Aan Yvonne

L.H.M. VROOMEN

IN VIVO AND IN VITRO METABOLIC STUDIES OF FURAZOLIDONE

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op dinsdag 24 november 1987
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

STELLINGEN

1. De conclusie van Van Esch et al., dat "voor een schadelijk effect
   van sterk reactieve intermediairen van furazolidon niet gevreesd
   behoeft te worden", is voorbarig gezien het reversibele karakter van
   de covalente binding aan eiwit van het reactieve acrylonitril derivaat
   <u>in vitro</u>.

   - G.J. van Esch, C.A. van der Heijden, B. van Klingeren en
     C.E. Voogd, Rijksinstituut voor de Volksgezondheid, Bilthoven,
     rapportnr. 358301001, 1983.
   - Dit proefschrift.

2. De suggestie van Mattammal et al., dat het N-hydroxyderivaat van de
   nitrofuraan N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide verantwoor-
   delijk is voor de interactie met glutathion, houdt geen rekening
   met een eventuele vorming van het reactieve acrylonitrilprodukt uit
   dit derivaat.

   - M.B. Mattammal, T.V. Zenser, M.O. Palmier and B.B. Davis,
     Cancer Research, 45 (1985) 149-156.
   - Dit proefschrift.

3. Het "surface walks" mechanisme voor de conjugatiereacties gecata-
   lyseerd door glutathion-S-transferases kan niet als algemeen worden
   aanvaard omdat van de meeste iso-enzymen bekend is dat deze niet,
   de voor dit mechanisme benodigde, vier cysteine residuen bevatten.

   - R.E. Ridgewell and M.M. Abdel-Monem, Drug Metabolism and Disposition,
     15 (1987) 82-90.
   - C.B. Pickett, C.A. Telakowski-Hopkins, G.J.F. Ding and V.D.H. Ding,
     Xenobiotica, 17 (1987) 317-323.

4. Gezien het feit dat verschillen in biotransformatie en toxische
   effecten tussen enantiomeren inmiddels goed gedocumenteerd zijn,
   moet het onaanvaardbaar worden geacht dat grote aantallen biolo-
   gisch actieve verbindingen zoals pesticiden nog steeds op de markt
   worden gebracht als racemisch mengsel.

   - E.J. Ariëns, in: Stereochemistry and Biological Activity of
     Drugs, E.J. Ariëns et al. (eds). Blackwell Sci. Publ., Oxford,
     1983, p. 11-32.
   - Stereoselectivity in pesticide action, a notable source of
     problems, E.J. Ariëns, J.J.S. van Rensen and W. Welling (eds),
     Elsevier/North-Holland, Amsterdam, in press.

VOORWOORD

Op deze plaats wil ik een woord van dank uitbrengen aan allen die een
bijdrage hebben geleverd aan het tot stand komen van dit proefschrift.
Op de eerste plaats aan de promotor prof. dr. J.H. Koeman voor de gebo-
den gelegenheid, begeleiding en goede samenwerking.
Aan de co-promotor dr. ir. H.A. Kuiper voor zijn intensieve begeleiding,
steun en alles wat ik dank zij hem geleerd heb.
Aan M.C.J. Berghmans voor al het verzette werk en geboden steun.
Aan dr. P.J. van Bladeren en ir. L.A.P. Hoogenboom voor hun bijdrage aan
de discussies.
Aan de directies van RIKILT, met name ir. M. Heuver en ir. G.S. Roosje
en IGMB-TNO, afdeling ILOB voor de geboden mogelijkheden.
Aan allen van het RIKILT die dit proefschrift mede mogelijk hebben
gemaakt, in het bijzonder J.C. van der Nagel.
Aan allen van de Vakgroep Toxicologie voor de goede samenwerking
gedurende de afgelopen jaren, in het bijzonder dr. ir. B. van Ommen.
Aan P. van Leeuwen, J. de Jong, drs. K. van Hellemond, dr. P. Hekman,
J. Haas, J. van Harn, A. Hok, M. van Baak, T.D.B. van der Struijs
en W.D.M. Driessen-Van Lankveld voor hun steun bij het dierexperimen-
teel onderzoek en de daaruit voortvloeiende werkzaamheden.
Aan A. van Veldhuizen, C. Teunis, J.M. Weseman, W.M.J. Beek, H.J.
Keukens en W.A. Traag voor hun bijdrage aan de identificatie van
metabolieten.
Aan Y.M. van Brakel-Louwers en J.M. Holtrust voor het typen van het
manuscript.
Aan de doctoraalstudenten J.M.M. Cruijsen, K. van Muiswinkel, J.P.
Groten en H.C.G. Wissink voor hun bijdrage aan het onderzoek.
Aan Harry, Marcel, Melle, Margot, Marcel, Ron, Folke, Sjoerdtje,
Saskia en Maryvon voor de plezierige en goede samenwerking binnen de
afdeling Toxicologie.
Tot slot wil ik Y.M.H. Monsewije bedanken. Yvonne, zonder jou was dit
nooit tot stand gekomen; moge dit proefschrift een aanvaardbare com-
pensatie zijn voor mijn veelvuldige afwezigheid.

Wageningen, november 1987.
L.H.M. Vroomen

PART I INTRODUCTION

CHAPTER 1: Introduction

INTRODUCTION

1. General introduction

During the last decennia considerable intensification has taken place
in the Netherlands in animal husbandry in terms of total number of
animals involved and of the number of animals raised per farm. For
instance since 1960 a twentyfold increase of the number of swine per
farm has been registered in the Netherlands (Table 1).

Table 1: Numbers of food-producing animals in the Netherlands.

Species	1960	1970	1983	1986
cattle (x 10^3)	3507	4314	5411	5123
cattle/farm	-	33	67	70
swine (x 10^3)	2955	5533	10656	13481
swine/farm	20	73	284	373
laying hens (x 10^6)	37.9	25.3	40.8	45.5
slaughter chickens (x 10^6)	4.5	30.1	35.1	39.4

According to the Dutch Central Office for Statistics (CBS), Voorburg
(Meitellingen)

This development appeared to have consequences for the conditions of
the animals, for instance abnormal behaviour was observed in swine
such as tail-biting and cannibalism (Van Putten, 1968). Swine also
became more susceptible to stress. Eikelenboom (1972) reported an
increased incidence of death animals occurring during transport to the
slaughterhouse and a pale, soft and exudative quality of the meat of
these pigs. Furthermore the chance of outbreak of infectious diseases
increased. Several measures had to be taken to either cure or prevent
these anomalies. Abnormal behaviour was cured by improving the housing
conditions with regard to factors like ambient temperature, humidity

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and the availability of fresh straw. Stress was in the beginning

treated by the application of tranquillizers but in recent years the

selection of stress resistant strains has been emphasized. For the

control and prevention of infectious diseases both the prophylactic

and therapeutic use of veterinary drugs increased markedly. Especially

administration of drugs via feed and drinkingwater has become common

practice since the last two decades with the result that large numbers

of animals are treated during most of their life-time with veterinary

drugs. It should be noticed that relatively little attention has been

payed to the prevention of these diseases through efforts to improve

the hygienic conditions under which animals are housed.

The number of available veterinary drugs increased. Hundreds of drugs

are presently on the market and the number of formulations runs into

the thousands. As most of these has not yet been adequately registered,

it is virtually impossible to give an accurate overview of all veteri-

nary drug preparations. The top five compounds used for mass medica-

tion of swine and poultry through the feed include oxytetracycline,

furazolidone, sulfadimidine, lincomycin and spectinomycin (Table 2).

Table 2: Production of medicated feed (x 1000 kg) for swine and
poultry in the Netherlands in 1985.

Drug	Swine	Poultry
Oxytetracycline	51,099	4,601
Chlortetracycline	–	3,676
Furazolidone	8,152	11,925
Oxytetracycline/Furazolidone	1,267	1,750
Sulfadimidine	13,134	214
Lincomycin/Spectinomycin	18,952	334
Amprolium	–	3,653

Data represent 62.5% of total production in the Netherlands.
Data from an internal annual report of the Dutch Commodity Board for
Feeding Stuffs, The Hague.

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The large use of veterinary drugs through mass medication poses a number of risks. First, pathogenic microorganisms may become resistant. Secondly, long term exposure to these compounds and/or its metabolites represent a health hazard for the animals concerned as well as for the human consumer of edible tissues originating from animals treated with veterinary drugs through the possible presence of residues. Consequently, in various countries laws and regulations have been adopted in order to assure the good veterinary use of drugs and the consumer safety of food products from treated animals. In the Netherlands such a law, based on the EEC-guidelines 81/851 and 81/852 (Anonymous, 1981 a,b) has become effective by the first of May 1986. For registration of drugs intended for mass medication of food producing animals, data have to be submitted concerning analytical, toxicological and pharmacological tests as well as results of clinical trials. As many drugs were already in use and quite a few of them not protected by patents anymore at the time the legal system was enforced, information of such compounds did not meet the new legal requirements. A typical example is the antimicrobial drug furazolidone, which is widely used in veterinary practice and of which essential information on metabolism and residu behaviour was lacking at the time the present study was started. The objective of the present investigation was to obtain a better understanding of the elimination kinetics and the biotransformation of furazolidone in swine, in relation to the safety of veterinary residues.

2. Furazolidone

2.1 General properties

Furazolidone [N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone (see Figure 1) belongs to the group of the 5-nitrofurans.
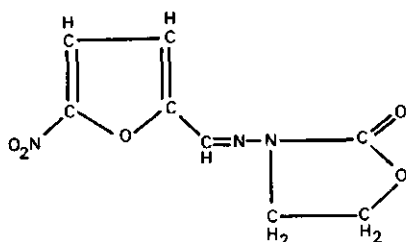
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Figure 1: Chemical structure of furazolidone.

Nitrofurans are synthetic products which are used worldwide for a
variety of purposes. The first 5-nitrofuran synthesized was 5-nitro-
2-furaldehyde (Gilman and Wright, 1930) followed by other ones (Bryan,
1978). The antimicrobial properties of the 5-nitrofurans have been
described for the first time by Dodd and Stillman (1944), who
discovered nitrofurazone, which was widely used in World War II as a
antimicrobial drug against wound infections.
One of the nitrofurans which became relatively popular was furazolidone.
The pure compound is a yellow, crystalline solid with a molecular
weight of 225, exhibiting absorption maxima at 259 nm and 367 nm; the
aqueous solubility is limited (40 mg/l) and independent of pH (Paul
and Paul, 1964). The drug is however readily soluble in dimethyl-
formamide or acetonitrile: 8.30 mg/ml and 3.75 mg/ml respectively
(Cohen, 1979). In solution furazolidone is unstable under the
influence of light (Kalim, 1985). Furazolidone displays a wide
spectrum of antimicrobial activity both in vitro and in vivo (Yurchenko
et al., 1953; Rogers et al, 1956; Paul and Paul, 1964), which was
believed to be attributable to metabolic activation of the 5-nitro
moiety of the furan ring (Dodd and Stillman, 1944; Dodd et al., 1950;
Paul and Paul, 1964). The susceptibility of a number of micro-
organisms for furazolidone is shown in Table 3.
The growth of most of these bacteria is inhibited in a concentration
range of approximately 1-10 µg/ml, although for Proteus and Pseudomonas

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Table 3: Antibacterial activity of furazolidone

Organism	Minimal Inhibitory Concentration (μg/ml)	Reference
Escherichia coli	0.66-1.5	Chamberlain, 1976; Hamilton-Miller and Brumfitt, 1978; Van Esch et al., 1983
Staphylococcus aureus	0.8-6.3	Chamberlain, 1976; Devriese, 1980; Van Esch et al., 1983
Staphylococcus epidermidis	1.3	Hamilton-Miller and Brumfitt, 1978
Streptococcus pyogenes	3-6	Chamberlain, 1976
Streptococcus faecalis	1.7-25	Chamberlain, 1976; Hamilton-Miller and Brumfitt, 1978; Van Esch et al., 1983
Clostridium spp	0.3-1.5	Chamberlain, 1976
Proteus spp	20-100	Chamberlain, 1976; Hamilton-Miller and Brumfitt, 1978
Proteus vulgaris	100	Van Esch et al., 1983
Klebsiella-Enterobacter spp	1.5-6	Chamberlain, 1976
Klebsiella aerogenes	3.5	Hamilton-Miller and Brumfitt, 1978
Salmonella spp	0.1-5	Chamberlain, 1976; Van Esch et al., 1983
Treponema hyodysenteriae	<0.1-1.56	Kitai et al., 1979
Shigella spp	0.2-1.2	Chamberlain, 1976
Pseudomonas aeruginosa	>200	Chamberlain, 1976; Van Esch et al., 1983
Vibrio cholerae	3	Chamberlain, 1976
Bacteriodes spp	6-12	Chamberlain, 1976
Campylobacter fetus subsp jejuni	0.09	Vanhoof et al., 1980
Campylobacter jejuni	<10	Vanhoof et al., 1982

much higher concentrations are needed. The difference in susceptibility of a species as reported by different authors may be explained by the absence of standardized determination methods.

Furazolidone has been widely used for 30 years for the effective and specific treatment of bacterial or protozoal diarrhoea and enteritis caused by susceptible organisms (Phillips and Hailey, 1986). The therapeutic use of furazolidone in man has been banned since 1980 in the Netherlands because of carcinogenic properties of the compound (Van Loenen, 1981).

Furazolidone is approved in the Netherlands for the treatment of intestinal infections caused by E.coli in swine (300 mg/kg of feed) and for the treatment of intestinal infections caused by entero-bacteriaecae and bronchial infections in poultry except laying hens (200 mg/kg of feed) (Landbouwkwaliteitsbesluit Gemedicineerd Voeder, 1975).

Furazolidone may improve growth and feed conversion in amounts of 25-220 mg per kg of feed (Clawson and Alsmeyer, 1973; Czarnecki et al., 1974; Villadelgado and Lasanas, 1975; Wahlstrom and Libal, 1975; Balayut and Tibay, 1976; Karunajeewa, 1978). In the EC the use of furazolidone for growth promoting purposes in swine is not allowed.

