dose showed no effect on fertility. Further study is needed to elucidate reproductively toxic effects of nitrofurantoin at low doses.

Table 8. Summary on reproductive toxicity studies including teratogenicity of nitrofurantoin

Species	Dose (mg/kg BW)	Duration	Effects	Reference
Rat	10 or 80	1 month	↓spermatogenesis	Yunda <i>et al.</i> (1974)
Rat, rabbit	10, 20 or 30	?	No effects	IARC (1990)
Mouse	100 or 250	3 days, between day 9 & 11 of gestation	↓birth-weight, ↑malformations	IARC (1990)
Rat	50-850	13 weeks	Testicular & ovarian degeneration	NTP (1989)
Mouse	96-1600	13 weeks	Testicular degeneration	NTP (1989)

3.4.4 Mutagenicity and genotoxicity

3.4.4.1 *In vitro* studies

In an Ames test, without metabolic activation, *S. typhimurium* TA 100 and TA 100FR was incubated for three days with 0.3 and 3 µg/plate, respectively (Wang & Lee 1976). The number of his⁺ revertant colonies was then counted and the results showed that nitrofurantoin was mutagenic in both strains at these concentrations. In another experiment, the urine of rats fed feed containing 0.5 % nitrofurantoin was tested in the same manner. The urine contained nitrofurantoin and tested positive for mutagenic properties.

The study does not follow the guidelines but shows that nitrofurantoin is mutagenic in S. typhimurium strains TA 100 and TA100FR.

The Ames test was used by Ni *et al.* (1987) to investigate the mutagenicity of nitrofurantoin. Three strains of *Salmonella typhimurium*; TA 98, TA 98NR and TA 98/1,8-DNP₆, were exposed to nitrofurantoin both with and without rat-liver hepatic S9 activation enzymes. The doses of nitrofurantoin tested were 1, 2, 10, 20 and 100

µg/plate. Nitrofurantoin tested negative for mutagenicity in all strains at the 1 µg/plate dose, both with and without S9. Nitrofurantoin was only mutagenic at the highest dose in strain TA 98NR. In the TA 98 strain, nitrofurantoin was mutagenic at the doses 2 and 10 µg/plate, both in the presence and absence of S9, and also mutagenic at 20 µg/plate with S9. At the highest dose, nitrofurantoin was toxic to bacteria and was also toxic at 20 µg/plate without S9. Nitrofurantoin was mutagenic in the strain TA 98/1,8-DNP₆ at the doses of 2, 10 and 20 µg/plate, both in the presence and absence of S9.

Nitrofurantoin was toxic to the bacteria at the highest dose.

The study follows OECD guidelines to some extent and the results show that nitrofurantoin is mutagenic in all tested strains both with and without metabolic activation. In all test strains, except TA 98NR, the mutagenicity of nitrofurantoin appeared to be dose-dependent.

In another Ames test, nitrofurantoin (0.1-5.0 µg/plate) was incubated with *S. typhimurium* TA 98 and 100 for 48 hours without metabolic activation (Goodman *et al.* 1977). The revertant his⁺ colonies were then counted and it was concluded that nitrofurantoin was mutagenic.

The study mostly follows guidelines and shows that nitrofurantoin is mutagenic.

An *in vitro* mammalian cell gene mutation test was performed on nitrofurantoin in CHO cells (Gao *et al.* 1989). Cells were incubated with four different concentrations of nitrofurantoin (0-200 µg/mL) for five hours with or without S9-mix. Benzo[a]pyrene was used as positive control when S9 was present. The results showed that nitrofurantoin was weakly mutagenic.

The study for the most part follows guidelines but does not follow GLP. From the results in the study the authors conclude that nitrofurantoin is mutagenic.

An *in vitro* chromosome aberration test was performed on nitrofurantoin using human peripheral blood (Slapsytè *et al.* 2002). Lymphocytes were incubated with nitrofurantoin (0, 5, 20 or 40 µM) for 24 hours. Colchicine was added three hours before harvest and metaphasic cells (50 or 25 cells per culture) were examined for chromosome aberrations and SCEs, respectively. Nitrofurantoin increased the number of chromosome aberrations and SCEs at 20 and 40 µM.

The study follow the guidelines to some extent and shows that nitrofurantoin is genotoxic in vitro.

E. coli WP2 and WP2 uvrA were plated on agar with limited tryptophan and incubated with nitrofurantoin (10 or 100 µg/plate) for 48 hours at 37 °C to evaluate its capacity to mutate tryp⁻ cells into tryp⁺ cells (McCalla & Voutsinos 1974). The number of colonies were then counted and compared to controls. The results showed that nitrofurantoin induced mutations in *E. coli* WP2 and WP2 uvrA at 10 µg/plate and in WP2 at 100 µg/plate.

The study does not completely follow guidelines and does not follow GLP. The results indicate that nitrofurantoin is mutagenic without metabolic activation, although no positive controls were included.

The effect of nitrofurantoin on DNA was also tested *in vitro* using rat liver nuclei and human foreskin fibroblasts (HuF₂₂) (Parodi *et al.* 1983). Cells were incubated with nitrofurantoin (112 mg/mL) for 30 minutes at 37 °C. The cells were then evaluated for DNA fragmentation. At 112 mg nitrofurantoin per mL the amount of DNA fragmentation was significantly increased compared to controls in both HuF₂₂ cells and liver nuclei.

The experiments presented in this study demonstrate that nitrofurantoin damages DNA both in vivo and in vitro and may therefore be mutagenic.

3.4.4.2 *In vivo* studies

The mutagenicity of nitrofurantoin was tested in a bone marrow micronucleus test using Sprague-Dawley rats (Goodman *et al.* 1977). Rats were intraperitoneally injected with nitrofurantoin (50, 100 or 200 mg/kg BW). Half of the dose was given 30 hours before sacrifice and the other half was given six hours before sacrifice. Triethylenemelamine served as positive control. Between 2000 and 3000 cells per rat were examined and the number of reticulocytes with micronuclei was counted. Nitrofurantoin did not increase the number of reticulocytes with micronuclei at any dose and was concluded to be non-mutagenic *in vivo*.

The study does not follow current guidelines and according to the authors the results show that nitrofurantoin is not mutagenic under these conditions. However, the sampling only occurred six hours after the last dosing and that is not long enough for

micronucleated reticulocytes to be formed. Therefore, the study is not that reliable since the results may be false negative. There is also no mention of any other signs of toxicity being observed or not observed during the study.

In a patient study, the number of sister chromatid exchanges (SCEs) in peripheral lymphocytes was examined in women (n=15) treated with nitrofurantoin for urinary tract infection (Sardas *et al.* 1990). Blood samples were collected before and after oral treatment with nitrofurantoin (10 mg/kg or 400 mg) for 10 days. Division of the lymphocytes was induced by phytohaemagglutinin and they were allowed to divide for two cell cycles at 37 °C for 72 hours. Two hours before harvesting, colcemid (10 µg/mL) was added. Around 30 lymphocytes in metaphase were analysed for SCEs. Treatment with nitrofurantoin did not increase the number of SCEs.

The results from the study indicate that nitrofurantoin is not genotoxic in vivo at doses used to treat urinary tract infections.

Blood from children (n=69) suffering from urinary tract infections were collected after treatment with nitrofurantoin (Slapsytè *et al.* 2002). The treatment period was between 0 and 12 months long with the treatment consisting of 5-8 mg/kg BW per day for the first seven days and after that 1-2 mg/kg BW per day for the remainder of the treatment period. Blood samples were collected 1, 3, 6 and 12 months after the start of the treatment. Blood from control children were retrieved before the start of treatment. Blood samples before treatment was available from 13 patients. Around 100 lymphocytes were then analysed for chromosome aberrations and between 25 and 50 cells were examined for SCEs. No significant increase in chromosome aberrations or SCEs compared to the control could be seen after nitrofurantoin treatment. But in the children who had available blood samples from before the treatment there was a significant increase in SCEs after nitrofurantoin treatment.

The results from the study are equivocal. On the one hand nitrofurantoin did not increase SCEs and chromosome aberrations compared to controls. On the other hand there was an increase in SCEs in children from which blood samples were available before and after treatment.

Young and adult BALB/C mice (n=64), three and eight weeks old respectively, were intraperitoneally injected with nitrofurantoin (5, 10 or 50 mg/kg BW) or cyclophosphamide (75 mg/kg BW) (Fucic *et al.* 2005). Each treatment group consisted

of four male and four female mice. Peripheral blood was collected before and 48, 96, 168 and 336 hours after administration, and the frequencies of micronuclei in 1000 reticulocytes were analysed. The results showed that in young mice nitrofurantoin caused a significant increase in the frequency of micronuclei at all doses and time points. In old mice nitrofurantoin caused a significant increase in the frequency of micronuclei at all doses, but two weeks after the administration the micronucleus frequency was the same as before administration.

The study mostly follows guidelines but does not follow GLP. Only 1000 instead of 2000 cells were examined for micronuclei which can cause the results to be false negative or positive. The results show that nitrofurantoin is genotoxic in vivo at these doses.

Transgenic Big Blue C57BL/6[LIZ] mice (n=10) were orally administered nitrofurantoin (0 or 167 mg/kg BW per day) for five consecutive days (Quillardet *et al.* 2006). The mice were sacrificed 20 days after the last administration and organs; stomach, colon, kidney, cecum, bladder, lung, intestine and spleen, were collected. The frequency of mutants in the *cII* gene in these tissues was examined. The results showed that there was only a weak mutagenic effect of nitrofurantoin on genes expressed in the kidney.

The study does not follow GLP or guidelines from 2011 or 2013. The results, however, do show that nitrofurantoin is slightly mutagenic to genes expressed in kidney of treated mice. However, the doses were much higher than the doses used to treat urinary infections in humans.

Nitrofurantoin was also investigated using the *Drosophila* wing somatic mutation and recombination test (SMART) (Graf *et al.* 1989). Three-day-old larvae of *Drosophila melanogaster* were exposed to 5 mL of nitrofurantoin (5 or 10 mM) dissolved in 5 mL medium for 48 hours. The larvae were allowed to pupate and hatch and their wings were then examined for spots. Compared to controls, nitrofurantoin significantly increased the frequency of large and twin spots at both and the highest concentration, respectively. The frequency of small spots was not affected.

The study shows that nitrofurantoin is mutagenic in Drosophila under these test conditions.

Parodi *et al.* (1983) tested the ability of nitrofurantoin to produce DNA damage in tissues of male Swiss mice and Sprague-Dawley rats. Rats and mice were administered nitrofurantoin (14-112 mg/kg BW) intraperitoneally and killed between six and 168 hours after administration. DNA damage in rat liver, spleen, kidney and lung was examined while only the bone marrow was examined in mice. The results showed that compared to controls the amount of damaged DNA was significantly increased in the tissues of treated animals. Effects were seen in the kidney at the lowest dose tested (14 mg/kg BW).

The experiments presented in this study demonstrate that nitrofurantoin damages DNA in vivo and may therefore be mutagenic.

Parodi *et al.* (1983) also investigated the potential of nitrofurantoin to induce sister chromatid exchanges in bone marrow cells of male Swiss mice. Mice were injected intraperitoneally with nitrofurantoin (32 or 64 mg/kg BW) and bone marrow cells were extracted 24 or 72 hours after administration. Thirty cells in metaphase were examined for SCEs per animal and dose. The results showed that there was a dose-dependent increase in the frequency of SCEs after treatment with nitrofurantoin.

The study shows that nitrofurantoin is capable of inducing SCEs in mice in vivo.

3.4.4.3 Conclusions on mutagenicity and genotoxicity studies

The results from the mutagenicity and genotoxicity studies are summarised in Table 9. Nitrofurantoin has shown to be mutagenic in bacterial tests both in the presence and absence of an exogenous metabolic system. It has also been shown to be mutagenic and genotoxic in other *in vitro* tests. In IARC (1990) there are some other *in vitro* studies mentioned reporting both negative and positive results. The *in vivo* studies stated above have reported both negative and positive results and most of the *in vivo* studies reported in IARC (1990) have shown negative results. From all of the studies it appears that nitrofurantoin is mutagenic in bacteria and mammalian cells *in vitro* but not *in vivo*. However, more *in vivo* studies should be performed to be able to declare this with certainty.

Table 9. Summary of mutagenicity and genotoxicity studies on nitrofurantoin

Test	Species/strain	Dose/concentration	Result	Reference
Ames test	<i>S. typhimurium</i> TA 100 & TA100FR	0.3 or 3 µg/plate	+	Wang & Lee (1976)
Bacterial reverse mutation test	<i>E. coli</i> WP2 and WP2 uvrA	10 or 100 µg/plate	+	McCalla & Voutsinos (1974)
Ames test	<i>S. typhimurium</i> TA 98, TA 98NR, TA 98/1,8-DNP	0.1-2.5 µg/plate*	+	Ni <i>et al.</i> (1987)
Ames test	<i>S. typhimurium</i> TA 98 & 100	0.1-5.0 µg/plate	+	Goodman <i>et al.</i> (1977)
<i>In vitro</i> DNA damage test	Rat liver nuclei & HuF ₂₂ cells	112 mg/mL	+	Parodi <i>et al.</i> (1983)
<i>In vitro</i> mammalian cell gene mutation test	CHO cells	0-200 µg/mL*	+	Gao <i>et al.</i> (1989)
<i>In vitro</i> chromosome aberration test	Human lymphocytes	5-40 µM	+	Slapsytè <i>et al.</i> (2002)
<i>Drosophila</i> wing spot test	<i>D. melanogaster</i>	5 or 10 mM	+	Graf <i>et al.</i> (1989)
Transgenic rodent somatic cell gene mutation assay	Transgenic Big Blue C57BL/6[LIZ] mice	167 mg/kg BW	+(in kidney genes)	Quillardet <i>et al.</i> (2006)
<i>In vivo</i> SCE assay	Swiss mice	32 & 64 mg/kg BW	+	Parodi <i>et al.</i> (1983)
<i>In vivo</i> SCE assay	Human lymphocytes	10 mg/kg or 400 mg	-	Sardas <i>et al.</i> (1990)
<i>In vivo</i> DNA damage test	Sprague-Dawley rats & Swiss mice	14-112 mg/kg BW	+	Parodi <i>et al.</i> (1983)
<i>In vivo</i> chromosome aberration test	Human lymphocytes	5-8 & 1-2 mg/kg BW	+/-	Slapsytè <i>et al.</i> (2002)
<i>In vivo</i> micronucleus test	BALB/C mice	5, 10 or 50 mg/kg BW	+**	Fucic <i>et al.</i> (2005)
<i>In vivo</i> bone marrow micronucleus test	Sprague-Dawley rats	50, 100 or 200 mg/kg BW	-***	Goodman <i>et al.</i> (1977)

+ positive, - negative, +/- equivocal

* With and without S9-mix, ** May be false negative/positive, *** May be false negative

3.4.5 Carcinogenicity and long-term toxicity

In a two year carcinogenicity study male and female Sprague-Dawley rats (50 per sex and treatment) were fed feed containing nitrofurantoin (Butler *et al.* 1990). The doses were for males; 12.4, 22.3 and 43.1 mg/kg BW per day, and for females; 16.8, 31.0 and

55.8 mg/kg BW per day. High-dose males showed increased mortality compared to controls. At the end of the study the rats were killed and necropsied. Tissues were sampled and histologically examined. The only reported effect observed was an increase in focal biliary proliferation in middle- and high-dose females.

The study follows guidelines but not GLP. The reported results indicate that nitrofurantoin is not carcinogenic. IARC (1990) showed that there was a significant increase in the incidences of malignant lymphomas in males. Therefore nitrofurantoin may be carcinogenic in male rats.