2.2 Toxicity

2.2.1 General

A well documented toxic action of furazolidone is its ability to inhibit the enzyme monoamine oxidase (MAO). This enzyme is involved in the metabolism of the neurotransmitter noradrenaline (Grobecker et al., 1980; Martindale, 1982). MAO inhibition after furazolidone administration has been observed in man (Pettinger et al., 1968), rats

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(Stern et al., 1966; Palm et al., 1967), chicken (Ali and Bartlet,
1980, 1982), ducklings (Ali and Bartlet, 1982) and turkeys (Powers et
al., 1983). As MAO was not inhibited in an in vitro model using rat
liver mitochondria (Stern et al., 1966; Pettinger et al., 1968) it has
been suggested that furazolidone required metabolic activation before
it may exert this toxic action. According to Stern et al. (1966) acti-
vation may take place by degradation of the oxazolidone-moiety to
2-hydroxyethyl hydrazine. Metabolic degradation may occur in the gut
because intramuscularly injected furazolidone in the chicken did not
affect MAO-activity in contrast to oral administration (Ali and
Bartlet, 1980). Additional evidence came from the observation that
pretreatment of chicken with neomycin reduced the effects of MAO-
inhibition by furazolidone (Ali and Bartlet, 1980). However, no dif-
ference in MAO activity in brain and liver could be observed between
conventional and germfree rats and therefore it has been proposed that
the intestine of rats can be excluded as the site for the formation of
a furazolidone metabolite which is inhibitory for MAO (Yeung and
Goldman, 1981). Thus, the mechanism of MAO-inhibition by furazolidone
is not fully understood till now and may also be species specific.

2.2.2 Toxicity in animals

When animals are exposed to relatively large amounts of furazolidone
(100-4000 mg/kg body weight), some acute adverse reactions could be
observed: anorexia, diarrhea, ataxia, depression and dyspnea (St.
Omer, 1978). There are large species differences in LD_{50}-value after
oral administration: mouse: 2086-3550 mg/kgbw (Dodd and Stillman,
1944; St. Omer, 1978), dove: 2375 mg/kgbw (St. Omer, 1978), rabbit:
2125 mg/kgbw (St. Omer, 1978), guinea pig: 1130 mg/kgbw and 1550
mg/kgbw for female and male animals respectively (Loeda et al., 1977),

chicken and turkey: 400-500 mg/kgbw (Cooper and Skulski, 1956).
Differences in LD_{50}-values might be explained by species differences
MAO inhibition.

Many adverse effects have been described in the literature caused by
furazolidone upon exposure during several days or weeks. In Table 4 a
short review is given of some adverse effects after administration of
furazolidone to animals.

2.2.3 Toxicity in man

As already mentioned, furazolidone is a monoamine oxidase inhibitor in
humans (Pettinger et al., 1968).

The most common effect of the drug in man appears to be gastro-
intestinal distress, characterized by nausea and/or vomiting.
Dizziness, drowsiness, ness, headaches and general malaise have also
been reported (Phillips and Hailey, 1986). Incidentally, allergic
contact eczema has been reported (Scharfenberg, 1967) as well as an
increased activity of alkaline phosphatase in faeces (Frolkis et al.,
1975), disulfiram (Antabuse)-like reactions when treatment with
furazolidone is combined with consumption of beer, characterized by
flushing, a slight elevation of temperature and occasionally a
sensation of tightness in the chest (Phillips and Hailey, 1986;
Paul and Paul, 1964) or liver damage (Van Esch et al., 1983; IARC,
1983).

2.2.4 Mutagenicity

Many members of the class of the 5-nitrofurans exhibit mutagenic acti-
vity (Tazima et al., 1975; Klemencic and Wang, 1978; McCalla, 1983).
Using bacterial strains which were deficient in nitro-reductase acti-
vity, no mutagenic activity could be observed, indicating that reduc-
tion of the nitro-group is an essential step for mutagenicity

Table 4: Effect of furazolidone in animals

Species	Exposure conditions	Effect	Reference
Swine	3,600 mg/kg feed	neurotoxic symptoms	Borland, 1979
	800 mg/kg feed, 14 days	neurotoxic symptoms, disturbance lactobacillae flora	Decuypere en Van der Heyde, 1972
	400 mg/kg feed, 3 weeks	growth retardation, increased numbers of death	Jansen, 1983
Calve	4-14 mg/kgbw/day, orally, 30-84 days	hemorrhagic diathetis, fatty degeneration of liver, bone marrow lesions	Glawischnig and Baumgartner, 1973; Hayashi et al., 1976; Hofmann et al., 1974; Hoffmann-Fezer et al., 1974
	2 mg/kgbw/day, orally, 19-20 weeks	growth retardation, decrease of neutrophils and thrombocytes, diminished myelopoesis and thrombopoesis	Hofmann et al., 1977
	20-30 mg/kgbw/day, orally, several days	spasms	Hofmann et al., 1977
	50 mg/kgbw, single oral dose	neurotoxic symtoms, increased death rate	Postema, 1983
Chicken	80 mg/kgbw for 5 days or 800 mg/kg feed for 10 days	decreased total plasma protein and cholesterol, increased plasma potassium, histological changes in liver, kidney, heart, cerebellum and testes, decreased weight of the testes, wattles and combs, decreased testicular and plasma testosterone	Ali, 1984; Ali et al., 1984
	400 mg/kg feed for 10 days	decreased weight of the testes, increased weight of the adrenal and thyroid glands, growth retardation, anorexia	Ali and Bartlet, 1982; Ali, 1983; Ali et al., 1984
	400 mg/kg feed, 3-6 week	depressed egg weight, growth retardation	Ehlhardt et al., 1975

Species	Exposure conditions	Effect	Reference
Chicken	300 mg/kg feed, 7 days	growth retardation	Vahl en Stappers, 1983
	500-700 mg/kg feed, 4 weeks	growth retardation, increased death rate	Jensen et al., 1975
	100 mg/kg feed, 3 weeks	increased plasma corticosterone	Sidorov and Lüders, 1971
	double or triple injec- tions of 60-100 µg per egg	growth retardation, increased mortality rate	Czarnecki and Sujarit, 1979
	220 mg/kg feed, 15 days	blocked spermatogenesis, histological changes in testes	Hernández-Jáuregui et al., 1983
Duck	500-1250 mg/kg feed, 4 weeks	dose related increased frequency of cardiomyopathy, ascites and mortality	Van Vleet and Ferrans, 1983 a,b,c
Turkey	300 mg/kg feed, 35 days	increased mortality and cardiac dilatation	Jankus et al., 1972
	500-700 mg/kg feed, 2-5 weeks	growth retardation, round-heart disease, myocardial lesions	Czarnecki et al., 1974; Good and Czarnecki, 1980
	500 mg/kg feed, 4 weeks	growth retardation, increased daeth rate, increased relative heart weight	Jensen et al., 1975
	700 mg/kg feed, 14-19 days	elevated levels of plasma creatine phosphokinase, glutamic oxalacetic transaminase and lactate dehydro- genase, depression of plasma protein, increased deposition of glycogen in myocardium and liver	Czarnecki et al., 1975, 1981
Goat	40-320 mg/kgbw/day, orally, 10 days	anaemia, neurotoxic symptoms, histopathological changes in liver, kidney, brain and adrenals, mortality	Ali et al., 1984

Species	Exposure conditions	Effect	Reference
Goat	10 mg/kgbw/day, orally, 5 days	decreased heart rate, pulse rate, rectal temperature, pseudocholinesterase activity and plasma total protein, increased adrenal cholesterol concentration; reduced seminal ejaculate volume, number of motile spermatozoa and of live spermatozoa per ejaculate, reduced fructose concentration and alkaline phosphatase activity	Mustafa et al., 1986, 1987
	80 mg/kgbw/day, orally between day 10 and day 16 of oestrus cycle	delayed luteolysis	Hassan et al., 1986
Dog	8.84 mg/kgbw, every 6 hours	neurotoxic symptoms	Van Esch et al., 1983
Rat	5–32 x 400 mg/kgbw during 11–101 days, orally	polyneuropathy	Klinghardt, 1967
	600 mg/kg feed, 7 days	liver enlargement, decreased activity of liver NADPH-cytcreductase and aminopyrine-N-demethylase, increased activity of cytochrome b_5 and aniline hydroxylase	Takabatake and Fukuhara, 1978
	3 x 100 mg/kgbw, orally per 2 days over 34 days	increase of chronaxy, decrease of conduction velocity	Glatt et al., 1979
	250–1000 mg/kg feed, 18 months	thrombosis, haemorrhagiae, changes in leucocytes, testes atrophy, necrosis of liver	Van Esch et al., 1983
	33–50 mg/kgbw/day, 14 weeks	testes atrophy, sterility, growth retardation, degenerative changes in liver and kidney	Van Esch et al., 1983

bw = body weight

(Rosenkranz and Speck, 1976). Once reduced to stable endproducts, mutagenic activity is not likely (McCalla, 1983; Skeggs et al., 1984). The mutagenic potency of nitrofurans is greatly influenced by the nature of the substituent at the 2-position of the furan ring (Ichikawa et al., 1986).

Furazolidone showed mutagenic activity in many prokaryotic and eukaryotic testsystems: single base substitutions in Salmonella typhimurium TA 98, TA 100 (Ames-test) or Escherichia coli WP 2 without the addition of a liver homogenate (S_9-mix) (Lu et al., 1979; Ebringer and Bencová, 1980; Ohta et al., 1980; Probst et al., 1981), rec-assay (repair-test) in Bacillus subtilis H17 (Ohta et al., 1980), the sex-linked recessive lethal test in Drosophila melanogaster (Blijleven et al., 1977; Kramers, 1982), the gen-conversion test in Saccharomyces cerevisiae D4 (Voogd et al., 1982), the fluctuation test in Klebsiella pneumoniae (Voogd et al., 1982), Unscheduled DNA synthesis test in primary rat hepatocyte cultures (Probst et al., 1981), chromosome breakage and sister-chromatid exchanges (SCE) in human peripheral lymphocytes (Cohen and Sagi, 1979), chromosomal anomalies in swine lymphocytes (Quéinnec et al., 1975; Babilé et al., 1978) and the hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) test in mice L 5178 Y lymphoma cells (Voogd et al., 1982).

Furazolidone stimulates E.coli to synthesize the rec A[+] gene product (protein X), an enzyme which is characteristic of error-prone repair functions of damaged DNA (Bryant and McCalla, 1980). Pal and Chatterjee (1985) reported the induction of λ prophage in E.coli by furazolidone, which is regulated upon induction and activation of rec A[+] protein (Klemencic and Wang, 1978). Ames-test results revealed genotoxic activity in urine and faeces of furazolidone treated rats (Crebelli

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et al., 1982; Willems and De Raat, 1985). Methanol extracts of rat
urine and faeces induced an increase in the number of SCE in Chinese
hamster ovary cells. In the case of methanol extracts of the faeces
a positive effect could only be observed in the presence of a metabolic
activation system (Willems and De Raat, 1985). Furazolidone was
negative in the Unscheduled DNA synthesis test in human fibroblasts
under aerobic conditions (Tonomura and Sasaki, 1973).

2.2.5 Carcinogenicity

Several 5-nitrofurans have been tested in long-term carcinogenicity
studies and proved to be positive (Morris et al., 1969; Cohen et al.,
1973, 1977; Cohen, 1978; Wang et al., 1984).

Furazolidone has been evaluated extensively by the Food and Drug
Administration of the U.S. Department of Health, Education and Welfare
for carcinogenic activity because of its widespread use in veterinary
medicine (Anonymous, 1976). In Table 5 the main results are summarized.
It was concluded that furazolidone induces cancer in rats and mice and
that "furazolidone must be assumed as a potential human carcinogen and
safeguards taken to assure no human exposure". To assure this no resi-
dues which may pose a risk of cancer may remain in edible animal
tissue at the time of slaughter. Since it is not known whether the
parent drug is carcinogenic and/or one or more metabolites, it was
stated that "all drug-related residues to which humans may be exposed
must be assumed to be potential carcinogens unless convincing evidence
is presented to the contrary". From the Mantel-Bryan extrapolation
model the upper bound on the total residue concentration in meat of no
carcinogenic concern was calculated based on a probability of the
occurrence of cancer of 1 in 1 million with a slope of 1 probit unit
per log increase in dose at the 99% confidence level: approximately

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Table 5: Results from carcinogenicity testing of furazolidone (Anonymous, 1976)

Species	Exposure conditions	Effects
Sprague-Dawley rats; 50 male and 50 female rats per group	250-500-1000 mg/kg feed, 18 months	Sex-linked differences in the development and types of tumors: a linear dose-relationship in the number of female rats with malignant mammary tumors (significant at the 0.1% dose level); a significant increase at the 0.025% and 0.05% dose levels in benign mammary tumors; a significant linear dose response in nonmammary tumor development in male rats (significant at the 0.1% dose level); these nonmammary tumors included squamous cell carcinoma, dermal fibroma, pituitary neoplasms and lymphoreticular neoplasms, with no one type or site singularly significant. A drug related effect on mortality: significant increase at the 0.05% and 0.1% dose in males and at the 0.1% dose in females.
Fisher rats; 50 male and 50 female rats per group	250-500-1000 mg/kg feed, 20 months	Sex-linked differences in the development and types of tumors: only in female rats a significant relationship between the dose and the proportion of rats with mammary tumors; at the 0.1% dose level a significant increase of the proportion of female rats with malignant mammary tumors. Thyroid adenomas were significantly increased in both males and females at the 0.05% and 0.1% dose levels. Sebaceous adenomas were significantly increased in males at the 0.05% dose level and in both males and females at the 0.1% dose level. Testicular mesotheliomas and basal cell epitheliomas were significantly increased in males at the 0.1% dose level. A significant effect on mortality in females at the 0.1% dose level and in males at the 0.05% and 0.1% dose level.
Sprague-Dawley rats; 40 male and 40 female rats per group	1-5-15 mg/kgbw/day, orally, 2 years (25-125-375 mg/kg feed)	No drug-effect on tumor development in the male rats. A significant linear dose-respons relationship for the number of female rats with tumors (significant at the highest dose level). The majority of tumors observed were in the mammary gland. Also pituitary adenomas and lymphoreticular neoplasms were diagnosed. A significant increase of the incidence of multiple mammary tumors in female rats for both the 0.0125% and 0.0375% dose groups. Early onset time of mammary tumor development in the female rats. No significant differences on the analysis of malignant tumors alone. A significant dose-response effect on mortality in female rats (significant at the 0.0125% and 0.0375% dose levels for a 24 month period).
Swiss MBR/ICR mice; 50 male and 50 female mice per group	75-150-300 mg/kg feed, 18 months	No sex-differences in the effect on tumor development in male and female mice. A dose related induction of malignant tumors in both male and female mice: malignant lung tumors (bronchial adenocarcinomas) were the primary effect (significantly increased at the 0.015% and 0.03% dose level for male mice and at the 0.03% dose level for female mice); early mortality in both sexes (the males at the 0.03% dose level and the females at the 0.015% and 0.03% dose levels differed significantly from the controls).

0.9 ng/g (Anonymous, 1984). Because of mutagenic and carcinogenic pro-
perties furazolidone is considered as a "category 1" carcinogen in the
Netherlands, i.e. in principle a non-carcinogenic dose cannot be
established and the compound should be absent in human food
(Anonymous, 1979).

The International Agency for Research on Cancer (IARC) could not eva-
luate the carcinogenic potential of furazolidone since this agency
does not consider classified data (Anonymous, 1983).

2.3 Residues and elimination kinetics of furazolidone

In general it can be concluded that upon administration of furazoli-
done to swine, chicken, turkey, rat, calve and trout the elimination
of the parent compound from plasma, muscle, kidney and liver is relati-
vely fast.

2.3.1 Swine

Orally administered furazolidone is absorbed from the digestive tract
by swine to a large extent and excreted in the form of a great number
of metabolic endproducts. Fourty-eight hours after a single oral dose
of 39 µCi $[^{14}C]$-furazolidone 70.5%, 3.2% and 19.1% of total dosed
radioactivity has be demonstrated in urine, expired air and faeces
respectively. In a similar study in rats these amounts were 81.5%, 3%
and 11% respectively of the total radioactive dose administered.
Radioactivity has been demonstrated in swine kidney, liver, thyroid,
bile, blood, muscle and fat (Tennent and Ray, 1971). No furazolidone
(< 0.2 mg/kg) has been detected in liver, kidney, muscle, lung and
heart 0.5-2 hours after dosing piglets (30-40 kg bw) or pigs (60-70
kg bw) with a high concentration of furazolidone in the feed (1000
mg/kg). In the piglets a plasma half-life of 0.45 hours was calculated
(Yamamoto et al., 1978).

Tissue samples taken from swine fed 300 mg furazolidone per kg feed for 2 weeks or 150 mg per kg feed for 5 weeks did not contain detectable furazolidone residues (< 0.5 µg/kg) 4 hours after feed withdrawal (Winterlin et al., 1984).

Addition of 0.02% Bifuran (a mixture of equal quantities of nitrofurazone and furazolidone) to the feed of swine for several weeks did not result in residues in tissues and blood (< 0.25 mg/kg) (Tapernoux et al., 1964).

Low levels of furazolidone (2-5 ng/g of tissue) have been detected in tissues of swine fed 150 mg and 300 mg per kg feed for 35 and 21 days respectively, 0 day withdrawal. In tissues taken after a 4 days withdrawal period, no residues have been detected (Heotis, 1974).

2.3.2 Other animals

Tissues of chickens and turkeys fed 200 mg per kg feed for approximately 7 weeks and 400 mg per kg feed for 14 days, respectively, contained no detectable residues of furazolidone (< 0.5 µg/kg) in any of the tissues except the skin (Winterlin et al., 1982, 1984). One day after starting treatment of laying hens with 400 mg furazolidone per kg feed for 14 days, residues have been detected in eggs. Maximum average values reached 0.7 mg/kg. Residues have been detected until five days after withdrawal of the drug (> 0.01 mg/kg) (Petz, 1984). In White Leghorn birds furazolidone residues have been measured in tissues in the order of 3-13 mg/kg of tissue following oral doses of 10 mg/kgbw/day for 1-10 days. The reported plasma half-life was 42 hours (Yadava et al., 1986).

Only traces of furazolidone have been detected in the urine of chickens medicated with a single oral dose of 30 mg/kgbw (Craine and Ray, 1972).

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Relatively low urinary levels of furazolidone have also been reported for goats (Pandey et al., 1978) and rats (Crebelli et al., 1982). Twenty-four hours after dosing calves with 10 mg furazolidone/kgbw for 3 days, no residues have been detected in plasma (< 1 mg/kg) (Pietsch et al., 1978).

Furazolidone has been detected in plasma and milk up to 24 hours and 12 hours respectively, after a single oral dose of 10 g/kgbw to goats (Pandey et al., 1980; Mustafa et al., 1985). Prolongation of the medication for 5 days, resulted in detectable residues in muscle and liver up to 7 days withdrawal (Mustafa et al., 1985). In buffalo, a plasma half-life of 2.52 hours has been reported after a single oral dose of 30 mg/kgbw (Mir and Uppal, 1984). During a 6-days treatment of trouts with medicated feed (3% furazolidone in the feed; amount of feed: 1% of bodyweight/day), 50 ng furazolidone could be measured per g of muscle. After 2.5 day withdrawal, no residues were detected (< 0.5 μg/kg) (Schmidt and Büning-Pfaue, 1985).

2.4 Biotransformation

2.4.1 Nitrofurans in general

In general, it is believed that reduction of the nitro-group is the most important metabolic pathway of nitrofurans (Swaminathan and Lower, 1978). The initial step involves the transfer of a single electron to the nitro-group, resulting in a nitro radical anion. Further reaction may lead to the nitroso-derivative and, under aerobic conditions, to the formation of the superoxide radical anion $O_2^{\bullet}$ which in the presence of superoxide dismutase may form hydrogen peroxide. In E. coli the presence of two different types of reductase have been postulated: an oxygen sensitive nitroreductase, which

transfers 1 electron to the nitro-group, and an oxygen-insensitive
nitroreductase which does not appear to transfer 1 electron to nitro-
furans, but which may catalyse a 2-electron transfer to nitrofurans,
resulting in the nitroso-derivative (Peterson et al., 1979).
This derivative is believed to be further reduced via the hydroxyl-
amino-derivative to the open-chain cyano-derivative (Fig. 2). The
highly reactive intermediate nitroso- and hydroxylamino-derivatives
are believed to be responsible for interaction with biological macro-
mulecules as DNA and protein (Swaminathan and Lower, 1978). For
several nitrofurans the open-chain cyano-derivative has been iden-
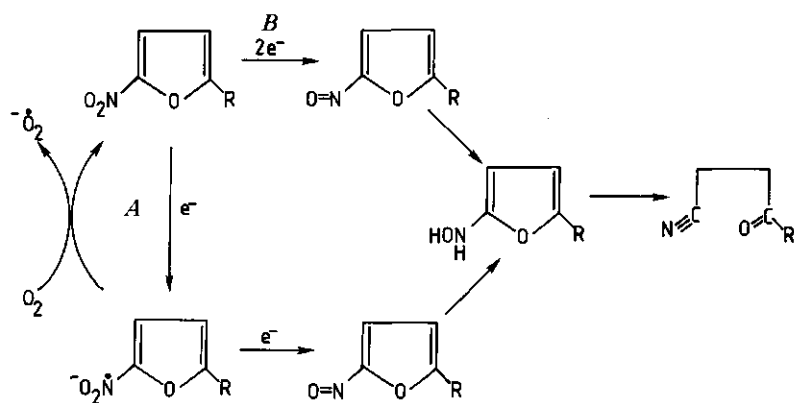tified (Swaminathan and Lower, 1978).

Figure 2: Proposed mechanisms of 5-nitrofuran reduction

      (Peterson et al., 1979)

      A: oxygen-sensitive

      B: oxygen-insensitive

Several enzymes can transfer electrons to the nitro-group: microsomal
NADPH cytochrome P-450-reductase, mitochondrial and microsomal
NADH-cytochrome-b$_5$-reductase, cytosolic xanthine oxidase, aldehyde
oxidase, DT-Diaphorase (NADPH-dehydrogenase) and Lipoyldehydrogenase
(Diaphorase II) (Swaminathan and Lower, 1978; Abraham, 1981; Hunder,
1985).

Although nitro-reduction is believed to be the main biotransformation
route, other routes cannot be excluded such as hydroxylation of the
furan ring as is shown in the case of nitrofurantoin (Jonen, 1980;
Jonen et al., 1980) and epoxidation of the furan ring, as is postulated
for methylfurans (Ravindranath et al., 1984). Biotransformation of the
side-chains of the 5-nitrofurans may also be possible (Swaminathan and
Lower, 1978).

2.4.2 Furazolidone

In the case of furazolidone, several metabolites have been reported to
be formed as a result of reductive metabolism _in vitro_ and _in vivo_.
The identification of the cyano-derivative has been reported in urine
of rats (Tatsumi and Takahashi, 1982; Tatsumi et al., 1984) and rabbits
(Tatsumi et al., 1978), as an excretion product in eels (Nakabeppu
and Tatsumi, 1984), in Escherichia coli (Abraham et al., 1984) and rat
liver fractions (Tatsumi et al., 1981, Abraham et al., 1984) and after
investigations using milk xanthine oxidase (Tatsumi et al., 1978, 1981).
Other identified metabolites from reductive metabolism are the
5-amino-derivative in a milk xanthine oxidase system (Tatsumi et al.,
1981), the monoacetyl derivative of the aminofuran, the open-chain
carboxylic acid derivative and α-ketoglutaric acid in the urine of
rats (Tatsumi et al., 1984) and 2,3-dihydro-3-cyanomethyl-2-hydroxyl-
5-nitro-1α,2-di (2-oxo-oxazolidin-3-yl)iminomethylfuro[2,3-b]furan

after in vitro metabolism (Tatsumi et al., 1981).

Although several metabolites have been identified, approximately 75% of urinary metabolites are not identified yet (Tatsumi et al., 1984). In urine of swine 2 peaks could be observed after ion exchange fractionation with absorption maxima at 415 nm (Tennent and Ray, 1971). This may be the result of 4-hydroxylation of the 5-nitrofuran ring, as the corresponding derivative of nitrofurantoin gave an absorption maximum at the same wavelength (Jonen et al., 1980). However, its structure has not yet been elucidated.

Under acid conditions hydrolysis of the azomethine linkage may occur, resulting in the formation of 3-amino-2-oxazolidone (Paul et al., 1960). Schmid (1985) suggested that this hydrolysis product is further metabolised into carbondioxide, ethene and hydroxyhydrazin.

3. Objective of the investigation

Considering the data available on furazolidone it can be concluded that essential information on its kinetics and biotransformation in swine is lacking. For instance the results of the studies made by Tennent and Ray (1971) suggest that residues may remain present in edible tissues, which could be attributed to possible metabolite(s). However, the identity of the metabolite(s) is yet unknown. Reduction of the nitro-group of furazolidone, which seems to be an important biotransformation route, may lead to the formation of unstable intermediate metabolites which subsequently may react with proteins or DNA. Covalent binding of drugs to tissue macromolecules may result in residues which are persistently present in edible tissues. Considering the carcinogenic properties of furazolidone and its widespread use in mass-medication of food producing animals, it is of

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the importance from the food consumer point to identify the reactive properties of possible metabolites present in the edible parts of animals treated with this drug.

The studies presented in this thesis were undertaken to obtain a better understanding of the biotransformation and kinetic behaviour of furazolidone in swine. For this purpose an analytical method has been developed for the determination of furazolidone in swine plasma, fat, muscle, kidney, liver and urine.

Subsequently, experiments were conducted using two different approaches:

- kinetic studies in the target animal to determine the elimination kinetics of furazolidone and its metabolites from plasma and tissues upon oral administration of furazolidone
- model systems using rat and swine liver microsomes to elucidate biotransformation routes and reactive intermediates responsible for the interaction with biological macromolecules.

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PART II EXPERIMENTS

CHAPTER 2: Determination of furazolidone in swine plasma, muscle,
liver, kidney, fat and urine based on high-performance
liquid chromatographic separation after solid-phase extrac-
tion on Extrelut^{R}1

Note

Determination of furazolidone in swine plasma, muscle, liver, kidney, fat and urine based on high-performance liquid chromatographic separation after solid-phase extraction on Extrelut® 1

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Furazolidone [N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone], which is a nitrofuran, has been described as a potent antimicrobial agent for therapeutic and prophylactic purposes in pig, poultry and cattle[1]. Since furazolidone exhibits mutagenic and carcinogenic properties[2-4], the amount of furazolidone residue in edible products of food-producing animals treated with furazolidone should be minimal. This requires sensitive analytical methods for the detection of furazolidone in liver, muscle, fat and kidney. In addition, methods for the detection of furazolidone in plasma or urine may be needed for screening purposes and pharmacokinetic studies. Many different methods have been described for the detection of furazolidone in tissues, milk, eggs, plasma and urine[5-16].

Up till now, no suitable sensitive method has been described for the detection of furazolidone in plasma, muscle, liver, kidney, fat and urine. In this study, a relatively fast and sensitive high-performance liquid chromatographic (HPLC) method is described for the determination of furazolidone in plasma, muscle, liver, kidney, fat and urine of swine using a simple clean-up procedure based on solid-phase extraction on a commercially available disposable column. Only 1 g of sample is required.

EXPERIMENTAL

Chemicals and solvents

Water was purified over a Millipore Milli-Q water purification system. Ethyl acetate (LiChrosolv 868), acetonitrile (LiChrosolv 30), n-hexane (4391), acetic acid (62) and sodium acetate (6268) were purchased from Merck (Darmstadt, F.R.G.). Furazolidone was a gift from Orphahell (Mijdrecht, The Netherlands).

Acetate buffer solution (1.0 M). The solution contained 41 g of sodium acetate and 30 g of acetic acid in 1 l of water.

Stock solutions. Stock solutions of furazolidone in acetonitrile were prepared. The final concentration range was 1–1000 ng/ml eluent.

Sample preparation

Plasma, urine. A 1-ml aliquot of plasma or urine was applied directly to a Merck Extrelut® 1 column (Merck 15371). After an equilibration period of 10 min, the drug was eluted with 6 ml of ethyl acetate. The eluate was collected in tubes and evaporated to dryness under nitrogen. The residue was dissolved in 300 μl of eluent.