Male and female F344/N rats and B6C3F₁ mice (50 per sex and treatment) were fed feed containing nitrofurantoin for 103 weeks (NTP 1989). The exposure to nitrofurantoin in the low and high dose groups were around 30 and 60 mg/kg BW per day for female rats and 60 and 110 mg/kg BW per day for male rats. For male and female mice the exposure levels were 300 and 570 mg/kg BW per day, and 280 and 580 mg/kg BW per day, respectively. At the end of the study all surviving animals were killed, necropsied and histological examinations were performed.

Male rats treated with nitrofurantoin had lesions in the kidney and testis (NTP 1989). Testicular degeneration and abnormal cells of the epididymis were seen in males of the high-dose group. Dosed male rats also had perivascular infiltration of mononuclear cells and fibrinoid necrosis of arterioles in the testis. In all treated male rats chronic nephropathy was seen but judged as most severe in high-dose males. There was also a significant increase in microscopic renal tubular cell adenomas in treated males compared to controls. Increased incidences of subcutaneous tissue neoplasms in dosed males were also observed. Rare osteosarcomas were also discovered in three rats, one from the low dose and two from the high dose. No effects were seen in female rats. It was concluded that there is some evidence of nitrofurantoin being carcinogenic in male rats.

In mice there was a reduced survival of control females compared to the dosed mice (NTP 1989). Males showed testicular degeneration in high dose males as well as mineralization of the renal medulla and dilation of the renal tubules. Almost all dosed female mice had ovarian atrophy, spindle cell hyperplasia of the adrenal cortex and there was also mineralization of the renal medulla in high-dose females. There was an increase in hepatocellular neoplasms (adenomas or carcinomas, combined) in high-dose females. There was a significant increase in tumours in the high dose group when

benign mixed ovarian tumours and tubular adenomas were combined ($p=0.01$) (NTP 1989). No effects were seen in male mice. It was concluded that there was clear evidence of nitrofurantoin being carcinogenic in female mice.

The study follows guidelines but it is not clear if GLP was followed. The results suggest that nitrofurantoin may be carcinogenic in male rats and female mice.

Morris *et al.* (1969) fed female albino Holtzman rats, 20 per treatment, nitrofurantoin for 44.5 weeks followed by a 15-17 weeks long non-medicated period. Rats that were 60 days old were fed 32 or 37 mg per day, corresponding to about 185 and 214 mg/kg BW per day respectively. Rats that were 22 days old were fed 35 mg per day, corresponding to 620 mg/kg BW per day. At the end of this period all animals were killed and examined. Nitrofurantoin did not cause tumours in this study.

The study is quite old and does not follow guidelines. It appears that female rats were not sensitive to any carcinogenic effects of nitrofurantoin but the low number of animals makes it difficult to draw any significant conclusions from the results.

The results from a study by Ito *et al.* (1983) are presented in IARC (1990). Mice (C57B1/6N×DBA/2N) F_1 (BDF₁) were fed nitrofurantoin (0, 750 or 3000 mg/kg feed), corresponding to about 37.5-150 and 150-600 mg/kg BW per day depending on the weight of the animals, for 104 weeks. The treatment groups contained around 50 animals per sex. No increase in tumour formation was found.

Only a summary was available. Therefore it is difficult to draw any substantial conclusions about the results in this study.

IARC (1990) reported the results of a study by Stitzel *et al.* (1989). In that study, female B6C3F₁ mice were orally administered nitrofurantoin (0, 350 or 500 mg/kg BW per day) for 64 weeks. At the end of the study almost all treated mice had ovarian atrophy, but there was no observed increase in the incidence of neoplasms in the reproductive system.

The results from this study could only be obtained as an abstract in IARC (1990). It does not follow GLP or guidelines. IARC reports that a small number of animals were used in this study.

A study by Cohen *et al.* (1973) is summarized in IARC (1990). Cohen *et al.* administered weanling female Sprague-Dawley rats nitrofurantoin for 75 weeks. For the first 16 weeks the rats were fed feed containing nitrofurantoin (1870 mg/kg). After this time the dose was lowered to 1000 mg/kg due to premature mortality and diminished growth. The doses corresponded to about 374 and 200 mg/kg BW per day. Five weeks after the medication period the rats were sacrificed and examined. There was no increase in tumour formation observed.

Only a summary of the study was available and the study does not follow guidelines. The change in dose during the study makes it not that reliable and IARC (1990) also reported that a small number of animals were used.

IARC (1990) also reported the results from a study by Wang *et al.* (1984). Wang *et al.* fed female weanling, germ-free Sprague-Dawley rats feed containing nitrofurantoin (0 or 1880 mg/kg), corresponding to roughly 94-204 mg/kg BW per day depending on the weight, for 104 weeks. The incidence of mammary fibroadenomas was significantly increased in treated rats.

Results from this study were only retrieved as a summary in IARC (1990), who reported that only a small number of animals were used. The study does not follow guidelines. The results indicate that nitrofurantoin may have a carcinogenic effect in the mammary of female rats.

3.4.5.1 Conclusions on the carcinogenicity and long-term toxicity of nitrofurantoin

The results from the carcinogenicity studies are presented in Table 10. Some long-term toxic effects have been reported in a few studies mentioned above. Female mice exposed to 350 and 500 mg/kg BW per day had ovarian atrophy and female rats exposed to 31 and 56 mg/kg BW per day had increased focal biliary proliferation. Male rats showed testicular degeneration when exposed to 110 mg/kg BW per day and male rats treated with 60 and 100 mg/kg BW per day had chronic nephropathy.

The results from the carcinogenicity studies have been equivocal. Four studies have observed a carcinogenic effect of nitrofurantoin while four others have not observed such an effect. Three out of the four studies that did not see an effect had a small number of test animals. This might have caused the authors to miss any carcinogenic effects of nitrofurantoin. Nitrofurantoin is possibly carcinogenic but more studies are needed to be certain of this.

Table 10. Summary of carcinogenicity and long-term toxicity studies on nitrofurantoin

Species	Dose (mg/kg BW)	Duration (medicated + non-medicated period)	Effects	Reference
Sprague-Dawley rats	~12-56	2 years	↑malignant lymphomas	Butler <i>et al.</i> (1990)
F344/N rats	30-110	103 weeks	↑microscopic renal tubular cell adenomas, ↑subcutaneous tissue neoplasms, testicular degeneration	NTP (1989)
B6C3F ₁ mice	280-580	103 weeks	↑tumours, testicular degeneration, ovarian atrophy	NTP (1989)
Holtzman rats	32-620	~60 weeks (44.5+~16 weeks)	No effects	Morris <i>et al.</i> (1969)
Mice	37.5-150 or 150-600	104 weeks	No effects	IARC (1990)
B6C3F ₁ mice	350 or 500	64 weeks	Ovarian atrophy	IARC (1990)
Sprague-Dawley rats	374→200	80 weeks (75+5 weeks)	No effects	IARC (1990)
Sprague-Dawley rats	94-204	104 weeks	↑mammary fibroadenomas	IARC (1990)

4 Furazolidone

Furazolidone has both antibacterial and antiprotozoal activity (Reynolds 1982, Sweetman 2002). In human medicine it was used to treat cholera, giardiasis, diarrhoea and gastro-enteritis (Reynolds 1982, Sweetman 2002). In veterinary medicine furazolidone has been used to treat enteritis in swine and rabbits, and also many diseases in poultry; coccidiosis, histomoniasis, synovitis, fowl typhoid etc. (Siegmund 1979 as cited in IARC 1983).

4.1 Physicochemical properties

International Non-proprietary Name (INN)

Furazolidone

Chemical Abstract Service (CAS) name

3-(5-Nitrofurfurylideneamino)-2-oxazolidone

CAS number: 67-45-8

Structural formula

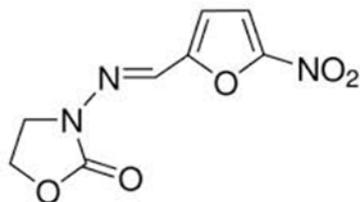


Figure 5. Structural formula of furazolidone

Molecular formula

$C_8H_7N_3O_5$

Molecular weight

225.2

Table 11. Physicochemical properties (Debnath *et al.* 1991, FAO 1993b)

Melting point	275 °C
Octanol/Water Partition Coefficient	Log K_{ow} = -0.04
Solubility in water (pH 6)	Slightly soluble (40 mg/L)
Solubility in ethanol	Slightly soluble (90 mg/L)
Solubility in dimethylformamide	Sparsingly soluble

4.2 Pharmacodynamics

The effects of furazolidone on human hepatoma G2 (HepG2)-cells were investigated by Jin *et al* (2011). In a comet assay HepG2 cells were exposed to furazolidone (0, 12.5, 25 or 50 $\mu\text{g/mL}$) or furazolidone (50 $\mu\text{g/mL}$), catalase (CAT) (100 $\mu\text{g/mL}$) and superoxide dismutase (SOD) (100 $\mu\text{g/mL}$) for three hours. H_2O_2 (40 μM) served as positive control and 0.1% DMSO as negative control. The positive control had significantly ($p < 0.01$) more DNA damage than the negative control. Furazolidone treated cells showed a dose-dependent ($p < 0.01$) increase in the amount of DNA strand breaks. Cells treated with furazolidone, CAT and SOD also had significantly ($p < 0.01$) more DNA damage than the negative control but significantly ($p < 0.01$) less DNA damage than cells treated with 50 $\mu\text{g/mL}$ furazolidone.

The production of reactive oxygen species (ROS) after treatment of cells with furazolidone (0, 12.5, 25 or 50 $\mu\text{g/mL}$) or furazolidone (50 $\mu\text{g/mL}$), CAT (100 $\mu\text{g/mL}$) and SOD (100 $\mu\text{g/mL}$) for 24 hours was examined using DCF-DA fluorescence. It was shown that ROS were induced in a dose-dependent manner and that ROS levels decreased when cells were exposed to furazolidone together with CAT and SOD. Cells, treated as above, were also stained for 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker for oxidative DNA damage, and it was found that the 8-OHdG staining intensity increased in a dose-dependent manner in the nucleus and cytoplasm after exposure to furazolidone. Simultaneous treatment with CAT and SOD decreased the

staining intensity. The levels of glutathione (GSH) and CAT in cells significantly decreased after exposure to furazolidone (25 and 50 µg/mL). The levels of SOD significantly decreased after exposure to 12.5, 25 and 50 µg/mL furazolidone.

Ali *et al.* (1988) orally administered furazolidone (7.5, 15 or 30 mg/kg BW) to male turkeys for two weeks and then measured the levels of luteinizing hormone (LH) and prolactin (PRL). No effects on the levels of PRL or LH were observed at any dose but at 30 mg/kg BW the hypothalamic levels of dopa, adrenaline and noradrenaline were significantly increased.

4.3 Pharmacokinetics

4.3.1 *In vitro* studies

Two metabolites were isolated after *in vitro* reduction of furazolidone using milk xanthine oxidase (Tatsumi *et al.* 1978). Only one metabolite could be identified, 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone. This metabolite was also found in the urine of four male albino rabbits following a single oral administration of furazolidone at a dose of 100 mg/kg BW.

The *in vitro* metabolism of furazolidone using milk xanthine oxidase and the S9-mix for 20 minutes was investigated by Tatsumi *et al.* (1981). Three metabolites were detected and identified; 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone, an aminofuran derivative N-(5-amino-2-furfurylidene)-3-amino-2-oxazolidone and 2,3-dihydro-3-cyanomethyl-2-hydroxyl-5-nitro-1 α ,2-di(2-oxo-oxazolidin-3-yl) iminomethylfuro[2,3-b]furan.

Vroomen *et al.* (1987a) examined the metabolism of ¹⁴C-furazolidone by rat liver microsomes under anaerobic and aerobic conditions. Around 2-3% of the total metabolites were covalently bound to proteins. Two major metabolites, 2,3-dihydro-3-cyanomethyl-2-hydroxyl-5-nitro-1 α ,2-di(2-oxo-oxazolidin-3-yl)iminomethylfuro[2,3-b]furan and 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone, were formed and accounted for 16.4 and 16.6 %, respectively, of the total extractable radioactivity. It was shown that the metabolism of furazolidone was not dependent on cytochrome P-450, but is reliant on another enzymatic NADPH-dependent process.

To study the degradation pathways in different cell lines (Caco-2, HEp-2 and V79) cells were incubated with ¹⁴C-furazolidone, labelled in the nitrofurantoin part, for 24 or 48 hours (de Angelis *et al.* 1999). The degradation of furazolidone led to the formation of several hydrophilic compounds which to a large extent differed between the cell lines. In HEp-2 cells small amounts of the open-chain cyano metabolite 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone was formed.

de Angelis *et al.* also evaluated the degree of protein-bound furazolidone metabolites by incubating cells with ¹⁴C-furazolidone, labelled in either the furan or oxazolidone ring, for 24 hours. Of the three cell lines, HEp-2 cells showed the most binding followed by Caco-2 and V79 cells. In Caco-2 and HEp-2 cells there was a higher degree of binding of molecules labelled in the furan ring. In V79 cells the degree of binding was the same.

Liver microsomes from a female swine weighing 90 kg were incubated with ¹⁴C-furazolidone (36.7 μM) for six minutes at 37 °C (Vroomen *et al.* 1987b). At the end of the incubation 53.6 % of the furazolidone had been metabolised, of which 11.6 % was covalently bound to protein. 27.4 % could be extracted in ethyl acetate and 61.1 % in water. Microsomes that had been inactivated by heating showed no furazolidone conversion when NADH or NADPH was added. Three major peaks were seen after HPLC-analysis of the ethyl acetate extract of which two metabolites were identified as 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone and 2,3-dihydro-3-cyanomethyl-2-hydroxyl-5-nitro-1 α ,2-di(2-oxo-oxazolidin-3-yl) iminomethylfuro[2,3-b]furan, constituting 10.5 and 6.4 % of the total metabolites formed respectively.

The amount of radioactivity in the ethyl acetate increased while the radioactivity in the water and protein-bound fraction decreased when 5 mM of mercaptoethanol was added to the incubation mixture. When the ethyl acetate fraction was analysed by HPLC three new peaks had appeared. One accounted for around 50 % of the total metabolites formed and the other two accounted for 4 and 8 %. The new major metabolite formed was tentatively identified as 3-(4-cyano-3(or 4)- β -hydroxyethylmercapto-2-oxobutylideneamino)-2-oxazolidone, a mercaptoethanol conjugate of furazolidone. This indicates that at least 50 % of the total metabolites of furazolidone are formed through reductive processes in swine liver microsomes.

Pig hepatocytes were incubated for 4 or 20 hours with furazolidone (2.5 or 25 μM), AOZ (2.5 or 25 μM) to investigate the formation of protein-bound metabolites (Hoogenboom *et al.* 2002). The results showed that the formation of protein-bound metabolites was dose and time related for AOZ. In a similar experiment pig hepatocytes were incubated for 4 or 20 hours with furazolidone or AOZ and the formation of protein-bound metabolites and their release under acidic conditions were examined. Protein-bound metabolites were formed in all dose and incubation groups. The fraction of released AOZ from proteins in cells incubated with furazolidone ranged between 31 and 59 % while in the cells incubated with AOZ the fraction ranged between 21 and 35 %.

Several other metabolites of furazolidone have also been discovered, e.g. 5-nitro-furaldehyde and 5-nitrofur-2-carboxylic acid (FAO 1993b).