Muscle, liver, kidney, fat. A 1-g quantity of frozen sample (liquid nitrogen) was pulverized and 2 × 6 ml of ethyl acetate were added and mixed for 1 min. After centrifugation for 5 min at 3000 g, the water phase was frozen (alcohol–dry ice). The ethyl acetate was collected in tubes and evaporated to dryness under nitrogen at room temperature. To remove fat from samples of liver, kidney and fat, the residue was dissolved in 5 ml of acetonitrile and 2 × 2 ml of hexane were added and mixed for 1 min. After centrifugation for 5 min at 3000 g, the hexane was removed and the acetonitrile was evaporated to dryness under nitrogen. The residues of samples of muscle, liver, kidney and fat were dissolved in 1 ml of a methanol–water mixture (25:75) and these solutions were applied to a Merck Extrelut 1 column. After an equilibration period of 10 min, the drug was eluted and prepared for HPLC as described for plasma and urine. Since furazolidone decomposes under the influence of daylight[17], brown glassware has been used and the experiments were performed under artificial yellow light (Pope FT 40W/16).

High-performance liquid chromatography

Liquid delivery system: Waters 6000A; automatic injection system: Waters 710B; detector: Kratos Spectroflow 773; absorbance recorded at 362 nm; recorder: Kipp BD 41.

Analytical column: Shandon Hypersil ODS 5 (250 × 4.6 mm I.D.) (Chrompack); precolumn: Brownlee Labs. OD-GU RP-18 (30 × 4.6 mm I.D.).

Eluent: water–acetate buffer–acetonitrile (675:75:250); flow-rate: 1 ml/min; run time: 15 min (urine: 20 min); injection volume: 50–100 μl.

RESULTS AND DISCUSSION

Because of the effective clean-up procedure, isocratic HPLC could be used. In blank samples of swine, no interfering peaks could be detected at 362 nm. Concentrations of furazolidone were calculated by comparing the peak heights from spiked samples with the peak heights from corresponding standard solutions, containing the same amounts of furazolidone. As shown in Fig. 1, linearity exists between the concentration of furazolidone and peak height in the range of 1–1000 ng/ml standard solution. Such a linearity was also found for spiked samples.

Recovery data for swine samples spiked with furazolidone in a concentration range of 1–1000 ng/ml (or ng/g) of sample are shown in Table I. Each result represents the average of six determinations. The mean overall recovery for plasma ($n = 30$) is 96.4 ± 3.3, muscle ($n = 30$): 98.8 ± 4.4, liver ($n = 30$): 86.7 ± 6.4, kidney ($n = 30$): 89.8 ± 4.9, fat ($n = 30$): 81.8 ± 5.1 and urine ($n = 24$): 91.2 ± 2.3.

The sensitivity of the method, expressed as three times the background of a blank sample, was 1 ng/ml for plasma, 2 ng/g for muscle, liver, kidney, fat and 25 ng/ml for urine.

In Fig. 2, the chromatograms of a blank and a spiked liver sample (5 ng/g) and a blank and spiked urine sample (25 ng/ml) are shown.
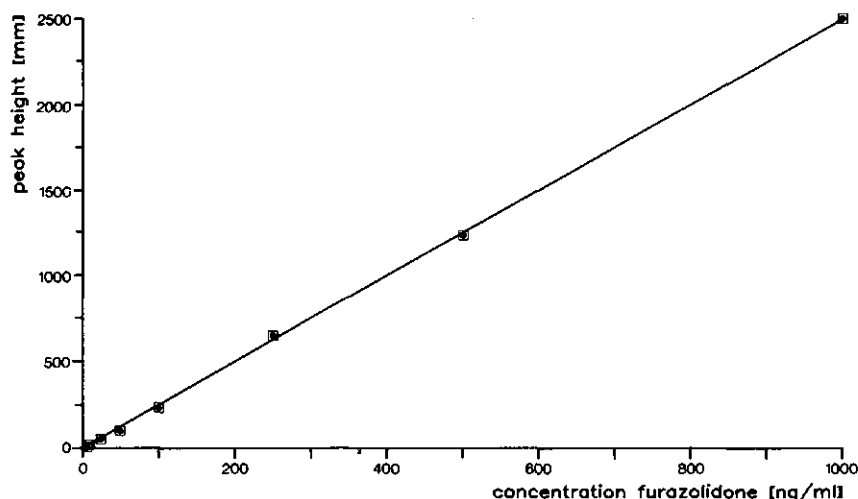
Fig. 1. Concentration of furazolidone in standard solution (ng/ml) as a function of peak height (mm) measured in the chromatogram.

The method proved its usefulness in a study in which a pig of approximately 90 kg body weight was treated with furazolidone by feed medication at a concentration of 300 mg/kg. Total daily feed intake was calculated on the basis of 4% of total body weight, dosing twice daily at 9.00 h and 16.00 h. Blood samples were collected via a syringe in the vena jugularis into heparinized tubes (Sarstedt 02.1065). Plasma was separated by centrifugation and immediately analysed, since a time-dependent decrease of furazolidone has been reported[16]. As an example, the chromatograms of furazolidone in plasma, 1 and 7 h after the last dosage at 9.00 h on day 10 of treatment, are shown in Fig. 3. Furazolidone concentrations were 104 and 6 ng/ml, respectively.

It can be concluded that the method described is suitable for the detection of furazolidone at the ng/ml or ng/g level in plasma, muscle, liver, kidney, fat and urine. The method is simple and requires only small amounts of sample and reagents. The

TABLE I

PERCENTAGE FURAZOLIDONE RECOVERED FROM SPIKED SAMPLES OF SWINE IN A FINAL RANGE OF 0–1000 ng/ml OR ng/g OF SAMPLE ($n = 6$; MEAN ± S.D.)

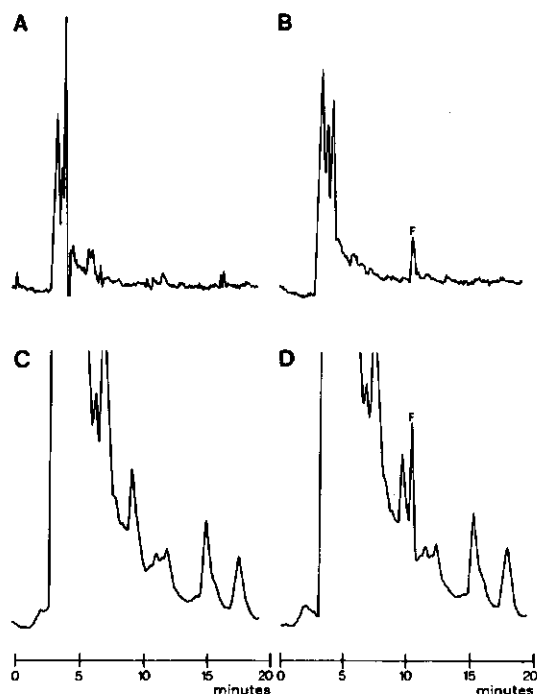| Sample	Concentration range of samples spiked with furazolidone					
	1 ppm	100 ppb	50 ppb	25 ppb	10 ppb	5 ppb
Plasma	92.3 ± 2.3	95.3 ± 0.6	101.0 ± 2.4	—	96.2 ± 1.5	97.4 ± 1.2
Muscle	101.6 ± 5.3	98.9 ± 4.0	95.4 ± 3.6	—	99.4 ± 3.3	99.2 ± 4.4
Liver	91.2 ± 2.5	82.7 ± 2.7	88.4 ± 4.7	—	82.2 ± 5.1	89.2 ± 9.8
Kidney	83.5 ± 3.0	86.5 ± 1.2	91.8 ± 2.3	—	95.1 ± 1.8	91.9 ± 4.0
Fat	84.2 ± 4.4	84.0 ± 3.5	76.1 ± 2.7	—	78.6 ± 4.7	86.3 ± 1.2
Urine	86.8 ± 2.1	93.4 ± 0.6	90.8 ± 2.3	91.8 ± 0.9	—	—

Fig. 2. High-performance liquid chromatograms of blank and spiked samples of swine liver (A: blank, B: 5 ng/g furazolidone) and swine urine (C: blank, D: 25 ng/ml furazolidone) (F = furazolidone). (A, B) λ = 362 nm; speed: 0.5 cm/min; sensitivity of detector: 0.002 a.u.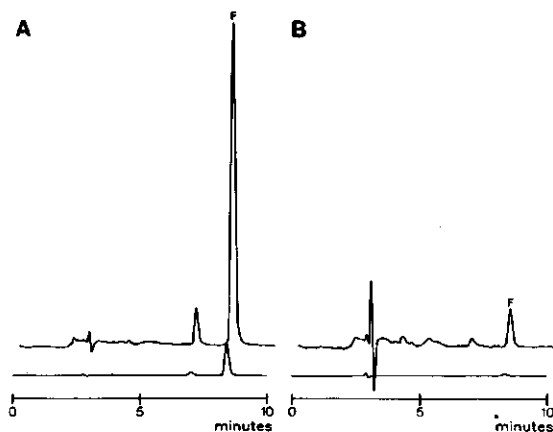f.s.; sensitivity of recorder: 10 mV; injection volume: 50 μl. (C, D) λ = 362 nm, speed: 0.5 cm/min; sensitivity of detector: 0.005 a.u.f.s.; sensitivity of recorder: 10 mV; injection volume: 50 μl.

Fig. 3. High-performance liquid chromatograms of furazolidone in plasma of a pig, 1 h (A) and 7 h (B) after the last administration of furazolidone on day 10 of treatment (F = furazolidone). (A) λ = 362 nm; speed: 1 cm/min; sensitivity of detector, underline: 0.1 a.u.f.s., upperline: 0.01 a.u.f.s.; sensitivity of recorder: 10 mV; injection volume: 50 μl. (B) λ = 362 nm, speed: 1 cm/min; sensitivity of detector, underline: 0.1 a.u.f.s., upperline: 0.005 a.u.f.s.; sensitivity of recorder: 10 mV; injection volume: 50 μl.

total time that elapsed between sample collection and final determination of the amount of furazolidone varied between 30 and 60 min. The method may be used for screening purposes in plasma or urine of food-producing animals treated with furazolidone, and in pharmacokinetic studies.

ACKNOWLEDGEMENTS

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CHAPTER 3: Kinetics of ^{14}C-furazolidone in piglets upon oral administration during 10 days and its interaction with tissue macromolecules

Kinetics of ^{14}C-furazolidone in piglets upon oral administration during 10 days and its interaction with tissue macro-molecules

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After dosing piglets orally with ^{14}C-furazolidone (75 mg/animal/day) for 10 days, no accumulation of the drug could be detected in the blood. The half life of furazolidone in blood after the last oral administration on day 10 was 60 minutes. In blood, radioactivity reached a plateau of approximately 10 ppm equivalents of parent compound at day 6 of the medication. After a relatively fast decrease of radioactivity during the first day of the withdrawal period, a much slower decrease could be observed in the blood with a half life of approximately 6 days. 1 day after stopping the medication almost all the radioactivity in plasma appeared to be associated with plasma proteins. Furazolidone could not be detected in muscle, fat, kidney and liver of piglets two hours after the last administration. However, the level of radioactivity was $5.7-7.2\mu$g equivalents per gram in muscle, $6.0-9.2\mu$g equivalents per gram in fat, 30.1μg equivalents per gram in kidney and 32.9μg equivalents per gram in liver. After a withdrawal period of 14 days $0.9-4.3\mu$g equivalents per gram could be detected in the analysed tissues. It also appeared that respectively 14.0%, 9.3% and 35.0% of the residual radioactivity could not be extracted from liver, kidney and muscle of piglets sacrificed 2 hours after the last administration of ^{14}C-furazolidone and 27.0%, 31.2% and 55.6% of the residual radioactivity from liver, kidney and muscle respectively of piglets sacrificed after a withdrawal period of 14 days. DNA-binding of ^{14}C-furazolidone in tissues of piglets varied from $87-382$ pmol equivalents of parent compound per mg DNA. 61% of total dosed radioactivity had been excreted via the urine and 18.1% via the faeces. No radioactivity could be measured in the expired air.

Introduction

Furazolidone [N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone] is a veterinary drug widely used as an antimicrobial agent for therapeutic and preventive purposes in pigs, poultry and cattle (Brander and Pugh 1971; Rossoff 1974). Because of the mutagenic (McCalla 1983) and supposed carcinogenic activities (Food and Drug Administration 1976) residues should be avoided in edible tissues of animals treated with furazolidone. The depletion of furazolidone from muscle, kidney and liver tissue upon oral administration to chickens and pigs is relatively fast: immediately after the last administration, residual concentrations of furazolidone are below the limit of detection (< 0.5 ppb) (Winterlin *et al.* 1984). However, information concerning the metabolism of furazolidone in food producing animals is scarcely available. In general, it is believed that reduction of the nitro group is the main metabolic pathway of nitrofurans (Swaminathan and Lower 1978). Several metabolites of furazolidone upon *in vitro* and *in vivo* reduction have been identified (Tatsumi *et al.* 1978, 1981, 1984;

Abraham *et al.* 1984; Nakabeppu and Tatsumi, 1984; Vroomen *et al.* in press). Information concerning the formation and depletion of metabolites of furazolidone and the kinetics of these processes upon oral administration of furazolidone to food producing animals during several days is lacking.

The results obtained in this study provide information on the metabolism of furazolidone and on the pharmacokinetic behaviour in blood and tissues of piglets upon oral administration of ^{14}C-furazolidone during 10 days at a dose level which is commonly used in veterinary practice (300 mg per kg feed). Furthermore the interaction of furazolidone with plasma protein and DNA and the amount of non-extractable residues of furazolidone or metabolites in edible tissues are reported.

Materials and methods

^{14}C-furazolidone

^{14}C-furazolidone was labelled in the methylene groups of the oxazolidone moiety (figure 1). The specific activity was 2·02 μCi/mg; the radiochemical purity was > 99% by TLC (toluene : acetic acid : acetone : water = 600 : 240 : 80 : 25) and HPLC.
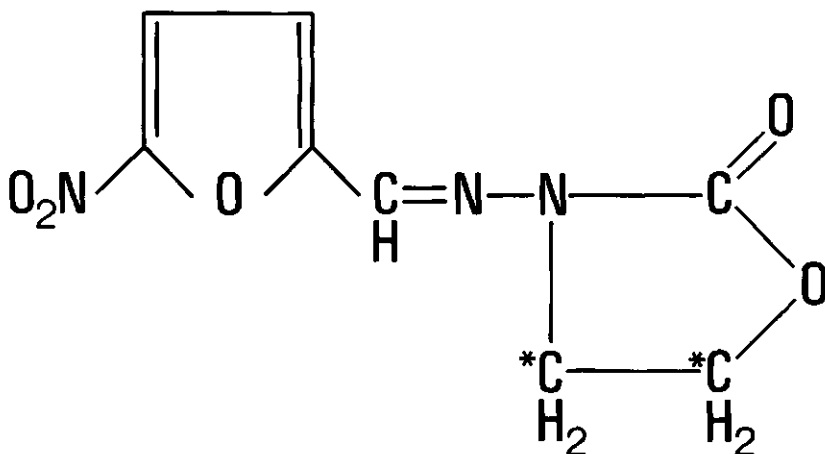

Figure 1. Chemical structure of furazolidone. ^{14}C-label in the methylene groups (*) of the oxazolidone moiety.

Animal handling and sample collection

The experiment was divided into three successive phases:

(1) an initial period of 2 weeks for acclimatization and surgical purposes;
(2) a medication period of 10 days followed by
(3) a withdrawal period of 0 and 14 days.

At the beginning six three-weeks-old male piglets (Dutch Landrace × Great Yorkshire) were placed in the radionuclide unit at IGMB/TNO-ILOB, Wageningen, The Netherlands. The animals were individually housed. After an acclimatization period of 1 week, catheters were implanted in the left vena jugularis which functioned satisfactorily during the whole experiment. After 4–5 days, when the animals were recovered, permanent stomach tubes for the administration of furazolidone preparations

were installed which were tolerated well by the animals. At the same time plastic bags were attached for the separate collection of faeces.

At the start of the medication period the animals (mean weight $\pm$ S.D.: 5900 ± 472 gram) were divided into 3 groups of 2 animals:

Group I: control animals 102 and 103.
Group II: ^{14} C-furazolidone treated animals 101 and 106.
Group III: unlabelled furazolidone treated animals 104 and 107.

A standard piglet diet without antibiotics was provided twice daily at 9·00 h and 16·00 h. The daily amount of feed to be administered was calculated as 4% of the body weight with a maximum of 250 gram/animal/day during the period of furazolidone administration. Feed restriction was applied in order to ensure minimal variations in the individual weights at the end of the medication period.

Furazolidone was dosed twice daily during feeding time via the stomach tube. It was given as a 1% suspension in a 5% gelatine gel. The total daily amount was 75 mg furazolidone/animal, which corresponded to approximately 12 mg per kg body weight. The animals had free access to tap water. The waste water was collected, the amount registered and samples of it were taken for radioactivity measurement.

Faeces and urine were collected separately. Twice daily, just before feeding time, total amounts of excreta were registered; immediately after collection, urine and faeces were homogenized and samples were taken for radioactivity measurement.

Each day the cages of the piglets of group II were cleaned by spraying water : methanol (50 : 50 v/v). The wash liquid was collected, the total volume registered and samples were taken for radioactivity measurement. In order to prevent contamination of the wash liquid of the cages with solid particles (feed, hair, scales of the skin, etc.) the wash liquid was filtered through a fibre glass filter during collection. This procedure ensured that the wash liquid contained only dried urine residues of the cages; at the end of the experiment approximately 5% of the total dose of radioactivity was present in this fraction. Two hours after each morning feeding period samples of blood were collected via the permanent catheter into heparinized tubes (Sarstedt 02·1065). Samples were immediately analysed for the concentration of furazolidone in blood and plasma since a time dependent decrease of furazolidone in blood has been reported (Veale and Harrington 1982). Plasma was separated by centrifugation. The protein fraction of the plasma was separated by ultrafiltration (Amicon Centrifree Micropartition System). Radioactivity in the protein fraction was calculated from the difference in radioactivity of the whole and protein free plasma.

During the medication period the expired air was qualitatively analysed for radioactivity.

After the last administration of furazolidone on day 10, samples of blood and plasma were collected at regular time intervals from the treated animals 107 and 106.

At· day 10 of the medication period, 2 hours after the last administration, one animal of each group (numbers 102, 106 and 104) was sacrificed by exsanguination via the permanent catheter in the vena jugularis; the other animals (numbers 103, 101 and 107) were sacrificed after a withdrawal period of 14 days. Samples were taken of the fat of the abdominal cavity, subcutaneous fat, liver lobus centralis dexter, liver lobus centralis sinister, kidneys, musculi longissimi dorsi, musculi psoas major, musculi biceps, musculi triceps and musculi quadriceps for analysis of the concentration of furazolidone and the level of radioactivity.

Samples were taken of the skin, bladder content, spleen, stomach, stomach content, small intestine, small intestine content, large intestine, large intestine content, gall bladder, bile, pancreas, lungs, heart, testicles, tongue and brains for radioactivity measurements only. All samples for furazolidone analysis were immediately frozen in liquid nitrogen and stored at $-40°C$. Samples for radioactivity measurements were stored at $-20°C$.

Analysis of furazolidone

Analytical procedures were performed under artificial yellow light (Pope FT 40 W/16), because of the instability of furazolidone in daylight (Kalim 1985).

Frozen tissue (1 g) was pulverized and extracted with ethyl acetate. After evaporation of ethyl acetate the residue was dissolved in water:methanol (75:25 v/v) and subsequently treated in a clean-up procedure by partitioning on a Merck Extrelut-1^R column. Blood (1 ml) and plasma (1 ml) were directly processed in this clean-up procedure. The concentration of furazolidone in the eluates was analysed by HPLC with UV-detection at 362 nm. The limit of detection was 2 ppb in tissue and 1 ppb in blood and plasma (Vroomen *et al.* 1986).

HPLC

Pump: Waters Associates 6000 A; automatic injection system: Waters 710 B; UV-detector: Kratos Spectroflow 773; recorder: Kipp BD 41. Column: Chrompack Hypersil 5 ODS (250 × 4·6 mm); precolumn: Brownlee Labs OD-GU RP18 (30 × 4·6 mm). Eluent: water-0·1 M acetate buffer pH 5·0-acetonitrile (675:75:250). Flow rate: 1 ml/min. Absorbance was recorded at 362 nm. Run time: 15 min.

Preparation of samples for radioactivity measurements

Tissue. Samples of 0·5–1·5 g were solubilized in a mixture of 5 ml Soluene-350^R (Packard) and 1 ml isopropanol at 60°C for 8–12 hours until a clear solution was obtained. After addition of an anti-foam agent, the solution was bleached by adding 0·5 ml 30% H_2O_2 and successively incubated for 15 minutes at room temperature and 45 minutes at 60°C. Then the samples were mixed with 15 ml Dimilume-30^R (Packard).

Blood and Plasma. 0·2 ml blood or 0·5 ml plasma were solubilized in a mixture of 1 ml Soluene-350^R and 1 ml isopropanol at 60°C for 1 hour. Bleaching procedures were the same as described for tissue.

Faeces. Samples of 0·1 g were solubilized in a mixture of 2 ml Soluene-350^R and 1 ml isopropanol at 60°C for 24 hours. After the standard bleaching procedure an additional bleaching with 0·2 ml 30% H_2O_2 at room temperature was necessary. Subsequently the samples were mixed with 19 ml Dimilume-30^R.

Urine, waste water and wash liquid. Samples of 1 ml were mixed with 15 ml Dimilume-30^R.

Radioactivity measurements. The radioactivity in all samples was measured in a Philips liquid scintillation counter (type PW 4700). Counting efficiency was calculated by means of an external standard-counting procedure. The amount of radioactivity is expressed as μg equivalents of parent compound per g of tissue. For all calculations it is assumed that no change in specific activity has taken place.

Non-extractable radioactivity

In order to determine the non-extractable radioactivity in liver, kidney and muscle, samples of 1 g of frozen material were pulverized and extracted with water (4 times), acetonitrile (2 times), ethyl acetate (2 times), 1 M urea (1 times), methanol (3 times), ethanol (2 times) and ethanol-chloroform-diethylether (2:2:1). The radioactivity measured in the tissue residue after these extraction steps was defined as non-extractable. Furthermore the amount of radioactivity was also measured in the ultrafiltrate of water extracts of the various tissues.

Isolation and purification of DNA

The DNA was isolated according to Jeffreys and Flavell (1977) with several modifications.

Frozen tissue (1 g) were pulverized and homogenized in 20 ml of ice-cold 150 mM NaCl, 100 M EDTA (pH 8·0); sodium dodecylsulphate was added to a final concentration of 1% and $NaClO_4$ to 1 M.

The lysate was extracted twice with an equal volume of chloroform isoamyl alcohol (24:1) and the DNA was precipitated by adding 2 ml of 2 M Na acetate (pH 5·6) and 20 ml of isopropanol.

After washing with ethanol, the DNA was dissolved in 12 ml TNE (TNE = 50 mM, Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 7·5). After incubation with pancreatic ribonuclease and proteinase, the solution was 10 times extracted with equal volumes of phenol/m-cresol/8-hydroxyquinoline (100:14:0·1). In order to remove residues of phenol, the water phase was subsequently extracted 5 times with diethylether. The DNA was precipitated by addition of ethanol. After washing with ethanol and drying, DNA was dissolved in 1 ml 10 mM Tris HCl pH 7·5. The amount of DNA was determined by spectrophotometry based on $A_{260} = 20 \text{ mg}^{-1}$. The purity was determined by the ratio's A_{260}/A_{230} and A_{260}/A_{280}. Where necessary an additional purification was used by cesium chloride gradient centrifugation as described by Maniatis *et al.* (1982).

Results

Animal condition and body weight gain

At the start of the medication period animal 106 showed a decreased appetite which may have been caused by an inflammation in the left foreleg. It was treated with a single intramuscular injection of Depomycine 15/25[R] (Gist-Brocades, The Netherlands). Within 1 day the piglet recovered its appetite and within 3 days the inflammation disappeared. The piglets showed a regular weight gain. During the medication period the mean daily body weight gain was 156 ± 18 g (n = 6), and during the withdrawal period 239 ± 32 g (n = 3).

Kinetic studies

Blood and plasma. Mean levels of furazolidone in blood during medication varied between 0·31 and 0·53 μg/ml while in plasma similar values were observed. No accumulation of furazolidone during the medication period could be observed in blood or plasma.

Mean blood and plasma levels of furazolidone upon oral administration on day 10 are shown in figure 2. The concentration of furazolidone reached a maximum value of 835 and 955 ng·ml^{-1} in blood and plasma respectively within 30 minutes after

administration, followed by a decrease which resulted in levels below the limit of detection of 1 ng · ml^{-1} 3–4 hours after the last administration. The half life ($t_{1/2}$) of furazolidone in blood and plasma was approximately 60 minutes.

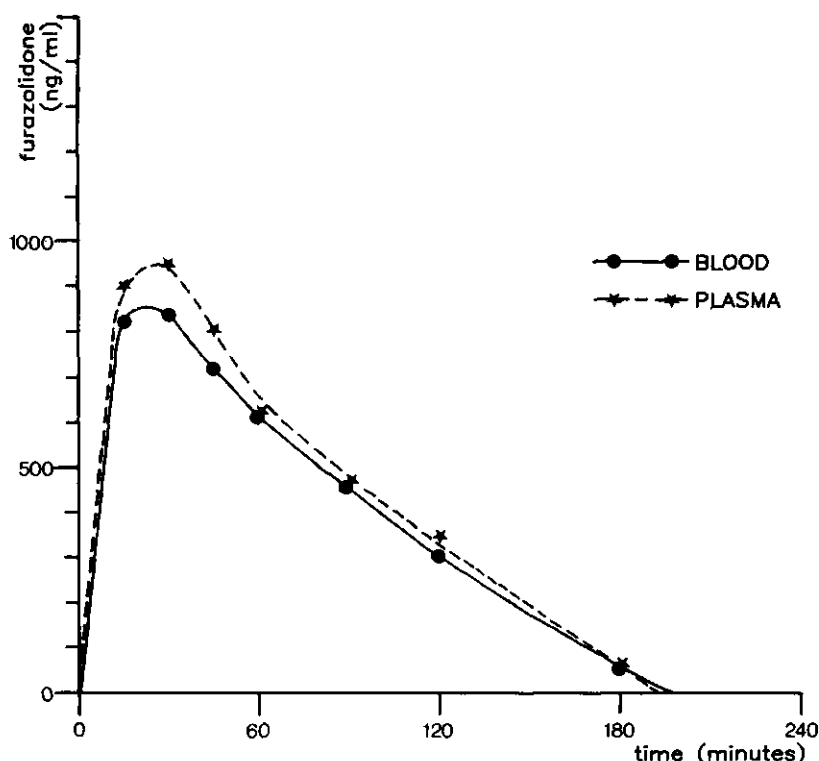Figure 2. Blood and plasma levels of furazolidone (ng per ml) after oral administration to piglets at day 10 ($n = 2$ except at $t = 180$ min.; $n = 1$ because animal 106 was slaughtered 2 hours after the last administration).

In contrast to the parent compound furazolidone, an increasing amount of radioactivity had been observed in blood and plasma during the medication period. Values of radioactivity as measured in plasma are shown in figure 3.