4.3.2 *In vivo* studies

4.3.2.1 In swine

Six individually housed three-weeks-old male piglets (Dutch Landrace \times Great Yorkshire) were divided into three groups; control, ^{14}C -furazolidone (labelled in the methylene group of the oxazolidone moiety), and unlabelled furazolidone (Vroomen *et al.* 1986). The piglets were fed twice daily and the furazolidone preparations (12 mg/kg BW) were administered, via stomach tube, at the same time for 10 days. A 14 days long withdrawal period then followed. Faeces and urine were collected throughout the entire experiment. Two hours after each morning feeding blood samples were retrieved and the concentration of furazolidone was analysed in blood and plasma. Expired air was analysed for radioactivity during the medication period.

At day 10 one piglet from each group was sacrificed and at the last day of the withdrawal period the three remaining piglets were sacrificed. Samples of fat, muscle and liver were taken and analysed for radioactivity and unlabelled furazolidone. Samples were also taken from several other organs and analysed for radioactivity. The concentration of furazolidone was analysed by HPLC-UV at 362 nm. Radioactivity (μg equivalents of parent compound per g tissue) was measured in a Philips liquid scintillation counter.

Mean levels of furazolidone in blood during the medication period were between 0.31 and 0.53 $\mu\text{g}/\text{mL}$. Similar values were seen in plasma, but no accumulation in blood

was seen during the experiment. After the last administration on day 10, the maximum concentrations of furazolidone in blood and plasma were reached within 30 minutes (0.84 µg/mL in blood and 0.96 µg/mL in plasma). After this point the levels decreased and were below the limit of detection (1 ng/mL) 3-4 hours after administration. The half-life of furazolidone in blood and plasma was around 60 minutes.

Radioactivity in blood and plasma increased during the medication period and reached maximum levels (9-10 µg equivalents of parent compound per mL) after 5-6 days. During the first day of the withdrawal period there was a fast decrease in radioactivity followed by a slower decrease during the next 13 days, with a half-life of approximately six days. At the end of the withdrawal period, residual values of 2.6 and 1.5 µg equivalents of parent compound per mL were still present in blood and plasma respectively.

The main excretion pathway of radiolabelled furazolidone was via urine. At the end of the experiment the total excretion of radioactive labelled material via the urine was 61 %. No radioactivity was measured in the expired air and 18-21 % of the radioactivity was excreted via the faeces. No parent furazolidone could be detected in muscle, liver, kidney or fat immediately after the medication period. The tissues with the highest radioactivity after the medication period were bile, liver and kidney (81.1, 32.9 and 30.1 mg/kg). The radioactivity in muscle was between 5.7 and 7.2 mg/kg. At the end of the withdrawal period the levels ranged between 1.1 and 3.1 mg/kg. The recovery of total dose radioactivity was 80%.

The percentage of non-extractable radioactivity increased during the withdrawal period in liver, kidney and muscle. At this time the non-extractable radioactivity levels were 0.8, 0.9 and 1.2 mg/kg. The interaction between furazolidone and DNA was also investigated. Immediately after the medication period there were marked interaction in the pancreas and kidney, 382 and 372 pmol equivalents of parent compound per mg DNA respectively. At the end of the withdrawal period the DNA binding was around 100 pmol equivalents of parent compound per mg DNA.

Pigs (n=18) were fed feed containing furazolidone (400 mg/kg feed) *ad libitum* for ten days, corresponding to around 24 mg/kg BW per day, followed by a withdrawal period of six weeks (Cooper *et al.* 2005). Three pigs were sacrificed each week of the withdrawal period and samples of muscle, liver and kidney were taken and analysed for furazolidone and AOZ using LC-MS/MS and high performance liquid chromatography-

UV (HPLC-UV). Furazolidone was only detected in one muscle sample (0.33 µg/kg BW) at week 0 of the withdrawal period. AOZ was detected in all samples and the levels found at week 6 of the withdrawal period were around 15 µg/kg BW in kidney and 50 µg/kg BW in muscle and liver. The depletion half-lives of AOZ in muscle, liver and kidney were 11.5±0.9 days, 7.3±0.3 days and 6.9±0.8 days respectively.

Adult swine (Dutch Landrace × Great Yorkshire) were orally administered feed containing around 7.8 mg/kg BW of furazolidone per day for 10 days followed by a withdrawal period of 14 days (Vroomen *et al.* 1987c). Ten swine were treated with furazolidone and two served as control. Urine was collected during the medication period and analysed for furazolidone. On day 10 of the medication period blood samples were taken after the last administration and analysed for furazolidone and 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone. On every other day of the withdrawal period two animals were sacrificed and samples of liver, kidney and muscle were retrieved and analysed for furazolidone and 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone. On the second day of medication the urinary excretion of furazolidone was at its highest, 447-1784 µg per animal, and then decreased during the rest of the medication period. The daily mean amount of excreted furazolidone in the urine was calculated to be 0.06% ± 0.04% of the daily dose (range: 0.01-0.26%). Five hours after the last administration of furazolidone over 60% of the total excreted amount of furazolidone had been excreted via the urine and after an additional 19 hours no furazolidone could be detected. The maximum plasma levels of furazolidone, 62-121 ng/mL, were reached within 60-75 minutes after the last administration and the elimination half-life was calculated to be approximately 45 minutes. 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone reached its maximum concentration, 24.9-45.3 ng/mL, in plasma within 1.5 and 4 hours. It could no longer be detected 17 hours after the administration. No furazolidone could be detected in any tissue samples two hours after the last administration on day 10, but 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone was detected in muscle samples at a mean level of 17 µg/kg.

McCracken *et al.* (1995) administered furazolidone to 15 pigs weighing around 30 kg via stomach tube, corresponding to 20 mg/kg BW. At 1, 2, 4, 6 and 12 hours after the exposure, three pigs were sacrificed and samples of the liver, muscle and kidney were collected. Additional muscle samples were taken from the pigs slaughtered after one

hour and stored at -20 °C, -70 °C and in liquid nitrogen to be analysed weekly for up to four weeks. The levels of furazolidone in the samples were examined using thermospray LCMS. It was found that the levels in liver and kidney were much lower than those in the muscle tissue. The half-life of furazolidone in muscle was calculated to be around one hour. The stability of furazolidone in muscle kept in storage at -20 °C was tested. Furazolidone proved unstable and after one week only 10 % of the original concentration remained. The levels of furazolidone in the samples stored at -70 °C had dropped to 50 % of the original concentration and samples stored in liquid nitrogen also showed decreased levels.

Ten twelve-weeks-old pigs were given feed two times per day containing 300 mg furazolidone per kg feed (corresponding to around 17 mg/kg BW) for seven days (Hoogenboom *et al.* 1992). Following the medication period was a withdrawal period of four weeks. At 0, 1, 2, 3 and 4 weeks of the withdrawal period two animals were slaughtered at each time point. Samples of kidney, liver and rump muscle were taken and stored at -20 °C before analysis. The samples were analysed for protein-bound metabolites containing the AOZ side chain. The results showed that the AOZ side chain was released from protein-bound metabolites in liver, muscle and kidney throughout the experiment. At the end of the medication period, the levels of AOZ released from liver, muscle and kidney were 915-1070, 103-144 and 434-765 µg/kg w.w., respectively. At the end of the four week withdrawal period, these levels had dropped significantly and were now 35-46, 6-13 and 6-7 µg/kg w.w. in liver, muscle and kidney samples, respectively.

Hoogenboom *et al.* (1991) studied the nature of protein-bound metabolites of furazolidone using pig hepatocytes, microsomes and liver samples. Hepatocytes were isolated from a liver sample from a sow between five and nine months old and cultured. These cells were then incubated for different time intervals with 25 or 50 µM ¹⁴C-furazolidone. There was a time-related increase in the amount of protein-bound metabolites and 36 hours after the exposure ended, the levels decreased to approximately 50 % of the maximal levels of bound metabolites. From a test using hepatocytes incubated with furazolidone for 24 hours it was shown that almost 70 % of the bound metabolites contained an AOZ side-chain that could be released under acidic conditions similar to those in the stomach.

Microsomes prepared from a sow between five and nine months old were incubated with 50 μM ^{14}C -furazolidone for 24 hours. Extraction of AOZ from microsome proteins showed that around 60 % of the bound metabolites contained an AOZ side-chain that could be released under acidic conditions.

Liver samples were collected from two male piglets with a bodyweight of 6 kg administered furazolidone (75 mg/day), corresponding to 12.5 mg/kg BW per day, for 10 days followed by a withdrawal period of 2 hours or 14 days. Levels of released AOZ from liver samples after 2 hours and 14 days withdrawal were 23 and 14 % of the total protein-bound metabolites respectively, corresponding to 3.8 and 0.3 μg furazolidone per g of tissue.

Castrated male pigs (n=24) weighing 15-18 kg were fed *ad libitum* feed containing furazolidone (400 mg/kg feed), corresponding to 23.5 mg/kg BW per day, for seven days (Liu *et al.* 2010). A control group of six pigs were fed untreated feed for seven days. At 0.5, 7, 21, 35, 56 and 63 days of the withdrawal period, four treated pigs and one control pig were euthanized and samples of blood, liver, kidney and muscle were taken. Following the last dosing urine was collected daily. The levels of AOZ in the samples were analysed using an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA). Twelve hours after the last dosing the highest concentration of AOZ was in liver ($2169.7 \pm 253.1 \mu\text{g}/\text{kg}$) and the lowest concentration was in muscle ($691.0 \pm 242.4 \mu\text{g}/\text{kg}$). This was the case throughout the withdrawal period. At the last day of the withdrawal period, day 63, AOZ was still present in all tissue samples (0.6-1.1 $\mu\text{g}/\text{kg}$) and urine and plasma samples (1.0-1.2 $\mu\text{g}/\text{L}$). The elimination half-lives of AOZ was calculated to be 13.6 days in liver and kidney, 13.7 days in plasma, 14.7 days in urine and 15.0 days in muscle.

Gottschall & Wang (1995) investigated the depletion and bioavailability of ^{14}C -furazolidone residues in swine tissues. Four swine (2 males and 2 females) served as control and six swine (3 females and 3 males) were administered a daily oral dose of furazolidone (16.5 mg/kg BW) for 14 days followed by a withdrawal period of 10 hours, 21 or 45 days. After the swine were euthanized samples of liver, fat, kidney and muscle were collected. The total radioactive residues at the first day of the withdrawal period were highest in liver (~41 mg/kg) followed by kidney (~34 mg/kg), muscle (~13 mg/kg) and fat (~6 mg/kg). After 21 days these levels had quickly decreased, between

50-90 %. The depletion of residues between days 21 and 45 of the withdrawal period was much slower than that between days 0 and 21. The levels of tissue residues at the last day of the longest withdrawal period were around 2 mg/kg in all tissues. The biphasic elimination may be due to rapid elimination of free drug and metabolites followed by elimination and turnover of residues bound to macromolecules.

The percentage of extractable residues from the tissue samples decreased with increasing withdrawal time. Of the residues in liver collected at the first day of the withdrawal period 44 % were extractable. From liver samples taken on day 45, only 8.3 % of the residues could be extracted. In muscle most of the residues were bound and only 21.8 % could be extracted on day 0 of withdrawal (Gottschall & Wang 1995).

4.3.2.2 In bovine

One cow was administered one capsule containing; 0.88 mg/kg BW furazolidone, nitrofurazone and furaltadone and 4.4 mg/kg BW nitrofurantoin (Chu & Lopez 2007). Milk samples were then collected for two weeks every 12 hours. Milk from non-treated cows was used as control. The levels of nitrofuran side-chain residues in the milk were determined using LC-MS/MS. The level of the side-chain of furazolidone, AOZ, in milk was highest 12 hours after dosing (~32 µg/kg) and decreased rapidly to below the detection limit (0.2 µg/kg) 72 hours after dosing.

Veal calves (n=6) were administered furazolidone and furaltadone (7-8 mg/kg BW) orally via the morning milk (Nouws & Laurensen 1990). Three hours after the administration four calves were slaughtered and samples of heart muscle, kidney, liver, *muscularis gracilis*, diaphragm, fat, lung, brain, urine and blood were collected and stored for analysis using HPLC. The remaining two calves were slaughtered four hours after administration. Only low levels of furazolidone could be detected in fat, brain and lung. The liver and kidney samples were analysed at 10, 40, 120 minutes and 24 hours after collection and the muscle and heart samples after 15, 60, 120 minutes and 24 hours. There were large variations between tissues and animals, but immediately after the slaughter furazolidone could be detected in the tissues. The highest levels were found in plasma (0.336-3.34 µg/mL) and urine (1.11-6.22 µg/mL). Two hours after the slaughter, trace amounts of furazolidone could be detected in the muscle and kidney samples, but not in liver samples. No limit of quantification (LOQ) was reported. After 24 hours, no levels could be detected. The postmortal degradation half-lives of

furazolidone were calculated to be 17 minutes in *muscularis gracilis* and kidney, 30 minutes in diaphragm and heart, and <7 minutes in liver.

4.3.2.3 In fish

The depletion of nitrofurans and their tissue-bound residues in channel catfish (*Ictalurus punctatus*) was investigated by Chu *et al.* (2008). Fish (n=55) were orally administered furazolidone, nitrofurantoin, nitrofurazone and furaltadone (1 mg/kg BW). After 2, 4, 8 and 12 hours, and 1, 4, 7, 10, 14, 28 and 56 days five fish were killed and muscle samples collected for analysis of parent nitrofurans and their tissue-bound residues. The highest concentration of furazolidone in muscle (30.4 µg/kg) was reached four hours after administration. Furaltadone could no longer be detected 96 hours after administration. The level of AOZ was the highest (33.7 µg/kg) 12 hours after administration. The elimination of all tissue-bound residues was biphasic and could still be detected 56 days after administration. The half-life for AOZ was calculated to be 32 days.

Chu *et al.* (2008) also examined the levels of nitrofurans and tissue-bound metabolites in muscle of fish after waterborne exposure to nitrofurans. Fish (5 per treatment) were exposed to either nitrofurantoin, nitrofurazone, furazolidone or furaltadone (10 mg/L) for eight hours. After this time the fish were killed and their muscle tissue was analysed for parent nitrofuran and tissue-bound metabolites. The concentrations of furazolidone and AOZ were around 401 and 203 µg/kg, respectively.

4.3.2.4 In rat

The bioavailability of AOZ residues to rats were investigated by McCracken & Kennedy (1997). Liver, kidney and muscle samples were taken from pigs administered furazolidone (~20 mg/kg BW) for five days and fed to female Sprague Dawley rats, four per feeding group, for three days. Another two groups of two rats were fed either pig liver, which had undergone solvent extraction, or control feed. Liver, kidney and muscle samples were collected on the third day and analysed for extractable and bound residues of AOZ. No AOZ was found in the control animals but was detected in all tissues from the rats fed pig liver or kidney. The rats fed liver had the highest levels of bound AOZ in liver (55 µg/kg) and kidney (40 µg/kg), and lowest levels in muscle (2 µg/kg). Extractable levels were highest in kidney (172 µg/kg) and liver (66 µg/kg), and lowest in muscle (5 µg/kg). The rats fed kidney had similar levels of bound AOZ in liver and kidney (33-34 µg/kg), and the levels in muscle were about 1 µg/kg.

Extractable levels were highest in kidney (318 µg/kg) and liver (42 µg/kg), and lowest in muscle (5 µg/kg). The rats fed muscle had similar levels of bound AOZ in kidney and liver (7-9 µg/kg). Extractable levels were highest in kidney (38 µg/kg) and liver (17 µg/kg). Trace amounts (<0.5 µg/kg) of bound and extractable AOZ was found in muscle. The two rats fed solvent-extracted pig liver had detectable levels of bound AOZ in their tissues; liver (52 and 79 µg/kg), kidney (45 and 48 µg/kg) and muscle (3 and 2 µg/kg). The levels of extractable AOZ were; 3 and 8 µg/kg in muscle, 175 and 260 µg/kg in kidney, and 94 and 116 µg/kg in liver.