5–6 Days after initiation of the medication, maximum levels of approximately 9–10 μg equivalents of parent compound per ml were measured. A relatively fast decrease of radioactivity in blood and plasma had been observed during the first day after the last administration of ^{14}C-furazolidone, followed by a much slower decrease during the next 13 days with a half life of approximately 6 days. During the withdrawal period the amount of radioactivity measured in blood was approximately 1 μg equivalent of parent compound per ml higher than that in plasma. 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 respectively plasma. The amount of radioactivity associated with the plasma protein fraction is shown in figure 4. One day after the start of the medication approximately 40% of total radioactivity in plasma was measured in the protein fraction; at the end of the medication period approximately
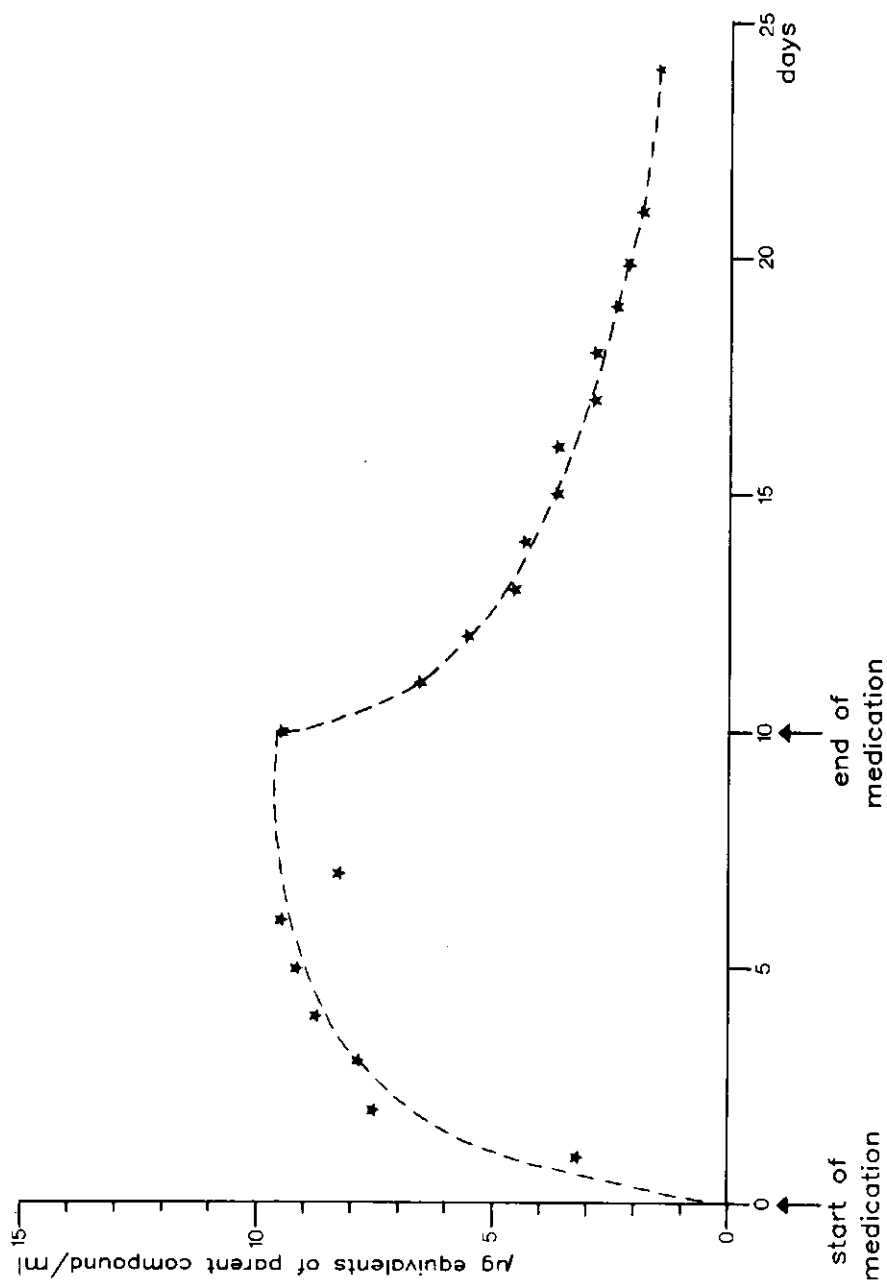
Figure 3. Radioactivity (μg equivalents of parent compounds per ml) in plasma of piglets during the medication period ($n = 2$) and the withdrawal period ($n = 1$).
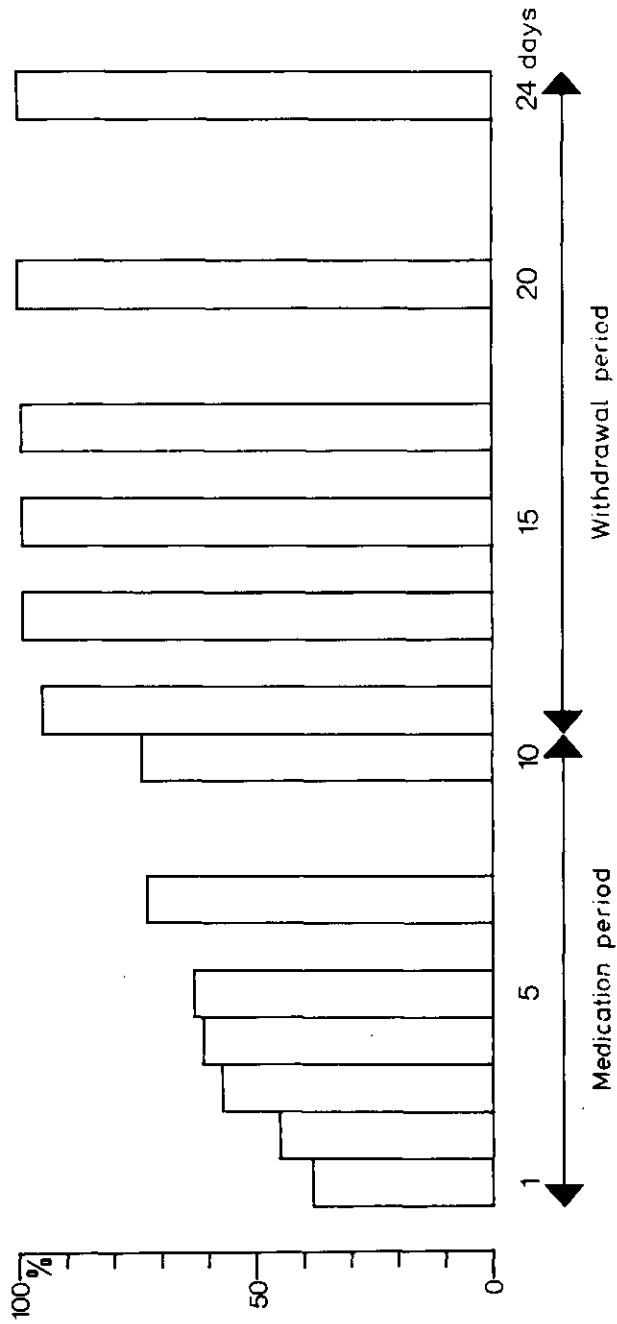
Figure 4. Percentage of radioactivity associated with plasma protein during the medication period ($n = 2$) and the withdrawal period ($n = 1$).

75% and during the withdrawal period nearly all radioactivity was found in the plasma protein fraction. No radioactivity could be detected in the ultrafiltrates of the plasma protein fraction of samples taken at day 5 of the withdrawal period which repeatedly had been washed with water and methanol. Furthermore, no absorption of ^{14}C-furazolidone to the membrane could be detected.

Urine, faeces, expired air. The main excretion pathway of radioactive labelled material proved to be the urine. At the end of the medication period 52% of the total dose of radioactivity was excreted via the urine while during the withdrawal period an additional 4% was collected (figure 5).

Taking the radioactivity of the wash liquid of the cages into account the total excretion of radioactivity via the urine at the end of the experiment was found to be 61%.

Negligible amounts of radioactivity were measured in the waste drinking water (< 0·5% of total dose).

At the end of the medication period 15% (animal 106) and 18% (animal 101) of the total dosed radioactivity was excreted via the faeces and during the withdrawal period an additional 3% (figure 5). No radioactivity associated with volatile metabolites or endogeneously formed $^{14}CO_2$ was found in expired air.

Tissues. None of the animals showed abnormalities at autopsy. The parent compound furazolidone could not be detected in muscle, fat, kidney and liver (< 2 ng per g) immediately after the medication period of 10 days even if 5 g of tissue instead of 1 g were taken for analysis.

Mean levels of radioactive residues in various tissues, calculated as μg equivalents of the parent compound per g immediately after the medication period and after the withdrawal period of 14 days are shown in table 1.

Immediately upon cessation of the medication highest levels of radioactivity were found in bile, liver and kidney (81·1, 32·9 and 30·1 μg equivalents per g respectively). In muscle 5·7–7·2 μg equivalents per g were measured. After the withdrawal period of 14 days, radioactivity levels detected in the tissues had decreased substantially and varied between 0·9–3·1 μg equivalents per g. Recovery of total dosed radioactivity in this experiment was 80%.

Non-extractable radioactivity. Table 2 shows that 14% of the radioactivity (corresponding to 4·8 μg equivalents of parent compound per g) is non-extractable from liver tissue immediately after the medication period of 10 days.

37·8% of total residual radioactivity in liver tissue was found in the ultrafiltrate of water extractable material, indicating that the remaining 44·1% of total residual radioactivity (corresponding to 53·8% of radioactivity in water extract) was associated with water soluble material which did not pass through the membrane. After the withdrawal period of 14 days 0·8 μg equivalents of parent compound per g were present as non-extractable material representing 27·0% of total residual radioactivity. In kidney 2·9 and 0·9 μg equivalents per g (9·3% and 31·2% of total residual radioactivity respectively) and in the musculus biceps 2·3 and 1·2 μg equivalents per g (35·0% and 55·6% respectively of total residual radioactivity) were non-extractable after 0 and 14 days respectively withdrawal time.

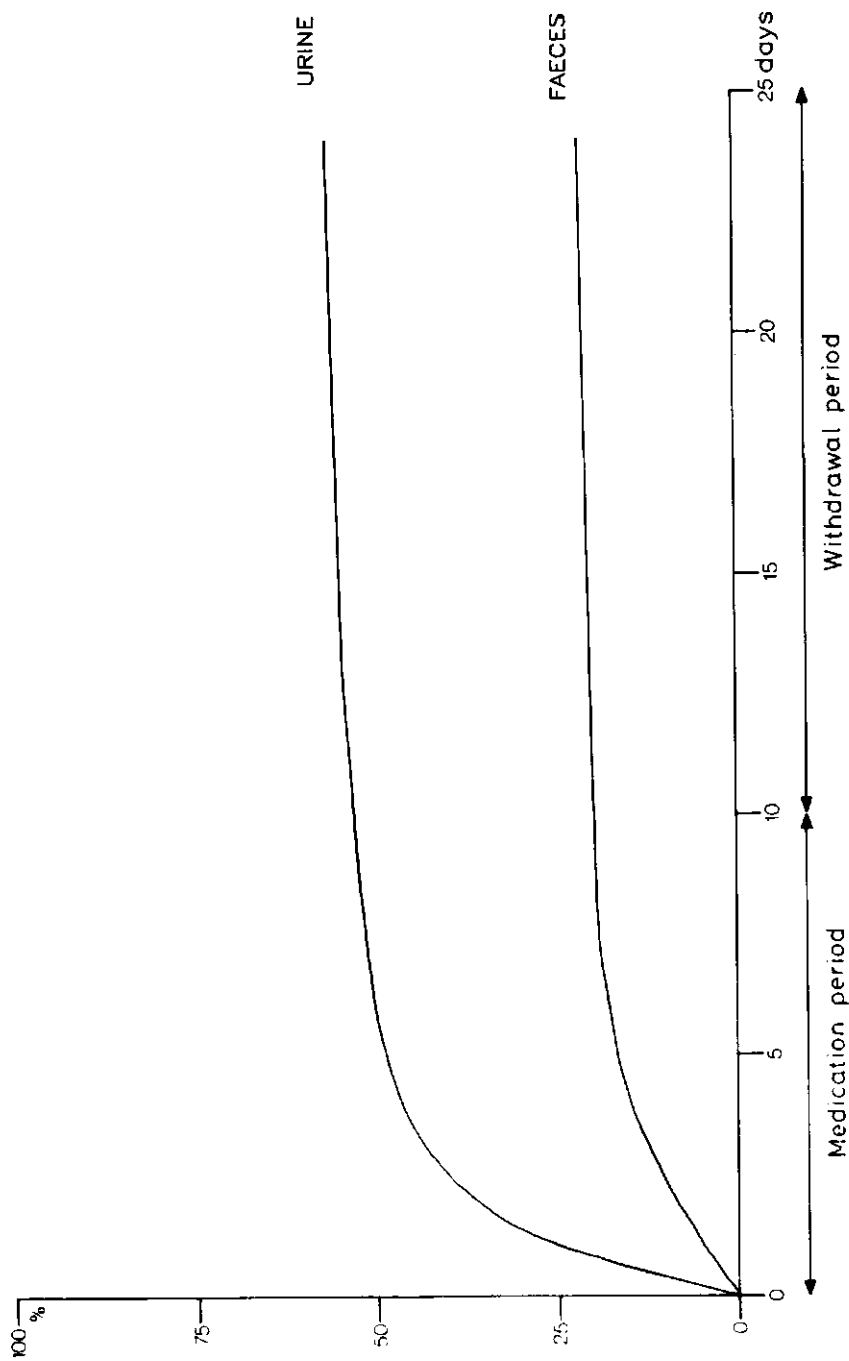Recoveries (mean ± S.D.; n = 6) of radioactivity after the extraction steps were 100% ± 4%.

Figure 5. Cumulative percentage of total administered radioactivity excreted in urine and faeces of piglets treated with ^{14}C-furazolidone during 10 days.

Table 1. Mean level of radioactivity (ppm-equivalents of parent compound) in tissue 0 and 14 days after oral administration to piglets of ^{14}C-labelled furazolidone during 10 days.

Tissue	n	0 Days after the medication period (animal 106)	n	14 Days after the medication period (animal 101)
Skin, back	2	7·3	2	3·5
Skin, abdomen	2	9·1	2	2·9
Subcutaneous fat	2	6·0	2	4·3
Fat of the abdominal cavity	2	9·2	2	2·9
Liver	4	32·9	4	3·1
Kidney	4	30·1	4	3·0
Musculus longissimus dorsi	6	6·4	6	2·0
Musculus psoas major	6	7·2	6	2·1
Musculus biceps	6	6·5	6	2·1
Musculus triceps	6	6·4	6	2·1
Musculus quadriceps	6	5·7	6	2·0
Tongue	2	8·1	2	1·9
Brains	2	7·6	2	1·6
Bladder	2	9·7	2	1·9
Spleen	2	11·4	2	2·1
Stomach	2	20·6	2	1·9
Small intestine	2	12·6	2	0·9
Large intestine	2	11·0	2	1·3
Gall bladder	1	50·3	2	2·0
Bile	2	81·1	2	1·1
Pancreas	2	10·4	2	1·9
Lung	4	10·9	4	2·3
Heart	2	8·3	2	1·9
Testicle	4	11·5	4	2·3

Table 2. Radioactivity in several extraction fractions of tissues of piglets as percentage of the total residual radioactivity after a withdrawal period of 0 and 14 days.

Tissue Days of withdrawal		Liver 0	14	Kidney 0	14	Muscle 0	14
Water	(4 ×)	81·9	66·0	88·1	59·5	59·9	39·9
in ultrafiltrate		37·8	19·4	53·9	15·2	36·5	15·0
Acetonitrile	(2 ×)	0·7	0·7	0·5	2·8	1·4	0·6
Ethyl acetate	(2 ×)	0·7	1·1	0·6	2·0	1·4	0·9
1 M Urea	(1 ×)	0·5	2·8	0·5	0·9	0·9	0·8
Methanol	(3 ×)	1·4	0·9	0·8	3·3	1·1	1·7
Ethanol	(2 ×)	<0·1	<0·1	<0·1	0·1	<0·1	0·2
Ethanol/chloroform/ether 2/2/1	(2 ×)	0·1	<0·1	0·1	0·2	0·2	0·3
Non-extractable		14·0	27·0	9·3	31·2	35·0	55·6
		4·8†	0·8†	2·9†	0·9†	2·3†	1·2†
Recovery		104·3%	96·2%	105·0%	99·3%	96·3%	99·4%

† ppm-equivalents of parent compound

Interaction of furazolidone with DNA. The purity of the isolated DNA was determined by the A_{260}/A_{230} and A_{260}/A_{280} ratio's. These were 2.41 ± 0.09 and 1.86 ± 0.05 respectively (n = 18) indicating that total protein amount was $< 0.01\%$ (Markov and Ivanov, 1974). Table 3 shows interaction of furazolidone with DNA expressed as pmol equivalents of parent compound per mg DNA.

Table 3. DNA binding (pmol equivalents of parent compound/mg DNA) in tissue of piglets 0 and 14 days after oral administration to piglets of ^{14}C-labelled furazolidone during 10 days.

Tissue	0 Day after the medication period	14 Days after the medication period
Spleen	240	102
Pancreas	382	119
Liver	186	106
Kidney	372	114
Lung	279	87
Heart	130	106
Muscle	139	118
Brains	57	89
Tongue	165	82

Discussion

The main excretion pathway of ^{14}C-material proved to be the urine. At the end of the experiment 61% of total dosed radioactivity had been detected in the urine of which approximately 90% already had been excreted at the end of the medication period of 10 days. 15–18% of total dosed radioactivity had been excreted via the faeces by piglets at the end of the medication period, with an additional 3% during the withdrawal period. Similar results have been reported in a study with a Yorkshire barrow after oral treatment with ^{14}C-furazolidone, in which 70.5% had been excreted via the urine and 19.1% via the faeces and in a similar test with rats: 81.5% in urine and 11.0% in faeces (Tennent and Ray 1971).

Maximum levels of furazolidone in plasma of piglets were achieved within 30 minutes after oral administration, reached approximately 1 μg per ml and disappeared very fast (calculated half life: 60 minutes). Furazolidone inhibits the growth of micro-organisms in a minimal concentration range of 1–10 μg per ml; for *Proteus* species and *Pseudomonas aeruginosa* minimal concentrations up to 300 μg per ml are necessary (Van Esch *et al.* 1983). So after oral administration to piglets furazolidone seems not to be effective against other than gastrointestinal infections. In older piglets, weighing 30–40 kg, maximum levels in the serum after oral treatment with furazolidone in the feed (1000 mg per kg feed) were achieved within 30–60 minutes and reached 3.8 μg per ml. The half life was 27 minutes (Yamamoto *et al.* 1978). This difference in half life may be due to changes with age in both pathways contributing to excretion and metabolism. Nitroreduction is believed to be the major metabolic pathway of nitrofurans (Swaminathan and Lower 1978). Short *et al.* (1972) reported that the rate of nitroreduction of p-nitrobenzoic acid in liver of swine increased from 655 nmol per g of tissue for a 6 weeks old piglet to 905 nmol per g of tissue for an adult swine.

In buffalo the half life of furazolidone in plasma after a single oral dose of 30 mg/kg body weight was 2.52 hours (Mir and Uppal 1984).

Compared with furazolidone itself, the half life of radioactivity in plasma upon oral administration of ^{14}C-furazolidone to piglets during 10 days was much higher indicating that the depletion of metabolites of furazolidone from plasma was much slower than that of furazolidone itself. It appeared that one day after stopping the medication almost all the radioactivity was associated with the plasma proteins.

No furazolidone could be detected in liver, kidney, fat and muscle 2 hours after stopping the medication. Comparable results have also been reported by Winterlin *et al.* (1984). However, radioactivity could be detected in all tissues examined at the $\mu g/g$ level (radioactivity expressed as equivalents of parent compound), even 14 days after stopping the medication. The major part of extractable radioactivity could be extracted by water. Previous studies have identified several metabolites of furazolidone. The open chain cyano-derivative 3-(4-cyano-2-oxobutylideneamino)-2- oxazolidone appeared to be an important end product of reductive metabolism in rabbits (Tatsumi *et al.* 1984), (rat liver fractions (Tatsumi *et al.* 1981; Abraham *et al.* 1984; Vroomen *et al.*, in press) and *Escherichia coli* (Abraham *et al.*, 1984). In preliminary experiments we obtained evidence for the presence of this open chain cyano-derivative in plasma of piglets as well. Further studies presently are being undertaken to obtain more information concerning the identity and kinetics of metabolites of furazolidone in blood and tissues of swine.

It also appeared that considerable amounts of the radioactivity in liver, kidney and muscle could not be extracted from the tissues after several consecutive extraction procedures. Persistent radioactivity might be covalently bound as is shown for other compounds, like the nitro imidazole compound ronidazole after administration to swine (Wolf *et al.* 1983). Also non-extractable radioactivity might be covalently bound as described for trenbolone-acetate (Ryan and Hoffmann 1978) and cambendazole (Baer *et al.* 1977) in cattle tissues and for ^{14}C-toluoylchloride phenyl hydrazone in erythrocytes of sheep (Jaglan *et al.* 1977).

Covalent binding may be the result of:

(1) Activation to highly reactive intermediates which may covalently bind to biological macromolecules such as proteins, DNA or lipids. In the case of furazolidone such intermediates may be formed after reduction of the nitro group. The nitroso and N-hydroxylamino derivatives formed may covalently bind to macromolecules (Olive 1978; Swaminathan and Lower 1978). *In vitro* covalent binding of furazolidone to microsomal protein has been reported (Abraham *et al.* 1984; Vroomen *et al.* in press) and for other nitrofurans to protein and DNA (Mattammal *et al.* 1985; Tatsumi *et al.* 1977). The possible role of the nitroso or N-hydroxylamino derivatives can be shown by trapping the reactive intermediates with nucleophilic agents such as glutathione. A glutathione conjugate has been described for the nitrofuran N-[4-(5-nitro-2-furyl)2-thiazolyl]acetamide (Mattammal *et al.* 1985).

(2) Metabolic degradation to non-drug related common carbon fragments, which may be incorporated in cellular components which are formed in various normal synthesis reactions. In the case of furazolidone metabolism the formation of α-ketoglutaric acid has been described as a result of degradation of the furan moiety of furazolidone in rats (Tatsumi *et al.* 1984). The formation of α-ketoglutaric acid may occur after splitting of the furan moiety from the oxazolidone moiety. Such a cleavage may occur by hydrolysis under acid conditions (Paul *et al.* 1960), which are present in the stomach. α-Ketoglutaric

acid may be involved in several processes, but may also be catabolized to CO_2 (Lehninger 1971). Tennent and Ray (1971) reported that 3% of total dosed radioactivity was detected in the expired air of rats after oral dosing of formyl ^{14}C-furazolidone. This radioactivity may be the result of formed $^{14}CO_2$ or volatile metabolites. In our study with piglets no radioactivity could be measured in the expired air, which may be expected in the case of formation of volatile metabolites. Detection of $^{14}CO_2$ formed by degradation of the furan moiety was not possible because ^{14}C-furazolidone was labelled in the oxazolidone moiety. No data are available concerning metabolic degradation of the oxazolidone moiety to components which may be involved in normal anabolic or catabolic processes. Further studies are necessary to elucidate the molecular basis of cellular drug interactions.

The *in vivo* interaction of ^{14}C-furazolidone with DNA in tissues of piglets has been shown in this study. The interaction with DNA was especially marked in pancreas and kidney. In comparison for example the extent of binding to DNA of benzo(*a*)pyrene in mice after an oral dose of 2×3 mg per animal varied from $177 - 230$ pmol per mg DNA in liver and from $22-32$ pmol per mg DNA in lung (Anderson *et al.* 1981). After the withdrawal period of 14 days radioactivity could still be measured in the DNA isolated from the several tissues. Taking into account the growth of the piglets, it seems that during a 14 days withdrawal period there are no signs of a quick and effective DNA-repair mechanism in piglets for the DNA-damage caused by furazolidone. Mutagenic activity of furazolidone has been described in many prokaryotic test systems (Ohta *et al.* 1980; Blijleven *et al.* 1977; Kramers 1982; Voogd *et al.* 1982) and in eukaryotic systems (Cohen and Sagi 1979; Babilé *et al.* 1978; Probst *et al.* 1981; Voogt *et al.* 1982).

Acknowledgements

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CHAPTER 4: The elimination of furazolidone and its open-chain cyano-
derivative from adult swine
Xenobiotica, in press.

THE ELIMINATION OF FURAZOLIDONE AND ITS OPEN-CHAIN CYANO-DERIVATIVE

FROM ADULT SWINE

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ABSTRACT

1. A sensitive method for the determination of 3-(4-cyano-2-oxobuty-
lidene amino)-2-oxazolidone, the open-chain cyano-derivative of the
veterinary drug furazolidone, in swine plasma and tissues is described.

2. After dosing adult swine orally with furazolidone (690 mg/ animal
per day) for 10 days no furazolidone was detected in liver, kidney and
muscle (< 2 ng/g). The half life of furazolidone as measured from the
terminal phase of the plasma curves was 45 minutes.
In urine small amounts (< 0.3% of total dose) of furazolidone were
detected.

3. The cyano-derivative of furazolidone, 3-(4-cyano-2-oxobutylidene
amino)-2-oxazolidone is a minor metabolite in plasma and tissues of
swine with a plasma half life of 4 h. No cyano-derivative was detected
in liver and kidney (< 5 ng/g) 2 h after the last administration of
furazolidone; 24 h after the last administration, the concentration in
plasma was < 2 ng/ml and in muscle < 5/g.

4. The cyano-derivative was not mutagenic in the Salmonella/microscome
test, with or without metabolic activation.

INTRODUCTION

Furazolidone [N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone] is a
veterinary drug widely used as an antimicrobial agent for prophylactic
and therapeutic purposes in swine (Brander and Pugh, 1971; Rossoff,
1974). Because of its mutagenic and carcinogenic activities (Klemencic
and Wang, 1978; McCalla, 1983; Food and Drug Administration, 1976) its
use is of public health concern. Earlier studies showed that no resi-
dues of furazolidone could be detected in edible tissues, 2-4 hours
after dosing (Winterlin et al., 1984; Vroomen et al., 1986a).
However, up to 14 days after dosing, radioactivity of the order of
µg-equivalents per gram could still be detected in tissues of piglets
after oral administration of ^{14}C-furazolidone (Vroomen et al., 1986a).
No information is available concerning the identity, depletion kine-
tics and toxicity of metabolites of furazolidone in swine tissues.
However, reduction of the nitro group, resulting in the formation of
an open-chain cyano-derivative, is an important metabolic pathway of
nitrofurans (Swaminathan and Lower, 1978). No analytical method is
available for the determination of the cyano-derivative of furazoli-
done in plasma and tissues.

In this paper a sensitive HPLC-method is described for the determina-
tion of 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone the cyano
derivative of furazolidone in plasma, muscle, liver and kidney of
swine. The method showed its value in a study of the rate of
disappearance of furazolidone and its open-chain cyano-derivative from
plasma and tissues upon oral administration of furazolidone for 10
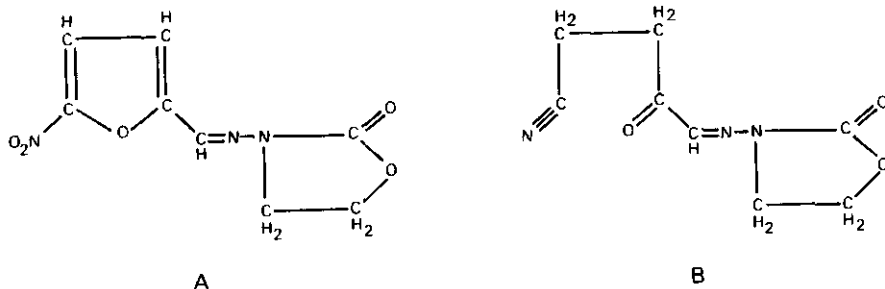days to swine.

Figure 1: Furazolidone (A) and its open-chain cyano-derivative (B).

EXPERIMENTAL

Chemicals and solvents
L-asparagine, ethyl acetate and acetonitrile were purchased from Merck
(Darmstadt, F.R.G.) and dextrose was purchased from Difco Laboratories
(Detroit Michigan, U.S.A.). Water was purified over a Millipore Milli-
Q water purification system. Furazolidone was a gift from Orphahell
B.V. (Mijdrecht, The Netherlands).

Biosynthesis of the cyano-derivative of furazolidone using E.coli
The cyano-derivative of furazolidone, 3-(4-cyano-2-oxobutylidene
amino)-2-oxazolidone (figure 1), was synthezised using E. coli
according to Abraham (1981) with several modifications.
After an incubation period of 75 min at 37°C, the aqueous layer con-
taining E.coli was extracted twice with equal volumes of ice-cold
ethyl acetate. The ethyl acetate extracts were combined and gently
evaporated to dryness under vacuum. The resulting residue was
dissolved in acetonitrile/ water (20/80). Separation of the cyano-
derivative was achieved on a semi-preparative Hibar prepacked column
RT 250-10 Lichrosorb RP-18 (7 μm) (Merck 50994) using a linear 22 min
gradient of 2% - 100% actonitrile in water/acetonitrile (975/25) at a
flow rate of 4 ml per min. The eluent was monitored at 254 nm. The
fractions with the cyano-derivative (retention time 6.2 - 6.8 min)
were collected and after evaporation to dryness under vacuum and
redissolving in acetonitrile/water (20/80), the cyano-derivative was
isocratically chromatographed four times (water-acetonitrile 92/8,
retention time: 10.4 - 11.0 min). White crystals were obtained with
similar spectral properties as described earlier for a metabolite of
furazolidone which was identified as 3-(4-cyano 2-oxobutylidene amino)
-2-oxazolidone (Tatsumi et al., 1978; Abraham et al., 1984; Vroomen et
al., 1987); characteristics of the mass spectrum (field desorption,
m/z): 195 (molecular ion) and of the infra red spectrum in KBr (cm^{-1}):
2250: cyano-group, 1778: oxazolidone, C=0 and 1688: α,β unsaturated
C=0 stretching.