Tatsumi *et al.* (1984) orally administered furazolidone (100 mg/kg BW) daily to six male Wistar-strain rats for four days. Urine was collected during the experiment and urinary metabolites were isolated. Simultaneously, five rats were given ¹⁴C-furazolidone (100 mg/kg BW) orally for four days to investigate the metabolic pathways of furazolidone. Urine was also collected and analysed for urinary metabolites.

Four metabolites were isolated from the collected urine; an open-chain cyano derivative 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone, an acetamidofuran N-(5-acetamido-2-furfurylidene)-3-amino-2-oxazolidone, a methylated open-chain carboxylic acid N-(4-meth-oxycarbonyl-2-oxobutylideneamino)-2-oxazolidone, and α-ketoglutaric acid.

Four rats were injected into the stomach with an amount equivalent to 1 g of dry weight liver from pigs that were killed immediately after the last administration of furazolidone containing about 70 µg non-extractable residues per gram dry weight (Hoogenboom *et al.* 2002). The rats were killed three or six hours after the injection. The levels of AOZ in plasma of the rats were estimated to be between 3 and 5 ng/mL.

Three rats in three groups were fed with liver taken either from non-medicated pigs or pigs administered ¹⁴C-furazolidone followed by a withdrawal period of 0 or 21 days (Hoogenboom *et al.* 2002). After six hours the rats were killed and the levels of AOZ in the plasma were analysed. The results showed that rats fed liver from pigs killed immediately after the last administration had levels of AOZ in their plasma. The livers of these rats were also analysed and the total residue levels were 26 ng/g and the levels of non-extractable residues were 18 ng/g. In the rats fed liver from non-medicated pigs or liver taken from pigs 21 days after the last administration, no AOZ could be detected in plasma or liver.

The bioavailability of furazolidone and its residues in rats was also investigated (Gottschall & Wang 1995). Male rats were fed control lyophilized swine muscle, control lyophilized swine liver and control laboratory chow fortified with ¹⁴C-furazolidone. The bioavailability of furazolidone in the laboratory chow and swine muscle were around 90 %, while in swine liver the bioavailability was approximately 73 %. For the absorbed furazolidone, the major excretion pathways were via urine and bile. For the non-absorbed fraction it was via the faeces.

Rats were also fed tissues from the residue depletion study with swine by Gotschall & Wang (1995). The bioavailability of the furazolidone residues was around 40 % for muscle taken 45 days into the withdrawal period and for liver and muscle taken 0 days into the withdrawal period. For liver taken at day 45 the bioavailability was 20 %. Also here the major excretion pathway for absorbed furazolidone was via urine and for the non-absorbed fraction via the faeces.

The bioavailability of the non-extractable residues was also examined by feeding rats tissues that had been treated with organic solvents to remove extractable residues. It was shown that the bioavailability was the highest for muscle collected at day 45 (37 %) followed by liver collected at day 0 (31 %) and liver taken at day 45 (16 %). Once again the major pathways were via the urine and faeces.

4.3.2.5 In poultry

To investigate the transfer of furazolidone from hen to egg four White Leghorn hens were given 500 mg furazolidone per kg feed orally for 14 days, corresponding to about 31 mg/kg BW and day (Furusawa 2001). Five hens were used as control. Eggs were collected at two day intervals and albumen and yolk were separated immediately. The concentration of furazolidone was measured by HPLC with 80 and 82 % recovery in albumen and egg yolk, respectively. During the experiment there was no difference in body weight, egg production or feed intake between the control group and treated animals. The levels of furazolidone in eggs increased during the first days of the experiment and reached a plateau around day 4 and remained at those levels throughout the experiment. The mean concentration of furazolidone in eggs was 0.27 mg/kg (range: 0.10-0.29 mg/kg). The transfer rate was calculated to be 0.03 % of the drug intake.

Furazolidone was administered to 28-day-old broilers (n=20) in the feed (30, 100, 300, 1000 or 3000 µg/kg feed) corresponding to 6, 20, 60, 200 and 600 µg/kg BW, for 12 days (McCracken *et al.* 2005b). After this time the broilers were killed and samples of muscle and liver were collected and analysed for AOZ using LC-MS/MS. At the lowest dose AOZ was still detected in muscle (0.33 µg/kg) and liver (1.1 µg/kg) samples. At the highest dose the levels in liver and muscle were around 25 and 12 µg/kg, respectively.

Six untreated broilers were placed in a dirty pen, which had housed four broilers administered furazolidone (600 µg/kg BW) for 12 days, for 24 hours. Liver and muscle samples were then collected and analysed for AOZ. AOZ was detected in all samples and the mean level of AOZ in muscle was 0.13 µg/kg (range: 0.05-0.31 µg/kg) and in liver the mean level was 0.10 µg/kg (range: 0.04-0.21 µg/kg).

McCracken *et al.* (2005a) fed six broiler hens and one cockerel 120-140 g of feed containing furazolidone (400 mg/kg feed), corresponding to about 96-112 mg/kg BW per day. Eggs were collected and analysed for AOZ and when it was clear that furazolidone residues had transferred to the eggs, the eggs laid after this were collected and allowed to hatch. After hatching, four chicks were sacrificed at determined intervals and muscle and liver samples were analysed for AOZ. However, AOZ could only be detected up to day 21. The levels of AOZ in liver were around 6.5 µg/kg, 1.6 µg/kg, 0.3 µg/kg and 0.1 µg/kg in 1, 4, 12 and 21 days old chicks, respectively. In muscle the levels of AOZ were around 4.6 µg/kg, 0.7 µg/kg, 0.2 µg/kg and 0.05 µg/kg in 1, 4, 12 and 21 days old chicks, respectively.

Twenty-four laying hens were fed feed containing 300 mg furaltadone, nitrofurazone, nitrofurantoin or furazolidone per kg feed for one week, corresponding to approximately 60 mg/kg BW per day (McCracken & Kennedy 2007). Eggs were then collected for two days and analysed immediately for nitrofuran parent compound and their bound residues in albumen, yolk and shell using LC-MS/MS. The levels of furazolidone in yolk, albumen and shell were 410, 291 and 51.8 ng/g, respectively. The levels of AOZ in yolk, albumen and shell were 478, 491 and 114 ng/g, respectively.

4.3.2.6 In eel

The metabolism of furazolidone in eels and eel liver homogenate was examined by Nakabeppu & Tatsumi (1984). Liver homogenate was incubated with 20 µmol of furazolidone for one hour in an open vessel in the dark at 37 °C. The mixture was then extracted with ethyl acetate and dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue was chromatographed on a silica gel column and a metabolite, 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone was discovered.

Two eels were bathed in 2 L ¹⁴C-furazolidone (0.5 µL/L) for 24 hours and four eels were bathed in 3 L unlabelled furazolidone (15 µL/L) for four days. Analysis showed that water-soluble metabolites and two ethyl acetate-extractable metabolites, one of which was identified as 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone, were formed from nitrofurazone metabolism.

4.3.3 Conclusions on pharmacokinetics of furazolidone and its metabolites

Pharmacokinetic studies in swine, rat, calf and poultry have shown that furazolidone is quickly absorbed and distributes throughout the body, with the highest concentrations found in liver and kidney. It was shown that furazolidone is readily degraded after slaughter and is degraded even when frozen, making it difficult to monitor illegal use by measuring the parent compound in food stuffs. Furazolidone is excreted from the body with half-lives of about one hour. The excretion of furazolidone appears to be biphasic in swine with a fast excretion in the beginning of the withdrawal period followed by a slower excretion. The major excretion pathways are via the urine, bile and faeces. From the study on the bioavailability of furazolidone for rats fed liver and muscle from pigs administered furazolidone it seems that furazolidone has a quite high bioavailability even after 45 days of withdrawal. It was also shown that furazolidone could be transferred to eggs from poultry treated with furazolidone.

The identified metabolites of furazolidone are presented in Table 12. From the studies above the most common metabolites observed in swine and rat are 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone and AOZ, formed via reductive pathways. However, it is the metabolite AOZ that is the marker residue for furazolidone due to its long half-lives in tissues. In swine the half-lives of AOZ were shown to be between one to two weeks in kidney, muscle, liver and plasma. But it was still present in tissues 63 days after the last administration of furazolidone. AOZ has been shown to accumulate more in liver than in muscle. Residues of furazolidone (e.g. AOZ) were also shown to be bioavailable for uptake for rats fed muscle and liver from furazolidone treated pigs.

Table 12. Metabolites of furazolidone in different species found in the literature

Metabolite	Species/cells	Reference
3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone	Rabbit, eel, rat and pig liver microsomes, Caco-2, Hep-2, V79 cells	de Angelis <i>et al.</i> (1999), Nakabeppu & Tatsumi (1984), Tatsumi <i>et al.</i> (1981 & 1984), Vroomen <i>et al.</i> (1987a, 1987b)
3-amino-2-oxazolidone (AOZ)	Broiler, pig	Cooper <i>et al.</i> 2005, Hoogenboom <i>et al.</i> (1991 & 1992), Liu <i>et al.</i> (2010), McCracken <i>et al.</i> (2005b)
N-(5-amino-2-furfurylidene)-3-amino-2-oxazolidone	Rat, pig	FAO 1993b, Tatsumi <i>et al.</i> (1981)
2,3-dihydro-3-cyanomethyl-2-hydroxyl-5-nitro-1 α ,2-di(2-oxo-oxazolidin-3-yl)iminomethylfuro[2,3-b]furan	Rat, pig liver microsomes	Tatsumi <i>et al.</i> (1981), Vroomen <i>et al.</i> (1987b)
N-(5-acetamido-2-furfurylidene)-3-amino-2-oxazolidone	Rat	Tatsumi <i>et al.</i> (1984)
3-(4-cyano-3(or 4)- β -hydroxyethylmercapto-2-oxobutylideneamino)-2-oxazolidone	Pig liver microsomes	Vroomen <i>et al.</i> (1987b)
N-(4-meth-oxycarbonyl)-2-oxobutylideneamino)-2-oxazolidone	Rat	Tatsumi <i>et al.</i> (1984)
α -ketoglutaric acid	Rat	Tatsumi <i>et al.</i> (1984)
5-nitro-furaldehyde	Rat, pig	FAO 1993b
5-nitro-furan-2-carboxylic acid	Pig	FAO 1993b

4.4 Toxicology of furazolidone

4.4.1 Acute toxicity

The results from acute toxicity studies by Mitchell *et al.* (1990a, 1990b) are summarized in JECFA (1993b). The acute toxicity of furazolidone was tested by administering furazolidone orally to male and female mice and rats. The LD₅₀ was 1110 mg/kg BW for mice and 1508 mg/kg BW for rats.

4.4.1.1 Conclusions on acute toxicity of furazolidone

From this study it is concluded that furazolidone is slightly toxic to mice and rats.

However, it is not clear whether or not the study followed OECD guidelines and GLP.

4.4.2 Chronic toxicity

Male Wistar rats (six rats per group) were administered furazolidone (0, 0.5, 5 or 10 mg/kg BW) daily via food for 13 months (Aisio *et al.* 1962 as seen in JECFA 1993b). No effects were seen on mortality, body weight or food consumption. Blood samples

were taken from two rats per dose group at the end of the experiment. No changes in the number of leucocytes or erythrocytes were detected. At the highest dose the relative spleen and liver weights were increased slightly and during histological examinations some hypertrophy of liver cells was seen at all doses.

4.4.2.1 Conclusions on chronic toxicity of furazolidone

No NOAEL could be set in this study due to hypertrophy of liver cells at all doses. The study does not meet current standards set in the OECD guideline 452 for chronic toxicity studies.

4.4.3 Reproductive toxicity including teratogenicity

4.4.3.1 Mouse

JECFA (1993b) presented the results from a study by Jackson & Robson (1957). Pregnant albino C strain mice were given furazolidone (≤ 2 g/kg BW) starting at different times during pregnancy (day 1-11). All mice treated with furazolidone (≤ 1 mg/kg BW) before day 8 had abortions or foetal deaths. Abortions or foetal deaths only occurred in two out of nine mice when the treatment started at day 10. No congenital abnormalities were observed.

Only a summary was available. The abortions seen in mice treated before day 8 may be due to failure of embryo implantation. It is not clear how long the medication period was. The study is quite old and does not meet the current standard for reproductive toxicity studies.

4.4.3.2 Rat

The study by Larson *et al.* (1963a) is summarised in JECFA (1993b). Male rats (n=10) were fed furazolidone (16 or 33 mg/kg BW) in the diet for 14 weeks. Another six rats were fed furazolidone (16 or 33 mg/kg BW) in the diet for 12 weeks followed by two weeks recovery period. Four rats constituted the control group. No dose-related effects were seen in the rats of the lowest dose group. In the high-dose groups the testes weight of the rats was decreased compared to the control group but more pronounced in the group treated for 14 weeks. Degeneration of the sperm-producing tubules, atrophy and oedema of the interstitium were seen in the testes of the rats from the high-dose groups.

There is no mention of if the study was carried out according to OECD guidelines or GLP. Due to the study being dated it does not meet the current standards for reproductive toxicity studies, but it does indicate a deleterious effect of furazolidone on

male reproduction at doses of 33 mg/kg BW. A NOAEL can be set to 16 mg/kg BW in this study.

JECFA (1993b) presented an abstract of studies by Borgman & Prytherch (1964b, 1966). Sprague-Dawley rats (20 animals per sex) were used in a three-generation reproduction study in which only females were initially given feed containing furazolidone (500 mg/kg feed). The dose was then lowered to 400 mg/kg feed on day 16 and again to 250 mg/kg feed (corresponding to 12.5 mg/kg BW per day) on day 37 due to growth depression. Each generation were allowed to mate three times. The first litter was sacrificed on day 21 after birth; the second litter was used for the second generation and the third litter was used for teratological examination. No effects on reproduction could be seen and a NOEL was set to 12.5 mg/kg BW per day.

The study was only available as an abstract in JECFA (1993b). The study does not meet today's standard regarding reproductive toxicity studies. The fact that the authors decreased the dose twice during the study makes the results, or lack thereof, difficult to interpret.

4.4.3.3 Poultry

The results from a study by Francis & Shaffner (1956) are summarised in JECFA (1993b). New Hampshire chickens were administered furazolidone in the feed (55, 110 or 220 mg/kg feed), corresponding to 11, 22 and 44 mg/kg BW, for 4 to 16 weeks. There was no observed effect on hatchability of eggs, shell quality, egg production or body weight.

Based on the results presented in the study a NOEL-value of 44 mg/kg BW could be set for the above mentioned end points. It does not follow OECD guidelines or GLP and does not meet today's standard in testing.

4.4.3.4 Swine

JECFA (1993b) presented a summary of a study by Hughes & McMinn (1963). Hampshire and Duroc pigs (12 gilts and 9 sows) were given furazolidone (300 mg/kg) in feed, corresponding to 6 mg/kg BW, for two weeks at breeding and 150 mg/kg, corresponding to 3 mg/kg BW, for three weeks after giving birth. No effects on number of piglets born, weaned or weight gain could be observed.

The doses are lower than in the other studies and there is no mention of the study following OECD guidelines or GLP. Due to the age of the study it probably does not meet today's criteria.

4.4.3.5 Goat

Mustafa *et al.* (1987) performed a study and the results are summarised in JECFA (1993b). The biochemistry and semen morphology of male Nubian goats administered furazolidone suspended in distilled water (10 or 40 mg/kg BW) for five days was studied. Furazolidone significantly decreased the semen fructose concentration, the number of live and motile spermatozoa per ejaculate and ejaculate volume at both doses.

It is not clear if the study was conducted according OECD guidelines or GLP, since only an abstract was available. The results of the study indicate a detrimental effect of furazolidone on male reproduction system.

4.4.3.6 Rabbit

JECFA (1993b) summarised the results from a study by Borgmann & Prytherch (1964a). Furazolidone (30 mg/kg BW) was orally administered to New Zealand white rabbits on days 7-15 of pregnancy. The dams were sacrificed at day 29 and the foetuses were examined. No teratogenicity or embryotoxicity was found.

There is no mention of following OECD guidelines or GLP and the study most likely does not meet today's standards.

4.4.3.7 Conclusions on reproductive toxicity including teratogenicity

The results from the reproductive toxicity studies are presented in Table 13. Of the seven studies described above only three presented results showing reproductive toxicity of furazolidone. Treating pregnant mice with furazolidone (≤ 1 mg/kg BW) before day 8 caused abortions and foetal deaths. In rats treated with 33 mg/kg BW for 12 to 14 weeks testicular degeneration was found. Goats treated with 10 mg/kg BW for five days showed decreased semen quality. Due to all studies being quite old and not living up to today's criteria in reproductive toxicity testing, it is difficult to draw any clear conclusions. From these results it appears that the male reproductive system is sensitive to toxic effects of furazolidone.

Table 13. Summary on reproductive toxicity studies including teratogenicity of furazolidone

Species	Dose (mg/kg BW)	Duration	Effects	Reference
Mouse	? - <2	?	Abortions and foetal deaths	JECFA (1993b)
Rat	16 or 33	12 or 14 weeks	Testicular degeneration	JECFA (1993b)
Rat	12.5	?	No effects observed	JECFA (1993b)
Chicken	11, 22, 44	4-6 weeks	No effects observed	JECFA (1993b)
Pig	6 & 3	2 & 3 weeks	No effects observed	JECFA (1993b)
Goat	10 or 40	5 days	Decreased semen quality	JECFA (1993b)
Rabbit	30	9 days	No effects observed	JECFA (1993b)

4.4.4 Mutagenicity and genotoxicity

4.4.4.1 *In vitro* studies with furazolidone

In a forward mutation assay on *Vibrio cholera* by Chatterjee *et al.* (1983), cells were treated with up to 12 µg/mL furazolidone to induce streptomycin resistance. To allow for expression of streptomycin resistant mutants, untreated and treated cells were incubated in NB medium at 37 °C for 18 to 20 hours. The bacteria were then plated in selective medium (NA containing 100 µg/mL of streptomycin) or a non-selective medium to score the number of formed mutant colonies and assess the total viable count, respectively. After 24 hours of incubation the viable counts were recorded and after 72 hours the mutant colonies were counted. The ratio of mutant counts on the selective medium to the total viable counts on the non-selective medium gave the mutation frequency. The results showed that furazolidone induced forward mutation leading to streptomycin-resistance with the mutation frequency being positively correlated with the drug concentration up to ~7.0 µg/mL. Above this concentration the mutation frequency declined.

The study was performed in 1983 and no positive control was used but the results show that furazolidone is mutagenic.

Furazolidone was tested in a chromosome aberration assay by Cohen & Sagi (1979). Furazolidone was dissolved in 1 % dimethylsulfoxide (DMSO) to produce concentrations of 0.2, 2.0 and 20 µg/mL of culture. Phytohemagglutinin (PHA)-stimulated human lymphocyte cultures were incubated with the different furazolidone

concentrations for 24 hours before harvest. From each culture 100 cells in metaphase were examined for chromosome breaks. Cohen & Sagi (1979) also studied the sister-chromatid exchange (SCE). For 72 hours PHA-stimulated lymphocytes were incubated with 10^{-5} M BUDR. After 48 hours the different concentrations of furazolidone was added. The lymphocytes were then harvested and 50 cells from each treatment were examined and the number of SCEs per cell was recorded.

There was no difference in the number of chromosome breaks in lymphocytes treated with 0.2 $\mu\text{g}/\text{mL}$ furazolidone and the control. But lymphocytes treated with 2.0 $\mu\text{g}/\text{mL}$ furazolidone showed an increase in chromosome breaks. The lymphocytes treated with 20.0 $\mu\text{g}/\text{mL}$ furazolidone did not enter mitosis and could not be analysed for chromosome breakage. The number of SCEs per cell was significantly higher ($p < 0.001$) in cells treated with furazolidone (0.2 and 2.0 $\mu\text{g}/\text{mL}$) compared to the control. Cells treated with 20.0 $\mu\text{g}/\text{mL}$ furazolidone did not enter metaphase.

The study does not follow current OECD guidelines but shows that furazolidone causes SCEs (0.2 and 2.0 $\mu\text{g}/\text{mL}$) and other chromosome aberrations (2.0 $\mu\text{g}/\text{mL}$) in lymphocytes. It also shows that furazolidone interferes with mitosis at 20 $\mu\text{g}/\text{mL}$.

The Ames test was used by Ni *et al.* (1987) to investigate the mutagenicity of furazolidone. Three strains of *Salmonella typhimurium*; TA98, TA98NR and TA98/1,8-DNP₆, were exposed to furazolidone both with and without rat-liver hepatic S9 activation enzymes. The doses of furazolidone tested were 0.1, 0.5, 1.0 and 2.5 $\mu\text{g}/\text{plate}$. Furazolidone tested positive for mutagenicity in the TA98 strain at all doses, both with and without S9, except for the highest dose which was toxic to the bacteria. Furazolidone was also mutagenic to TA98NR at 0.5 $\mu\text{g}/\text{plate}$ without S9 and 1.0 $\mu\text{g}/\text{plate}$ with and without S9. Also in this strain the highest dose was toxic to the bacteria. In the TA98/1,8-DNP₆ strain furazolidone was mutagenic at the two lowest doses, with and without S9, and toxic at the two highest doses.

The study mostly follows OECD guidelines and the results show that furazolidone is mutagenic in all tested strains both with and without metabolic activation. In all test strains the mutagenicity of furazolidone appeared dose-dependent.

Furazolidone tested positive at all concentrations (0.01, 0.1, 1.0 and 10 $\mu\text{g}/\text{plate}$) in an Ames test performed with *S. typhimurium* TA 100, both with and without S9-mix (Vroomen *et al.* 1987c). A dose-response relationship could be observed.

The study follows guidelines to some extent but only the conclusion not the data is reported.

Tatsumi *et al.* (1978) examined the mutagenicity of furazolidone in an Ames test using *S. typhimurium* TA 100. The bacteria were incubated with furazolidone (0.03, 0.1, 0.3 or 1 µg/plate) for two days at 37 °C on plates lacking histidine. The revertant colonies were then counted in test and control plates. The results showed that furazolidone was strongly mutagenic.

The study follows OECD guidelines to some extent and the results indicate that furazolidone is mutagenic to S. typhimurium TA 100.

4.4.4.2 In vitro studies with metabolites of furazolidone

The mutagenicity of 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone was also tested by Tatsumi *et al.* (1978). The test followed the same procedure as stated above with the exception that the dose added to each plate was higher (1, 3, 10 or 100 µg/plate). The number of revertant colonies was far less than those seen on plates containing furazolidone and 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone was considered nonmutagenic.

The study follows OECD guidelines and the results indicate that 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone is not mutagenic.

3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone (10 and 100 µg/plate) tested negative in an Ames test using *S. typhimurium* TA98 and TA100, with and without S9-mix (Vroomen *et al.* 1987c).

The study mostly follows guidelines and the results show that 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone is not mutagenic.

AOZ (0.1, 0.33, 1, 3.33 or 6 mg/plate) was tested in an Ames test using several *S. typhimurium* strains (TA 98, TA 100, TA 1535, TA 1537), both with and without S9-mix (Hoogenboom *et al.* 2002). It was shown that AOZ was mutagenic to TA 1535 in a dose-dependent manner with rat liver S9 fraction. A positive response was also seen in strain TA 100, but not in TA 98 or TA 1537.

The experiments were carried out at NOTOX and RIKILT and most likely follow guidelines. The results show that AOZ is mutagenic in some strains of S. typhimurium.

AOZ was also studied in a chromosome aberration test with human lymphocytes (Hoogenboom *et al.* 2002). Cells were exposed to AOZ (1000, 3330 or 5000 µg/mL) for 24 or 48 hours without S9-mix and for three hours with S9. MMC (0.2 µg/mL) was used as positive control. One hundred cells in metaphase were examined for chromosome aberrations. The number of cells with chromosome aberrations increased significantly after exposure to AOZ in the absence of S9-mix. No effects were seen when S9-mix was present.

The study mostly follows OECD guidelines. The results from this study demonstrates that AOZ is genotoxic to human lymphocytes in the absence of S9-mix

4.4.4.3 *In vivo* studies with metabolites of furazolidone

In a bone marrow micronucleus-test male and female Swiss mice (n=120) were intraperitoneally administered with AOZ at different concentrations (32, 62.5, 125, 250 or 500 mg/kg BW in males and 250, 500, 750, 1000 or 1500 mg/kg BW in females) (Hoogenboom *et al.* 2002). Prior to this experiment two limit tests were performed. Cyclophosphamide (50 mg/kg BW) and vehicle (0.9 % NaCl) served as positive and negative control, respectively. Each dose group consisted of between three and five animals per sex. Sampling occurred at 24 and 48 hours. The number of micronucleated polychromatic erythrocytes was counted in 1000 cells. No dose-response relationship was found and significant increases in micronucleated cells was only seen in the positive controls and in male mice administered 500 mg/kg BW when sampling occurred at 48 hours.

The study mostly follows OECD guidelines and the authors conclude that AOZ is mutagenic due to increases in the fraction of animals with three or more micronucleated polychromatic erythrocytes per 1000 polychromatic erythrocytes. The results could be considered positive but since only one dose produced a significant increase in micronucleated cells, only 1000 cells were examined instead of 2000 and that the dose group only consisted of four animals the results should be interpreted as negative.

4.4.4.4 Conclusions on mutagenicity and genotoxicity studies

The results from mutagenicity and genotoxicity studies are summarised in Table 14. In the studies mentioned above furazolidone has been shown to be mutagenic and genotoxic in both bacterial tests and an *in vitro* chromosome aberration test. The bacterial tests were performed both with and without metabolic activation and using different species and strains. Furthermore furazolidone has also tested positive in several other *in vitro* assays; mammalian cell gene mutation test, HPRT test, sex-linked recessive lethal test etc. (JECFA 1993b). Of all the *in vitro* studies stated in JECFA (1993b) only four had results that were negative. These were; two chromosome aberration tests using human lymphocytes, an UDS assay using human lymphocytes and an Ames test. Only two *in vivo* tests for chromosomal effects, the micronucleus test, are mentioned in JECFA (1993b). Both tests were performed on mice. In the first one, mice were injected intraperitoneally with furazolidone (300 mg/kg BW) in methylcellulose which proved toxic to the animals. The reported results were negative. In the second test mice were orally administered furazolidone (100 or 500 mg/kg BW) and the results were equivocal since there was a slight induction of micronuclei but it was not dose-related. Furazolidone has shown to be mutagenic and genotoxic *in vitro* but does not appear to be genotoxic *in vivo*. To ascertain whether or not furazolidone truly is not mutagenic *in vivo* further study is required.

The metabolite 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone did not test positive in an Ames test using *S. typhimurium* TA 100 and might not be mutagenic, although more studies are needed to verify this. The metabolite AOZ tested positive *in vitro* but not *in vivo* and further study is needed to determine its mutagenic properties.

Table 14. Summary of mutagenicity and genotoxicity studies on furazolidone and its metabolites

Test	Compound	Species/strain	Dose/ concentration	Result	Reference
Forward mutation assay	Furazolidone	<i>V. cholera</i>	≤12 µg/mL	+	Chatterjee <i>et al.</i> (1983)
Ames test	Furazolidone	<i>S. typhimurium</i> TA 98, TA 98NR, TA 98/1,8-DNP	0.1-2.5 µg/plate*	+	Ni <i>et al.</i> (1987)
Ames test	Furazolidone	<i>S. typhimurium</i> TA 100	0.1-10 µg/plate*	+	Vroomen <i>et al.</i> (1987c)
Ames test	Furazolidone	<i>S. typhimurium</i> TA 100	0.03-1 µg/plate	+	Tatsumi <i>et al.</i> (1978)
<i>In vitro</i> chrom. aberration assay	Furazolidone	Human lymphocytes	0.2-20 µg/mL	+	Cohen & Sagi (1979)
Ames test	3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone	<i>S. typhimurium</i> TA 100	1-100 µg/plate	-	Tatsumi <i>et al.</i> (1978)
Ames test	3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone	<i>S. typhimurium</i> TA 98, TA 100	10 & 100 µg/plate*	-	Vroomen <i>et al.</i> (1987c)
Ames test	3-amino-2-oxazolidone (AOZ)	<i>S. typhimurium</i> TA 98, TA 100, TA 1535, TA 1537	0.1-6 mg/plate*	+/-	Hoogenboom <i>et al.</i> (2002)
<i>In vitro</i> chrom. aberration assay	3-amino-2-oxazolidone (AOZ)	Human lymphocytes	1000-5000 µg/mL*	+	Hoogenboom <i>et al.</i> (2002)
<i>In vivo</i> bone marrow micro-nucleus test	3-amino-2-oxazolidone (AOZ)	Swiss mice	32-2000 mg/kg BW	-**	Hoogenboom <i>et al.</i> (2002)

+ positive, - negative, +/- equivocal

* With and without S9-mix, ** Reported as positive by the authors

4.4.5 Carcinogenicity and long-term toxicity

4.4.5.1 Mouse

JECFA (1993b) summarised a study by Halliday *et al.* (1974b). Swiss MBR/ICR mice were fed furazolidone daily via the diet (0, 12, 24 or 47 mg/kg BW) for 13 months. Each dose group contained 50 animals of each sex. After this period the mice were kept on an untreated diet for 10 months. No effects on body weight or food consumption

could be seen during the experiment. For high-dose males and mid- and high-dose females the survival was decreased at the end of the experiment. Males in the mid- and high-dose group had a significantly increased incidence of lymphosarcomas. The incidence of bronchial adenocarcinomas was significantly increased in both female and male mid- and high-dose groups.

The study does not meet today's standard in testing but shows a carcinogenic effect in mice after furazolidone exposure. No effects were seen at the lowest dose level and a NOAEL value of 12 mg/kg BW can be set in mice although, further study is needed.

4.4.5.2 Rat

The results from studies by King *et al.* (1972a) and Halliday *et al.* (1973a) are summarised in JECFA (1993b). In a two year study Sprague-Dawley rats were daily administered furazolidone (0, 0.8, 4.3 or 14 mg/kg BW for females and 0, 0.7, 4 or 10 mg/kg BW for males) via feed with 35 animals per dose group and sex. Females from the mid- and high-dose group showed a dose-related decrease in haemoglobin, erythrocyte and haematocrit counts and an increased neutrophil/lymphocyte ratio. Females in all dose groups also had increased incidences of mammary tumours. At histopathology it was seen that; females from the highest dose group had increased thyroid atrophy, males from the highest dose group had increased parathyroid hyperplasia and both males and females from the highest dose group had increased adrenal cortical heperplasia.

The study does not follow guidelines but demonstrates an effect of furazolidone on haematological values. It also shows that exposure to furazolidone increased the number of observed mammary tumours in all female dose groups.

JECFA (1993b) also presented an abstract of the studies by King *et al.* (1972b) and Halliday *et al.* (1974a). Fisher 344 rats were treated with furazolidone (0, 12.5, 25 or 50 mg/kg BW) added to the feed for 20 months followed by four months of control diets. The dose groups contained 50 animals of each sex. Clinical chemistry and haematological examinations were done after one year and at the end of the treatment period. Histopathology examinations were performed on all rats. Male rats in the mid- and high-dose groups and females in the highest dose group had an increased mortality. The body-weight gain was significantly decreased at the end of the treatment period for

animals in the mid- and high-dose groups. Male rats showed significantly decreased haematocrit (25 and 50 mg/kg BW), haemoglobin (50 mg/kg BW) and erythrocytes counts (50 mg/kg BW). Females in the highest dose group also showed decreased erythrocytes counts. Male rats in the highest dose group had increased incidences of haemorrhage, lipolysis, adrenal cortical hyperplasia and congestion. Mid- and high-dose males had increased incidences of testicular atrophy. The incidence of thyroid adenomas and sebaceous gland adenomas was increased in both sexes exposed to the mid- and high-dose. For males in the highest dose group, basal-cell carcinomas and epithelioma were observed and for females in the same dose group there was an increase in mammary gland adenocarcinomas. At all dose levels the incidence of mammary neoplasms was increased in female rats but did not show a dose-response relationship.

The study mostly follows guidelines and the results show that furazolidone is carcinogenic and affects haematology values in rats..

Other studies described in JECFA (1993b) were carried out by King *et al.* (1972b) and Halliday *et al.* (1974a). They were carried out as described above but with Sprague-Dawley rats (as seen in JECFA 1993b). The body-weight gain was significantly decreased at the end of the treatment period for males in the mid- and high-dose groups and for females in the high-dose group. Females in the mid- and high-dose group had decreased number of erythrocytes. A decreased lymphocyte count and an increased neutrophil/lymphocyte ratio were seen in males from the high-dose group. Hepatic necrosis was seen in all treated rats and testicular atrophy was observed in males from the mid- and high-dose groups. Females from all dose groups had a dose-related increase in adrenal cortical hyperplasia. At all dose levels the incidence of mammary neoplasms was increased in female rats but did not show a dose-response relationship. Females from the highest dose group had a significantly increased incidence of mammary adenocarcinomas while males from the same group had significantly increased incidence of neural astrocytomas.

The study mostly follows guidelines and the results show that furazolidone is carcinogenic and affects haematology values in rats. No NOAEL value can be set since effects were observed at all dose levels.

4.4.5.3 Conclusions on the carcinogenicity and long-term toxicity of furazolidone

The results from the carcinogenicity studies are presented in Table 15. Male and female Swiss MBR/ICR mice administered furazolidone for 13 months had increased incidences of bronchial adenocarcinoma (24 and 47 mg/kg BW). Male mice (24 and 47 mg/kg BW) also showed increased incidences of lymphosarcomas.

Female Sprague-Dawley rats treated with furazolidone (0.8, 4.3 or 14 mg/kg BW) for two years developed mammary tumours to a higher extent than controls.

Female Sprague-Dawley and Fisher 344 rats treated with furazolidone (50 mg/kg BW) for 20 months had increased incidences of mammary gland adenocarcinomas. At all dose levels (12.5, 25 or 50 mg/kg BW) the incidence of mammary neoplasms was increased but did not show a dose-response relationship.

The studies above show that furazolidone is carcinogenic and causes predominantly mammary tumours in female rats. It is also shown that furazolidone can affect haematology values of the test animals and long-term exposure can cause testicular atrophy at doses of 25 to 50 mg/kg BW.

Table 15. Summary of carcinogenicity and long-term toxicity studies on furazolidone

Species	Dose (mg/kg BW)	Duration (medicated + non-medicated period)	Effects	Reference
Swiss MBR/ICR mice	12, 24 or 47	23 months (13+10 months)	↑bronchial adenocarcinomas, ↑lymphosarcomas	JECFA (1993b)
Sprague-Dawley rats	0.7-14	24 months	↑mammary tumours, ↓haemoglobin, erythrocyte and haematocrit counts	JECFA (1993b)
Fisher 344 rats	12.5, 25 or 50	24 months (20+4 months)	↑mammary gland adenocarcinomas, ↓haemoglobin, erythrocyte and haematocrit counts	JECFA (1993b)
Sprague-Dawley rats	12.5, 25 or 50	24 months (20+4 months)	↑mammary gland adenocarcinomas, ↓lymphocytes, erythrocytes, hepatic necrosis	JECFA (1993b)

5 Furaltadone

Furaltadone has been used as an antibacterial agent in both human and veterinary medicine (Reynolds 1982, Sweetman 2002).

5.1 Physicochemical properties

International Non-proprietary Name (INN)

Furaltadone

Chemical Abstract Service (CAS) name

5-Morpholinomethyl-3-(5-nitrofurfurylideneamino)-2-oxazolidinone

CAS number: 59-87-0

Structural formula

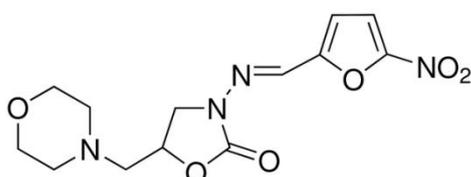


Figure 6. Structural formula of furaltadone

Molecular formula

C₁₃H₁₆N₄O₆

Molecular weight

324.3

Table 16. Physicochemical properties (Reynolds 1982)

Melting point	205 °C
Solubility in water	Very slightly soluble (1:2000)
Solubility in ethanol	Slightly soluble (1:1000)
Solubility in chloroform	Slightly soluble (1:300)

5.2 Pharmacodynamics

Furaltadone has antibacterial properties (Reynolds 1982, Sweetman 2002).

5.3 Pharmacokinetics

5.3.1 *In vitro* studies

The biotransformation of furaltadone by pig hepatocytes and *S. typhimurium* TA 100 was studied by Hoogenboom *et al.* (1994). Pig hepatocytes were incubated with furaltadone (50 µM) for 8, 24 or 48 hours. Furaltadone was metabolised into several minor metabolites and one major metabolite, an N-oxide (FAX).

When *S. typhimurium* TA 100 was incubated with furaltadone (25 µM) for 15 or 30 minutes one major metabolite, an open-chain metabolite (FACN), was formed. FACN was degraded further to several hydrophilic metabolites by hepatocytes.

It was also shown that proteins obtained from bacteria and hepatocytes exposed to furaltadone for 24 hours were bound to residues containing an intact AMOZ side-chain, of which 60-80 % could be released after mild acid treatment.

Furaltadone was incubated with a mixture of 8500g supernatant of liver homogenate taken from male rats aged 6-10 weeks (Akao *et al.* 1971). After 60 minutes of incubation under aerobic conditions there was a change in optical absorbance corresponding to a loss of a nitro group.

5.3.2 In vivo studies

5.3.2.1 In bovine

Veal calves (n=6) were administered furazolidone and furaltadone (6-8 mg/kg BW) orally via the morning milk (Nouws & Laurensen 1990). Three hours after the administration four calves were slaughtered and samples of heart muscle, kidney, liver, *muscularis gracilis*, diaphragm, fat, lung, brain, urine and blood were collected and stored for analysis using HPLC. The remaining two calves were slaughtered four hours after administration. The liver and kidney samples were analysed at 10, 40, 120 minutes and 24 hours after collection and the muscle and heart samples after 15, 60, 120 minutes and 24 hours. There were large variations between tissues and animals, but immediately after the slaughter furaltadone could be detected in the tissues, with the highest levels found in plasma (0.259-3.02 µg/mL) and urine (7.08-24.86 µg/mL). Two hours after the slaughter trace amounts of furaltadone could be detected in the muscle and kidney samples, but not in liver samples. After 24 hours no levels could be detected. Only low levels of furaltadone could be detected in fat, brain and lung. The postmortal degradation half-lives of furaltadone were calculated to be 22 minutes in *muscularis gracilis*, 29 minutes in diaphragm, 63 minutes in kidney, <43 minutes in heart, and <8 minutes in liver.

Furaltadone and nitrofurazone (14.0 mg/kg BW) suspended in milk were given orally to five preruminant MRY male calves (Nouws *et al.* 1987). Furaltadone was given three days before nitrofurazone. Blood samples were taken at different time points after each administration and urine was collected from three calves and analysed for the nitrofurans. The maximum concentration of furaltadone in plasma (2.5 µg/mL) was

achieved 2.5 hours after administration and the half-life of furaltadone was calculated to be around 2.5 hours.

A cow was administered a capsule containing; 0.88 mg/kg BW furazolidone, nitrofurazone and furaltadone and 4.4 mg/kg BW nitrofurantoin (Chu & Lopez 2007). Milk samples were then collected for two weeks at intervals of 12 hours. Milk from non-treated cows was used as control. The levels of nitrofurans side-chain residues in the milk were determined using LC-MS/MS. The level of the side-chain of furaltadone, AMOZ, in milk was highest 12 hours after dosing (~55 ng/g) and decreased rapidly and 72 hours after dosing the level of AMOZ was below the detection limit (0.2 ng/g).

5.3.2.2 In swine

Pigs (n=18) were fed feed, medicated with furaltadone (400 mg/kg feed), *ad libitum* for 10 days, corresponding to around 24 mg/kg BW per day, followed by a withdrawal period for six weeks (Cooper *et al.* 2005). Three pigs were sacrificed each week of the withdrawal period and samples of muscle, liver and kidney were taken and analysed for furaltadone and AMOZ using LC-MS/MS and HPLC-UV. Furaltadone was detected in one liver sample (0.7 µg/kg) at week 0 of the withdrawal period. AMOZ was detected in all samples and the levels found at week 6 of the withdrawal period were around 15 µg/kg in kidney and liver and 60 µg/kg in muscle. The depletion half-lives of AMOZ in muscle, liver and kidney were 8.7±1.2 days, 5.4±0.5 days and 5.7±0.7 days respectively.

5.3.2.3 In fish

The depletion of nitrofurans and their tissue-bound residues in channel catfish (*Ictalurus punctatus*) was investigated by Chu *et al.* (2008). Fish (n=55) were orally administered furazolidone, nitrofurantoin, nitrofurazone and furaltadone (1 mg/kg BW). After 2, 4, 8 and 12 hours, and 1, 4, 7, 10, 14, 28 and 56 days five fish were killed and muscle samples collected for analysis of parent nitrofurans and their tissue-bound residues. The highest concentration of furaltadone in muscle (35.2 µg/kg) was reached 12 hours after administration. Furaltadone could no longer be detected 96 hours after administration. The level of AMOZ was the highest (46.8 µg/kg) 12 hours after administration. The elimination of all tissue-bound residues was biphasic and could still be detected 56 days after administration. The half-life for AMOZ was calculated to be 27 days.

Chu *et al.* (2008) also examined the levels of nitrofurans and tissue-bound metabolites in muscle of fish after waterborne exposure to nitrofurans. Fish (5 per treatment) were exposed to nitrofurantoin, nitrofurazone, furazolidone or furaltadone (10 mg/L) for eight hours. After this time the fish were killed and their muscle tissue was analysed for parent nitrofuran and tissue-bound metabolites. The concentrations of furaltadone and AMOZ were around 46 and 37 µg/kg, respectively, and indicates uptake of furaltadone via the branchial route.

5.3.2.4 In poultry

Furaltadone was administered to 28-day-old broilers (n=20) in the feed (30, 100, 300, 1000 or 3000 µg/kg feed) corresponding to 6, 20, 60, 200 and 600 µg/kg BW, for 12 days (McCracken *et al.* 2005b). After this time the broilers were killed and samples of muscle and liver were collected and analysed for AMOZ using LC-MS/MS. At the lowest dose, AMOZ was detected in liver (0.6 µg/kg) samples. At the highest dose the levels in liver and muscle were around 40 and 18 µg/kg respectively.

Isa Brown chickens (10 per treatment) were treated with furaltadone (150 mg/kg feed) or furaltadone and nifursol (15 and 10 mg/kg, respectively feed), corresponding to around 30 mg/kg BW of furaltadone per day, 3 mg/kg BW of furaltadone per day and 2 mg/kg BW of nifursol per day, for five weeks followed by a withdrawal period of three weeks (Barbosa *et al.* 2011). At the end of the medication period five chickens were slaughtered and samples of muscle, liver and gizzard were collected and analysed for AMOZ using LC-MS/MS. The remaining chickens were slaughtered and sampled at the end of the withdrawal period, i.e. 21 days. Furaltadone was only measured in the samples taken at the end of the medication period. The levels in the tissues were around 50 µg/kg in muscle and liver, and 80 µg/kg in gizzard in the birds treated with furaltadone (30 mg/kg BW). The levels in birds treated with approximately 3 mg/kg BW of furaltadone and 2 mg/kg BW of nifursol per day were around 17 µg/kg in all tissues. Directly after the medication period the levels of AMOZ in birds treated with furaltadone or furaltadone and nifursol were much higher than after three weeks of withdrawal. The levels of AMOZ in tissues retrieved after the withdrawal period were 270 µg/kg in muscle, 80 µg/kg in liver and 331 µg/kg in gizzard in birds treated with furaltadone. The levels in tissues from birds administered furaltadone and nifursol were

29 µg/kg in muscle, 17 µg/kg in liver and 32 µg/kg in gizzard after the withdrawal period.

Barbosa *et al.* (2012) also investigated the transfer of furaltadone and AMOZ from laying hens to eggs by administering 150 mg furaltadone per kg feed (treatment 1) or 15 mg furaltadone per kg feed and 10 mg nifursol per kg feed (treatment 2), corresponding to around 30 mg/kg BW of furaltadone per day, 3 mg/kg BW of furaltadone per day and 2 mg/kg BW of nifursol per day, for five weeks followed by a withdrawal period of three weeks. Five hens per treatment were used and a minimum of five eggs were collected on the last two days of weeks 3-8. The levels of furaltadone and AMOZ were measured using LC-MS/MS. The levels of furaltadone in yolk and white peaked at week 4 and could not be detected at week 6. The maximum levels of furaltadone, for treatment 1, measured in yolk and white were 445 and 253 µg/kg, respectively. For treatment 2, the maximum levels were 64 µg/kg yolk and 45 µg/kg white. AMOZ could be detected in all egg tissues at all weeks except for in white (treatment 2) the last two weeks of the study. Like furaltadone the maximum levels of AMOZ was reached at week 4 and were for treatment 1 712 and 613 µg/kg in yolk and white, respectively. For treatment 2, the levels were 89 and 68 µg/kg in yolk and white, respectively. Three weeks after the last treatment the levels of AMOZ in egg yolk and white were 3.1 and 2.9 µg/kg for treatment 1. For treatment 2, AMOZ could only be detected in yolk at a level of 0.2 µg/kg.

McCracken *et al.* (2005a) fed six broiler hens and one cockerel 120-140 g of feed containing furaltadone (400 mg/kg feed), corresponding to around 96 and 112 mg/kg BW per day. Eggs were collected and analysed for AMOZ and when it was clear that furaltadone residues had transferred to the eggs, the eggs laid after this were collected and allowed to hatch. After hatching four chicks were sacrificed at determined intervals and muscle and liver samples were analysed for AMOZ. However, AMOZ could only be detected up to day 10. The levels of AMOZ were 11.4 µg/kg liver and 1.0 µg/kg muscle in one day old chicks, ~3 µg/kg liver and ~0.5 µg/kg muscle in five days old chicks, and ~0.7 µg/kg liver and ~0.2 µg/kg muscle in 10 days old chicks.

Twenty-four laying hens were fed feed containing 300 mg furaltadone, nitrofurazone, nitrofurantoin or furazolidone per kg feed, corresponding to approximately 60 mg/kg

BW per day, for one week (McCracken & Kennedy 2007). Eggs were then collected for two days and analysed immediately for nitrofurantoin parent compound and their bound residues in albumen, yolk and shell using LC-MS/MS. The levels of furaltadone in yolk, albumen and shell were 593, 486 and 35.7 ng/g, respectively. The levels of AMOZ in yolk, albumen and shell were 590, 624 and 56.3 ng/g, respectively.

5.3.3 Conclusions on pharmacokinetics of furaltadone and its metabolites

In the studies above it has been shown that furaltadone is absorbed and rapidly distributed in the body. It is quickly eliminated with a half-life in plasma of 2.5 hours in calves and can be excreted via the urine. Post mortal degradation of furaltadone in calves was shown to be very fast. The shortest half-life was less than eight minutes in liver and the longest was one hour in kidney. After broilers were administered furaltadone orally the highest levels were found in the gizzard (80 µg/kg). It was also demonstrated that furaltadone can be transferred from laying hens to eggs. Furaltadone have been shown to be metabolised into protein-bound residues containing the AMOZ side-chain in cow, pig, fish and poultry. It was also shown that in pig hepatocytes, furaltadone was metabolised to an N-oxide, FAX, and in bacteria it was metabolised to an open-chain metabolite, FACN.

AMOZ was shown to be transferred into milk from cows administered furaltadone. It was also shown that eggs from furaltadone treated hens contained AMOZ and that it could still be detected in chicks hatched from those eggs. In poultry administered furaltadone the highest levels of AMOZ were found in the gizzard (331 µg/kg) followed by muscle (270 µg/kg) and liver (80 µg/kg). In pigs fed furaltadone, the highest levels of AMOZ were found in muscle, followed by kidney and liver (60 and 15 µg/kg, respectively). The half-lives were determined to be around nine days in muscle and five days in kidney and liver. In channel catfish muscle the half-life of AMOZ was calculated to be 27 days but could still be detected 56 days after the last treatment.

5.4 Toxicology of furaltadone

5.4.1 Acute toxicity

No studies could be found.

5.4.2 Chronic toxicity

No studies could be found.

5.4.3 Reproductive toxicity including teratogenicity

No studies could be found.

5.4.4 Mutagenicity and genotoxicity

An Ames test using *S. typhimurium* TA 100 was carried out on furaltadone and the N-oxide metabolite (FAX) (Hoogenboom *et al.* 1994). *S. typhimurium* TA 100 was incubated with furaltadone or FAX (10, 100 or 1000 ng/plate) without any metabolic activation. Furaltadone tested positive at all doses and FAX tested negative at all doses.

The study conducted follows guidelines to some extent. Mutagenicity with metabolic activation was not tested and no positive control was used. It is possible that FAX could be mutagenic after metabolic activation.

5.4.4.1 Conclusions on mutagenicity and genotoxicity studies

Furaltadone tested positive in the only mutagenicity test found, while its metabolite FAX tested negative. It is difficult to draw any conclusions from just one study. To be able to say whether or not furaltadone and FAX are mutagenic and genotoxic more studies are needed.

5.4.5 Carcinogenicity and long-term toxicity

No studies could be found.

6 Summary of the effects of nitrofurans

The conclusions that can be drawn about the reproductive toxicity, mutagenicity and genotoxicity, as well as carcinogenicity of the reviewed nitrofurans are presented in Table 17. Regarding the reproductive toxicity, nitrofurazone and nitrofurantoin have been shown to be toxic to the reproductive system. Observed effects after exposure to these substances were decreased fertility, altered oestrus cycle, malformations, aberrant sperm and testicular degeneration. A reduction in the concentration of spermatozoa was seen at the lowest dose of nitrofurazone (10 mg/kg BW) tested in rats, while the most commonly observed effect was testicular degeneration. For furazolidone, a clear conclusion cannot be drawn and a lack of studies on furaltadone makes it impossible to determine if it is toxic to the reproductive system.

All nitrofurans reviewed, except for furaltadone, have been shown to be mutagenic and genotoxic *in vitro*. Conclusions cannot be drawn regarding the genotoxicity *in vivo* due to there being few studies with conflicting results. Further

research regarding their genotoxicity and mutagenicity *in vivo* therefore needs to be performed.

As to the carcinogenicity of the substances, nitrofurazone and furazolidone have proven to be carcinogenic. Nitrofurantoin is possibly carcinogenic and since no carcinogenicity studies have been performed with furaltadone no conclusions can be drawn about its potentially carcinogenic effects. Mammary tumours were the most commonly observed effect in carcinogenicity studies. The lowest daily dose where this effect occurred was with furazolidone at 0.8 mg/kg BW, but this study was only seen as an abstract.

Table 17. Classification of nitrofurazone, nitrofurantoin, furazolidone and furaltadone

	Reproductive toxicity	Mutagenic and genotoxic <i>in vitro</i>	Mutagenic and genotoxic <i>in vivo</i>	Carcinogenic
Nitrofurazone	X	X	Possibly	X
Nitrofurantoin	X	X	Possibly	Possibly
Furazolidone	Possibly	X	Possibly	X
Furaltadone	Not known	Possibly	Not known	Not known
Effects	Altered oestrus cycle, testicular degeneration, ↓fertility			↑mammary tumours
Lowest dose with effect	10 mg/kg BW			0.8 mg/kg BW

7 Risk assessment of nitrofurans

There are several methodologies that can be used when carrying out risk assessments, all with their own advantages and disadvantages. The currently available risk assessment methodologies and approaches have been reviewed in (SCHER/SCCP/SCENIHR 2009). Some of these are: quantitative risk characterisation by linear extrapolation, margin of exposure (MoE), threshold of toxicological concern (TTC), benchmark dose lower confidence limit (BMDL), 25 % increase of a certain tumour incidence (T25), and no observed adverse effect level (NOAEL). EFSA recommends the MoE-approach when assessing the risk of substances that are genotoxic and carcinogenic and this method will therefore be used in this risk assessment (EFSA 2005).

The MoE describes a ratio between the dose descriptor for tumour formation (T25 or BMDL) in animals and human exposure (SCHER/SCCP/SCENIHR 2009).It is

calculated by first deriving a benchmark dose (BMD), corresponding to a response level (benchmark response, BMR), and its lower 95%-confidence bound (BMDL) for different endpoints from carcinogenicity studies. Then, the model that adequately fits the data and has the lowest BMDL is selected. The BMDL of this model is then divided by the dietary exposure. EFSA considers that the risk to public health is of low concern if the MoE is equal to, or higher than, 10000 (EFSA 2005).

7.1 Benchmark dose (BMD)

The BMD and BMDL for different endpoints in carcinogenicity studies using nitrofurazone, nitrofurantoin and furazolidone was derived using the BMD software developed by the US EPA (US EPA 2014 <http://www.epa.gov/ncea/bmds/>). Datasets which produced a BMD and BMDL are presented in Table 18. Most of the carcinogenicity studies did not expose the test animals for the normal two year period and it is assumed that the number of tumours formed would have been higher if the studies had been performed for two years. To account for this the doses were adjusted to what they would have been if the study was carried out over a two year period. The doses were adjusted using the formula (1) presented by Carthew *et al.* (2010) and the individual BMD and BMDL were derived.

$$(1) \quad d_1 = \left(\frac{w_1}{104}\right) \times \left(\frac{w_2}{104}\right) \times d_0$$

w_1 = weeks dosed, w_2 = weeks observed, d_0 = dose used in study

Table 18. Benchmark doses for different endpoints in carcinogenicity studies with furazolidone

Tumour types	Substance	Species (Sex)	BMD*	BMDL*	Extrapolation	Reference
Bronchial adenocarc. **	Furazolidone	Swiss MBR/IC R mice (male)	4.36	1.08	Yes	JECFA (1993b)
Bronchial adenocarc. **	Furazolidone	Swiss MBR/IC R mice (female)	8.06	1.36	No	JECFA (1993b)
Thyroid adenomas **	Furazolidone	Fisher 344 rats (male)	14.5	8.51	No	JECFA (1993b)
Sebaceous gland adenomas **	Furazolidone	Fisher 344 rats (male)	21.4	13.95	No	JECFA (1993b)
Sebaceous gland adenomas **	Furazolidone	Fisher 344 rats (female)	23.8	12.2	No	JECFA (1993b)
Neural astrocytomas **	Furazolidone	Sprague-Dawley rats (male)	40.6	30.2	No	JECFA (1993b)
Mammary adenocarc. **	Furazolidone	Sprague-Dawley rats (female)	34.05	25.4	No	JECFA (1993b)
Tubular adenoma or mixed benign tumour	Nitrofurantoin	B6C3F ₁ mice (female)	558.5	443.7	No	NTP (1989)

*Doses in mg/kg BW and day

** Adjusted for shorter exposure time

All models that did not have a dose-response trend or where extrapolation occurred, i.e. when the BMD was lower than the lowest dose tested, were disregarded for use in calculating the MoE. The lowest BMDL, 1.36 for bronchial adenocarcinomas in female mice, was used for the calculation of MoE (Figure 7).

Log-Logistic Model, with BMR of 10% Extra Risk for the BMD and 0.95 Lower Confidence Limit for the BMDL

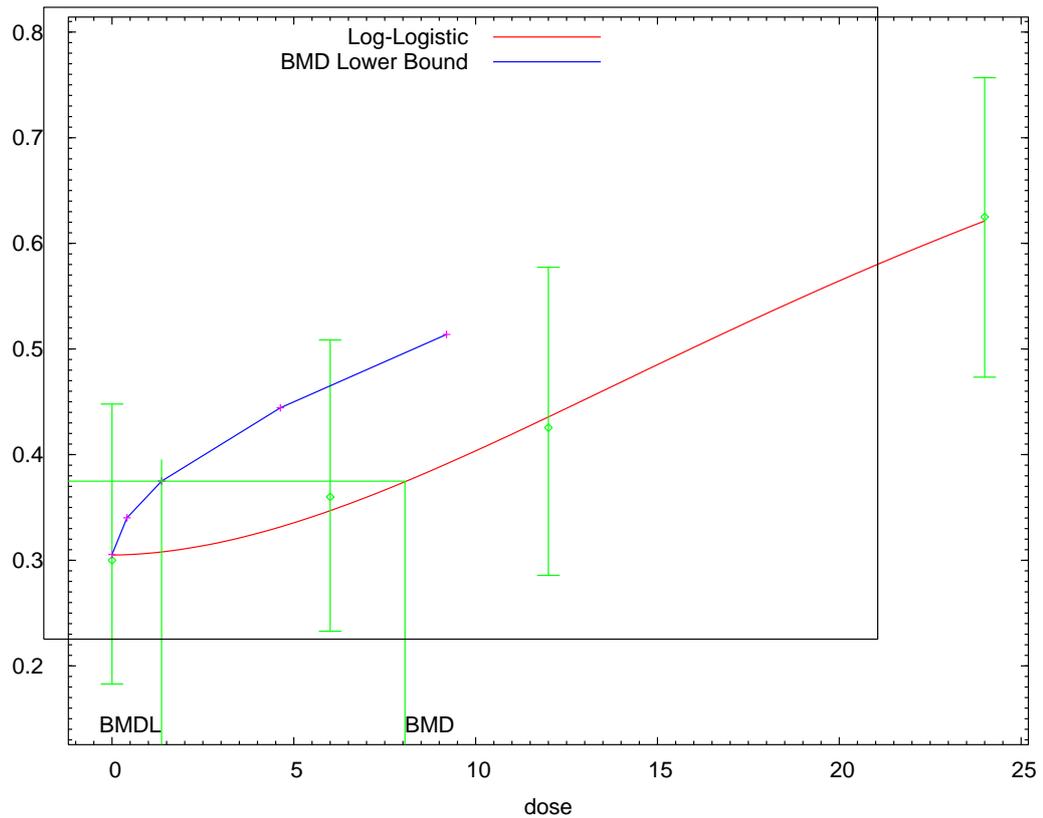


Figure 7. Log-logistic model with BMR of 10 % extra risk for the BMD and 0.95 lower confidence limit for the BMDL for bronchial adenocarcinomas in female Swiss MBR/ICR mice. Doses are in mg/kg BW per day.

7.2 Exposure assessment

No exposure assessment has been performed on nitrofurans and it is consequently hard to know exactly how much people are exposed to. In order to calculate the Swedish population's exposure to nitrofurans it is assumed that all consumed foodstuffs of animal origin in Sweden contain levels of nitrofurans at 1 µg/kg, the current MRPL. Based on intake data from Riksmaten, a national dietary survey, the average consumption of foodstuffs of animal origin (meat, bird, sausage, organ meats, blood products, fish and shellfish, caviar and egg) and dairy products in Sweden is 0.392 and 0.486 kg per day and person for adult women and men, respectively (Amcoff *et al.* 2012). For the 95th percentile (high consumer) it is 1.024 and 1.357 kg per day and person for women and men, respectively (Amcoff *et al.* 2012). Assuming this, then the mean exposure to nitrofurans for an average Swedish woman and man per day is 0.392 and 0.486 µg, respectively. For a high consumer the exposure is 1.024 and 1.357 µg for women and men, respectively. The mean bodyweight for women in Riksmaten (Amcoff *et al.* 2012) was 69 kg and for men the mean bodyweight was 84 kg. The exposure for average women and men is 5.68 and 5.79 ng/kg BW and day, respectively (Table 19).

For a high consumer the exposure is 14.8 and 16.2 ng/kg BW for women and men, respectively (Table 19).

In Riksmaten from 2003 (Enghardt Barbieri *et al.* 2006) the food consumption of children in Sweden was studied. The children looked at were four year olds and children in 2nd and 5th grade. The average consumption of foodstuffs of animal origin (meat, bird, sausage, organ meats, blood products, fish and shellfish, and egg) and dairy products was 0.490 and 0.556 kg per day and person for four year old girls and boys, respectively (Enghardt Barbieri *et al.* 2006). For boys and girls in 2nd grade the consumption was 0.711 and 0.608 kg per day and person, respectively. Children in 5th grade had an average consumption of 0.525 and 0.651 kg per day and person for girls and boys, respectively. If again it is assumed that all consumed foodstuffs of animal origin in Sweden contain levels of nitrofurans at 1 µg/kg the mean exposure to nitrofurans is; 0.490 and 0.556 µg per day for four year old girls and boys, 0.711 and 0.608 µg per day for boys and girls in 2nd grade, and 0.525 and 0.651 µg per day for girls and boys in 5th grade. The mean bodyweight for four year olds was 18 kg, for girls and boys in 2nd grade 31 and 32 kg respectively, and for 5th graders 42 kg (Enghardt Barbieri *et al.* 2006). The exposure for average four year old girls and boys is 27.2 and 30.9 ng/kg BW and day, respectively (Table 19). For girls and boys in 2nd grade the exposure is 19.6 and 22.2 ng/kg BW and day, respectively (Table 19). And lastly, for girls and boys in 5th grade the exposure is 12.5 and 15.5 ng/kg BW and day, respectively (Table 19).

Another source of exposure to nitrofurans can be via the use of pharmaceuticals. Nitrofurantoin is regularly used to treat urinary tract infections in men and women. The normal treatment is 50 mg, three times per day for five days, which amounts to a total dose of 150 mg per day (FASS 2010). Since this dose is so high, the added exposure via food is negligible and therefore, only the medicinal exposure will be considered in a separate assessment. Assuming the same mean bodyweights as before the exposure per day is 2.17 and 1.79 mg/kg BW per day for women and men, respectively. Assuming that people only undergo one treatment, for five days, in their lifetime (70 years), the lifetime exposure is 425 and 349 ng/kg BW per day for women and men, respectively (Table 19).

Table 19. The food consumption, bodyweight and exposure for adult female and male (18-80 years) average and high consumers of nitrofurans via food in Sweden, child average consumers of nitrofurans via food and men and women treated with nitrofurantoin for five days

	Consumption of animal food products and dairy products (kg/day)	Exposure via food (µg/day)	Medicinal exposure (mg/day)	Bodyweight (kg)	Exposure to nitrofurans (ng/kg BW per day)
Women (average)	0.392	0.392		69	5.68
Women (high)	1.024	1.024		69	14.8
Men (average)	0.486	0.486		84	5.79
Men (high)	1.357	1.357		84	16.2
Four year old girls	0.490	0.490		18	27.2
Four year old boys	0.556	0.556		18	30.9
Girls in 2nd grade	0.608	0.608		31	19.6
Boys in 2nd grade	0.711	0.711		32	22.2
Girls in 5th grade	0.525	0.525		42	12.5
Boys in 5th grade	0.651	0.651		42	15.5
Women (medicinal)			150	69	425
Men (medicinal)			150	84	349

7.3 Margin of exposure (MoE)

The MoEs for the average and high consumer of foodstuffs of animal origin in Sweden are presented in Table 20. None of the MoEs are lower than the margin of 10000 when only the exposure to nitrofurans via food is considered, indicating a risk of low concern. However, if only the medicinal exposure is considered the MoEs are below 10000 for both men and women, indicating that there may be a risk.

Table 20. Calculated margin of exposure (MoE) for adult female and male (18-80 years) average and high consumers of nitrofurans via food in Sweden, child average consumers of nitrofurans via food and men and women treated with nitrofurantoin for five days

	Average consumer	High consumer
Women	240000	92000
Men	230000	84000
Four year old girls	50000	
Four year old boys	44000	
Girls in 2nd grade	69000	
Boys in 2nd grade	61000	
Girls in 5th grade	110000	
Boys in 5th grade	88000	
Women (medicinal)	3200	
Men (medicinal)	3900	

8 Discussion and conclusions

Genotoxic compounds exert their effects at all concentrations and can therefore pose a health risk at even very low concentrations. However, if the compounds are only genotoxic *in vitro* and not *in vivo* the risk for potential adverse effects is reduced and will affect the risk assessment of these compounds. The mutagenicity and genotoxicity of nitrofurazone, nitrofurantoin, furazolidone and furaltadone have been studied in many tests, both *in vitro* and *in vivo*. The results from these tests have shown that nitrofurazone, nitrofurantoin and furazolidone are genotoxic *in vitro*. The results in the *in vivo* tests have been inconclusive and therefore no definite conclusions can be drawn. More studies are needed to elucidate their mutagenicity and genotoxicity *in vivo*. Since the *in vivo* tests have shown both negative and positive results the possibility of genotoxic effects *in vivo* cannot be overlooked and must therefore be considered in the risk assessment. Only one mutagenicity test was performed with furaltadone. That test was positive but due to a lack of studies no conclusions regarding the mutagenicity and genotoxicity of furaltadone can be drawn.

Some of the metabolites of these nitrofurans have also been tested for mutagenic and genotoxic properties. FAX, a metabolite of furaltadone, tested negative in the only mutagenicity study found. Due to a lack of studies no conclusions can be drawn. SEM,

a metabolite of nitrofurazone, tested positive in bacterial tests but the results from *in vitro* and *in vivo* tests are inconclusive. To safely determine the mutagenicity and genotoxicity of SEM more studies are needed. The metabolite of furazolidone, AOZ, tested positive in *in vitro* tests but not in the only *in vivo* study found. To further elucidate AOZ's mutagenic and genotoxic properties more studies are needed. Another metabolite of furazolidone, 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone, tested negative in an Ames test and must be examined more before a conclusion can be drawn.

Nitrofurazone and nitrofurantoin have in several studies been shown to be toxic to the reproductive system of experimental animals and no NOAELs could be set. Such substances should be extensively reviewed, especially when they are used in human medicine. Nitrofurantoin, which is currently being used to treat urinary tract infections, had a somewhat reversible adverse effect on the number of spermatozoa in rats at the lowest dose tested (10 mg/kg BW). This study does not meet today's standard of testing, but the results should still be treated as reliable and an effect at quite a low dose is troubling since both children as well as adults are taking drugs in which nitrofurantoin is the active substance.

Nitrofurazone and furazolidone have been shown to be carcinogenic in rats and mice, mainly causing mammary tumours. No conclusion regarding the carcinogenicity of furaltadone can be drawn since no studies were found. Contradicting results from studies with nitrofurantoin makes it difficult to determine whether or not it is carcinogenic. To ascertain the carcinogenicity of nitrofurantoin additional research is required. One can argue that compounds which are both carcinogenic and genotoxic *in vivo* pose a greater health risk than compounds which are only genotoxic, or carcinogenic and genotoxic *in vitro*. This should therefore be taken into account when performing risk assessments. Nitrofurazone and furazolidone are carcinogenic, nitrofurantoin and furaltadone might be and all, except for furaltadone, have been shown to be genotoxic *in vitro*. Since it cannot be excluded that the nitrofurans are genotoxic *in vivo* the precautionary approach must be used and it should be assumed that the nitrofurans are genotoxic *in vivo*.

The BMDL is based on a carcinogenicity study on furazolidone in mice. However, this study was only seen as an abstract and the experimental animals were only treated for 13 months instead of the usual 24 months. Due to this there are some uncertainties concerning the results of the study and the number of bronchial

adenocarcinomas may be lower than they would be if the exposure was over 24 months. To account for this uncertainty the results were adjusted as stated previously.

The MoE after a five day long course of treatment with nitrofurantoin, stretched out over a lifetime, are below 10000 for both men and women. Furthermore, nitrofurantoin is used as prophylaxis for urinary tract infections with long treatment periods which would further decrease the MoE. This demonstrates that there may be an increased risk for bronchial adenocarcinomas for people treated with nitrofurantoin. Some side effects of nitrofurantoin affect the lungs. Long term treatment can cause pulmonary fibrosis, while some acute pulmonary reactions include; coughing, shortness of breath, rapid breathing and chest pains (FASS 2010). In regards to the reproductive toxicity of nitrofurantoin and that there is an increased risk of developing cancer after only one course of treatment, it might be wise to reevaluate the safety of nitrofurantoin as a drug used in humans.

The calculated MoEs for children and for adult male and female, average and high, consumers of foodstuffs of animal origin are above the margin of 10000. This shows that there is a negligible risk to the Swedish population's health from nitrofurans in food and that the MRPL of 1 µg/kg is a safe limit for nitrofurans in food. Furthermore, in the exposure assessment dairy products constitute a large portion of exposure but in the Swedish controls in 2011 and 2012, no milk samples tested contained nitrofurans (Gustavsson *et al.* 2012, Nordlander *et al.* 2013). Since it is assumed that all foodstuffs of animal origin and dairy products contain 1 µg/kg, which has not been observed during inspections, the risk from nitrofurans is overestimated. That being said, only a small percentage of total production is tested and there may also be a risk that imported milk contains nitrofurans. Furthermore, the exposure via food is based on results from Riksmaten in which young men underreported their food consumption, thereby underestimating the male food consumption and consequently the risk for adult males (Amcoff *et al.* 2012).

When calculating MRLs, as well as the MoE, the consumption of foods from animal origin is considered. For MRLs the food basket (i.e. exposure via food) is comprised of 300 g muscle, 50 g kidney, 50 g fat, 100 g liver, 1.5 L milk, 100 g eggs or egg products and 20 g honey. If this food consumption is used for the MoE exposure assessment, the subsequent MoEs are 44000 and 54000 for women and men, respectively. The increased risk is due to a higher estimated consumption of meat, egg and milk in the MRL assessment than the reported consumption in Riksmaten (Amcoff

et al. 2012). It appears that the food consumption used for MRL determinations is not consistent with that in Sweden and would therefore overestimate the risk. However, it is a good estimation to use when the precautionary approach is applied.

In order for nitrofurans to have a MoE of 10000 for male high consumers, all of the dairy products and consumed food of animal origin needs to contain on average around 8 µg/kg. According to RASFF Portal (2014) the mean nitrofuran level found in different food products (e.g. crayfish, shrimp, fish and meat) in border controls between January 1, 2005 and March 7, 2014 was 19.86 µg/kg. The highest reported concentration was 5000 µg/kg found in shrimp from Bangladesh.

SEM, a metabolite of nitrofurazone, has been shown to occur naturally in crayfish (Saari & Peltonen 2004, McCracken *et al.* 2013). In 2003 wild crayfish were caught in a Swedish lake and analysis showed that they contained between 2 and 5 µg/kg (Christina Elgerud, personal communication). The Swedish National Food Agency decided in 2006 to stop testing for, and reporting levels of SEM in European crayfish and other shellfish. This is due to SEM being a rather unspecific marker residue for nitrofurazone and therefore the found levels could not be connected to illegal use of nitrofurans (Christina Elgerud, personal communication, National Food Agency, unpublished report). In light of the MoE it is unlikely that naturally occurring SEM levels in crayfish would pose a risk to the population's health, since the consumption of crayfish is not that great and seasonal. The decision by the NFA to stop testing European crayfish for SEM is therefore justified.

In relation to the MoEs for nitrofurans, the minimum required performance limit (MRPL), which has been used as a reference point of action (RPA), provides enough protection for the Swedish population, both adults and minors, from nitrofurans in food. However, since no dietary intake and consequent risk assessment for infants were performed, there may be a risk for infants posed by nitrofurans in foodstuffs. Also, the current documentation on nitrofurans is incomplete and what is available is quite old. Most of the found studies do not follow guidelines. It is therefore not possible to perform a comprehensive risk assessment and be able to fully elucidate what impact nitrofurans in food have on the human health. Furthermore, the reliability of the carcinogenicity study, from which the BMDL is based on, can be questioned since it had to be corrected for not being performed over a two year period. Nonetheless, based on the results of this risk assessment the current RPA of 1 µg/kg, the MRPL, can be retained. All foodstuffs containing lower levels can be allowed to enter the market.

Food products containing higher levels should be removed from the market to protect the consumers. However, crayfish containing residues of SEM lower than 5 µg/kg could be accepted from a consumer health perspective. There is already an inherent safety margin in the proposed RPA since all animal based foodstuffs and dairy products eaten in a single day must contain, on average, 8 µg/kg to pose a risk to the population's health, which is unlikely. That being said, nitrofurans should not be allowed for use in food-producing animals due to their reproductive toxicity, genotoxicity and carcinogenicity.

Under normal circumstances, carcinogenic and genotoxic compounds are not permitted for use in food-producing animals but exceptions can be made when it is deemed necessary. Metamizole sodium is used as an anti-inflammatory, antipyretic, spasmolytic and analgesic compound in veterinary medicine (CVMP unpublished report). In the assessment report on metamizole sodium the compound is carcinogenic in mice but produces equivocal results in genotoxicity tests. It was cleared for use since a drug against colic was needed and therefore a quite arbitrary MRL of 100 µg/kg was set (Commission Regulation (EU) No 37/2010).

In the future less safe antibiotics, like nitrofurans, may need to be used due to an increased bacterial resistance to antibiotics. However, there is always a potential risk when using genotoxic substances. The risks of using nitrofurans have to be weighed against the potential benefits. At this time there are no great benefits in allowing nitrofurans to be used in food-producing animals. A risk-benefit analysis should be performed on nitrofurantoin to evaluate if it is absolutely necessary in human medicine or if its use can be reduced or eliminated completely. The use of nitrofurans should not be allowed until the benefits outweigh the potential risks. To safeguard the possible future use of nitrofurans it must be ensured that bacteria do not gain resistance to nitrofurans. Thus, nitrofurans should not be used in food-producing animals and the amounts in food should be limited. It should also be investigated if the metabolites of the nitrofurans have antibacterial properties like their parent compounds, but without their toxicity. If so, it is possible that they can be used as alternative antibacterial agents to today's antibiotics.

In conclusion, nitrofurans are genotoxic and carcinogenic substances which could cause adverse health effects in humans. It was shown that one course of treatment with nitrofurantoin might increase the risk for bronchial adenocarcinomas in humans. Therefore, the safety of nitrofurantoin ought to be reevaluated. In the future it may be

necessary to use nitrofurans, due to an increase in bacterial resistance to antibiotics, and they should therefore not be used today. It would also be prudent to investigate the antibacterial effects of the metabolites of nitrofurans and possibly use them as antibiotics in the future. In regards to nitrofurans in food, the Swedish population has been efficiently protected from the health risks posed by nitrofurans via the MRPL of 1 µg/kg. The RPA of 1 µg/kg sufficiently protects high consumers of foodstuffs of animal origin and should be retained. When dealing with food products containing nitrofurans at the RPA, or lower, the Swedish NFA should only notify this in RASFF but take no further action. However, any products containing nitrofurans over this level should not be allowed to enter the market, with the exception of SEM in crayfish, in order to protect the health of the population. At this time, countries within the EU have different views regarding residues of nitrofurans in foodstuffs and therefore differ in their treatment of foodstuffs containing nitrofurans. Implementing a RPA for nitrofurans in food would provide a more uniform treatment of food products containing nitrofurans above the current MRPL, which is currently lacking within the EU.

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11 Appendix

11.1 Bacterial reverse mutation test

This test is used to screen for genotoxic compounds able to cause point mutations. The principle is that strains of *Escherichia coli* and *Salmonella typhimurium*, who require an external supply of amino-acids, are incubated for around two or three days with the compound at 37 °C. The incubation occurs in the presence and absence of an exogenous metabolic system. Positive, e.g. Mitomycin C, and negative controls should be used. Depending on the method the bacteria can either be immediately plated on minimal agar plates, along with the test substance, and then incubated or first incubated with the test substance for around 20 minutes and then plated. After the incubation period the number of revertant colonies, colonies of bacteria who now can produce amino-acids on their own, are counted per plate. When evaluating and interpreting the results statistical methods can be used. If there is a dose-related increase in the number of revertant colonies or a reproducible increase at one or more concentrations the results should be considered positive.

11.2 Mammalian erythrocyte micronucleus test

The *in vivo* micronucleus test detects chromosomal damage and impairment of the mitotic apparatus in erythrocytes. In short, animals (5 per sex and dose) are exposed to a substance and samples of peripheral blood or bone marrow, are taken at certain time points after the exposure. Samples should be retrieved at a minimum of two time points between 24 and 48 hours for bone marrow and between 36 and 72 hours for peripheral blood. Rats are not recommended when peripheral blood is used due to micronucleated erythrocytes being effectively filtered in the spleen. Positive, e.g. Mitomycin C, and negative controls should be used. The erythrocytes are then stained and the proportion of immature erythrocytes is determined by counting either 200 cells from bone marrow or 1000 cells from peripheral blood. The frequency of erythrocytes is then determined by counting 2000 cells. When evaluating and interpreting the results statistical methods can be used. If there is a dose-related increase in the number micronucleated erythrocytes or a clear increase in a single dose group at a single time point the results should be considered positive.

11.3 *In vitro* mammalian chromosome aberration assay

The *in vitro* chromosome aberration test is used to test compounds ability to induce structural aberrations in chromosomes. Mammalian cell cultures are exposed to the

substance, with and without metabolic activation, for three to six hours and harvested after 1.5 cell cycles. A metaphase arresting agent, e.g. colchicine, should be added to the cultures around one to three hours before harvest. Positive, e.g. benzo(a)pyrene or methyl methanesulphonate, and negative controls should also be used. After harvesting the cells they should be fixated and stained. A minimum of 200 cells in metaphase should be scored for chromosome aberrations per treatment. The type and frequency of the aberrations should be listed along with the total percentage of cells with aberrations. The cytotoxicity should also be measured in all cultures. When evaluating and interpreting the results statistical methods can be used. If there is a dose-related increase in the number of cells with chromosome aberrations or a reproducible increase the results should be considered positive.