Analysis of the cyano-derivative

Sample preparation

Plasma. A 1-ml aliquot of plasma was applied directly to a Merck Extrelut[R] 1 column (Merck 15371) and after equilibration for 10 min, the cyano-derivative was eluted with 6 ml of ethyl acetate. The eluate was collected, evaporated to dryness under nitrogen and the residue dissolved in 300 µl of eluent.

Muscle. A 1-g frozen sample (liquid nitrogen) was pulverized and mixed with 4 ml of water for 2 min. After centrifugation at 3000 g for 15 min the water-extracts were applied to a Merck Extrelut[R] 3 column (Merck 15372) and after 10 min equilibration, the cyano-derivative was eluted with 15 ml of ethyl acetate. The eluate was collected, evaporated to dryness under nitrogen, the residue dissolved in 300 µl of eluent and ultrafiltered (Amicon Centrifree Micropartition System). No specific absorption of the cyano-derivative to the membrane occured. The ultrafiltrate was subjected to HPLC.

Kidney, liver. Quantities of 1 g of kidney and liver were prepared as described for muscle. Direct HPLC-analysis on a Hamilton PRP-1 column (see High Performance Liquid Chromatography, plasma and muscle) was not possible because of interference of other peaks absorbing at 254 nm. Therefore a first separation was achieved on a Hamilton PRP-1 column (250 x 4.1 mm int.diam.; 10 µm) using a linear 22 min gradient of 2% - 100% acetonitrile in water/acetonitrile (975/25) at a flow rate of 1 ml per min (injection volume: 150 µl). The fraction with the cyano derivative (retention-time: 11.5 - 12.3 min) was collected and after evaporation to dryness under nitrogen, the residue was dissolved in 300 µl of water/acetonitrile (80/20) and prepared for HPLC.

High-performance liquid chromatography

Liquid delivery system: Waters 6000 A; automatic injection system: Waters 710 B; detector: Kratos Spectroflow 773; absorbance was recorded at 254 nm; recorder: Kipp BD 41.

Plasma and muscle. Analytical column: Hamilton PRP-1 (250x4.1 mm int.diam.; 10 µm), precolumn: Brownlee Labs OD-GU RP-18 (30x4.6 mm int.diam.). Run-time: 17 min (after 5 min isocratic HPLC (water-acetonitrile, 75/25, v/v), the column was washed with water-acetonitrile, 50/50 (v/v) for 12 min; flow-rate 1 ml/min; injection volume: 25 - 100 µl. Retention time: 4.3 - 4.8 min.

Kidney and liver. Analytical column: Chrompack Hypersil ODS 5 (250 x 4.6 mm int.diam.) Precolumn: Brownlee Labs OD-GU RP-18 (30 x 4.6 mm int.diam.).
Eluent: water-acetonitrile (80/20, v/v); flowrate: 1 ml/min; injection volume: 50-100 µl; run-time: 10 min. Retention time: 4.9 - 5.6 min.

Analysis of furazolidone

Analytical procedures were performed under artificial yellow light (Pope FT 40 W/16), because of the instability of furazolidone in daylight (Kalim, 1985). The concentration of furazolidone in blood, plasma, urine, liver, kidney and muscle was analysed as described elsewhere (Vroomen et al., 1986b).

Animal handling and sample collection

Twelve male pigs (Dutch Landrace x Great Yorkshire; mean weight +
S.D.: 89.3 + 3.6 kg) were individually housed in metabolism cages at
IGMB/TNO- Department ILOB, Wageningen, The Netherlands. After an
acclimatization period of 1 week, the animals were divided into 2
groups:
Group I : 2 control animals
Group II: 10 animals treated with furazolidone during 10 days.
A standard swine diet without antibiotics was provided to group I
twice daily at 9.00 h and 16.00 h. For group II furazolidone was added
to the feed in a final concentration of 300 mg per kg.
The daily amount of feed corresponded to the amount of feed under
practical conditions: 2300 g/ animal per day.
The feed/water ratio was 1:2.
Urine was collected separately into containers to which an 1.0 M ace-
tate buffer (pH 5.0) was added. Each morning, just before feeding time
at 9.00 h, total amounts of urine were measured and immediately
samples were taken for analysis of the concentration of furazolidone.
No blood samples were taken during the medication period, since ear-
lier studies indicated a relatively short half life of furazolidone
(Vroomen et al., 1986a) and the cyano-derivative (data not published)
in plasma.
After the last administration of furazolidone on day 11 at 9.00 h,
blood samples were collected in heparinized tubes at regular time
intervals from the jugular vein. Plasma was separated by centrifuga-
tion. Samples were immediately analysed for the concentration of fura-
zolidone since a time-dependent decrease of furazolidone in blood has
been reported (Veale and Harrington, 1982). Plasma samples for the
analysis of the cyano-derivative were stored at -40°C.
On day 0, 1, 3, 7 and 14 of the withdrawal period, 2 animals of Group
II were killed 2 h after feeding at 9.00 h. The animals of Group I
were killed on day 0 and 14 of the withdrawal period, also at 11.00 h.
Samples were taken of the liver, kidney and muscle for analysis of the
concentration of furazolidone and the cyano-derivative, immediately
frozen and stored at -40°C.

Salmonella/microsome-test

The Salmonella/microsome test was carried out as described by Maron
and Ames (1983) using tester strains TA 98 and 100.
Furazolidone and the cyano-derivative were tested dissolved in DMSO in
quantities of 0.01, 0.1, 1.0 and 10 µg, respectively 10 and 100 µg per
plate, both in the presence and absence of 50 µl S9-mix (from livers
of Aroclor-treated rats) per plate.

RESULTS

Determination of 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone

The cyano-derivative of furazolidone as synthesized by E.coli was

stable for at least one month in the range of 1 - 1000 ng/ml standard

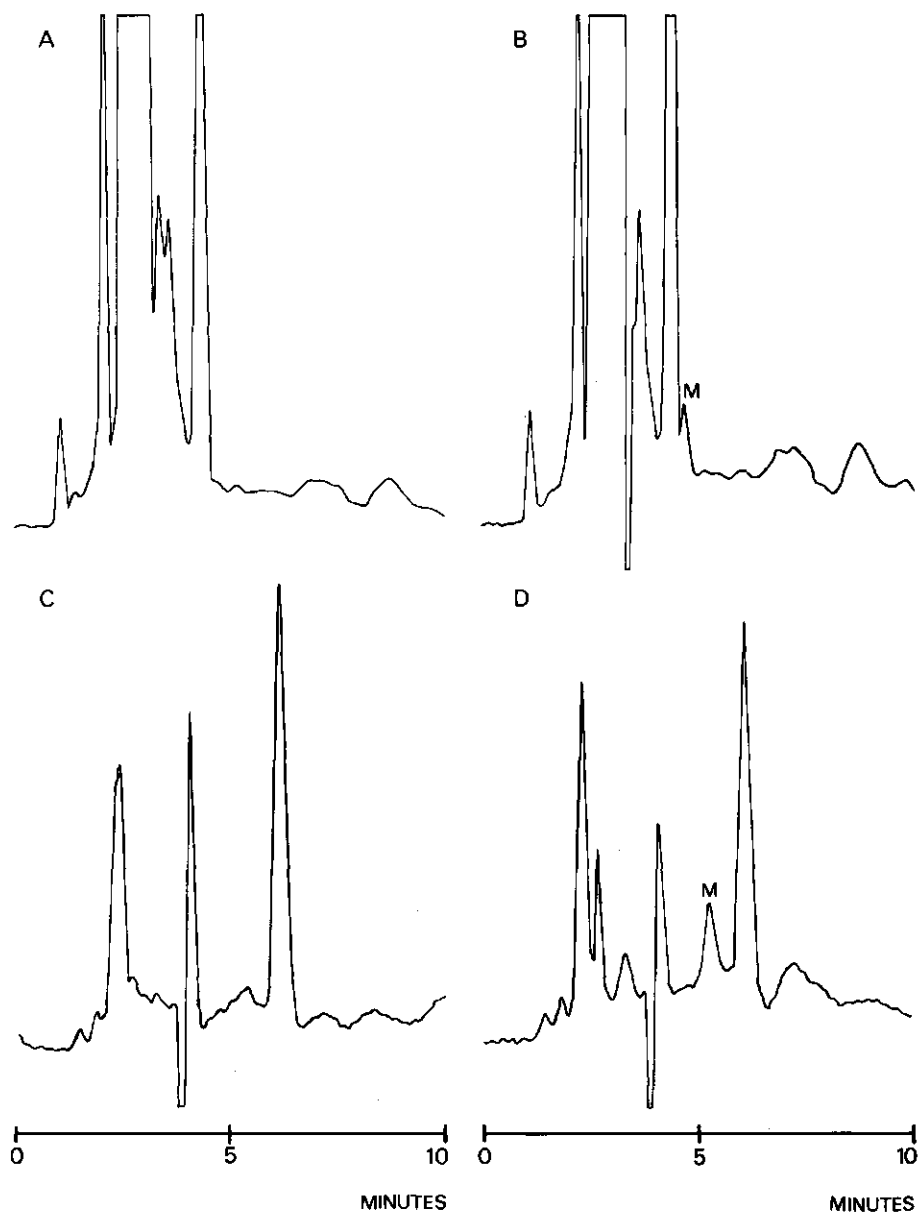solution, when stored in the dark at room temperature.

Figure 2: High-performance liquid chromatogram of the cyano-derivative in samples of swine muscle (A: blank, B: 10 ng/g cyano-derivative) and swine liver (C: blank, D: 10 ng/g cyano-derivative). (A,B) : λ = 254 nm; speed 1 cm/min; sensitivity of detector: 0.01 aufs; sensitivity of recorder: 10 mV; injection volume: 50 µl. (C,D): λ 254 nm; speed 1 cm/min; sensivity of detector: 0,002 aufs; sensitivity of recorder: 10 mV; injection volume: 50 µl (M = the cyano-derivative of furazolidone).

Concentrations of the cyano-derivative of furazolidone were calculated by comparing the peak heights from samples to which the derivative had been added with the peak heights from corresponding standard solutions, containing the same amounts of cyano-derivative. Linearity exists between the concentration of the cyano-derivative and peak height in the range of 1 - 1000 ng/ml standard solution. Linearity was also found for samples. In blank samples of swine plasma, liver and kidney no interfering peaks could be detected at 254 nm. Despite of an interfering peak in samples of swine muscle (figures 2A and 2B), the reproducibility of the separation at all tested levels was not influenced, so the additional HPLC-separation step as described for liver and kidney was not necessary. Recovery data for swine samples with added cyano-derivative of furazolidone in a concentration range of 1 - 1000 ng/ml (or ng/g) of sample are shown in Table 1.

Table 1: Percentage recoveries of the cyano-derivative of furazolidone from pig plasma and tissues. The cyano-derivative was added to pig plasma and tissues in a concentration range of 0 - 1000 ng/ml or ng/g of sample (n = 6; mean $\pm$ S.D.)

Cyano-derivative added (ng/g or ng/ml)	% Recovery of cyano-derivative			
	Plasma	Muscle	Liver	Kidney
1000	93.0 + 3.7	97.7 + 3.6	70.3 + 5.8	73.9 + 7.8
100	104.1 + 4.2	95.3 + 3.9	73.2 + 7.8	67.5 + 2.3
50	98.1 + 6.0	89.1 + 3.1	71.0 + 9.7	69.5 + 6.8
25	97.4 + 3.1	96.2 + 2.1	66.2 + 7.1	76.7 + 8.9
10	97.8 + 4.5	92.5 + 8.1	72.4 + 6.9	74.5 + 5.9

Each result represents the average of six determinations. The mean overall recovery for plasma (n = 30) is 98.1 $\pm$ 5.5, muscle (n = 30): 94.2 $\pm$ 5.1, liver (n = 30): 70.6 $\pm$ 7.4 and kidney (n = 30): 72.3 $\pm$ 7.0).

The sensitivity of the method, expressed as three times the background of a blank sample was 2 ng/ml for plasma, 5 ng/g for muscle, liver and kidney of swine.

In figure 2, the chromatograms of a blank and a muscle sample (10 ng/g) and a blank and a liver sample (10 ng/g) are shown.

Kinetic studies

Plasma. Neither furazolidone nor the cyano-derivative could be detected in plasma samples taken just prior to the last administration of fura-zolidone on day 10. Mean plasma levels of furazolidone and the cyano-derivative after the last oral administration are shown in figure 3. The concentration of furazolidone reached maximum levels of 62-121 ng/ml within 60-75 min after administration while similar values were observed in blood. Elimination half life of furazolidone as measured from the terminal phase of the plasma curves was approximately 45 min. The concentration of the cyano-derivative reached maximum levels of 24.9-45.3 ng/ml within 90-240 min after administration. Seventeen h after administration the concentration was < 2 ng/ml.

Urine. The mean total daily amounts of furazolidone excreted via the urine are shown in figure 4. Maximum excretion was measured after 2 days of medication: 447-1784 µg of furazolidone per animal. In the following days of the medication period a decrease was observed. After 10 days of medication the excreted amounts varied from 170-293 µg of furazolidone per animal. The daily amount of furazolidone excreted via the urine was only 0.06% $\pm$ 0.04% (n=108) of the daily administered dose of furazolidone with an individual maximum of 0.26% and a minimum of 0.01%.

Within 5 h after the last administration more than 60% of the total excreted amount was excreted via the urine; 24 h after the last admi-
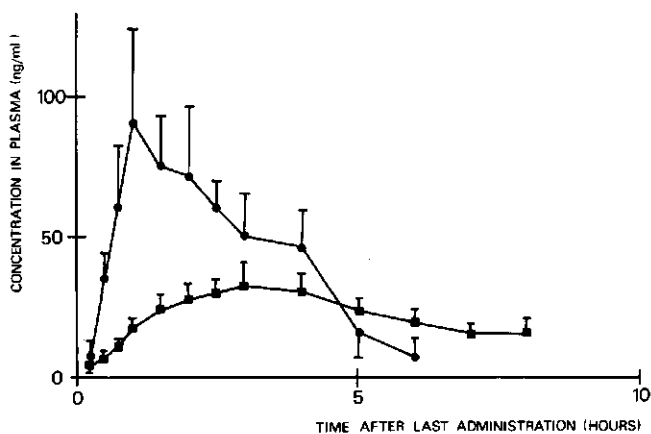
Figure 3: Mean plasma levels of furazolidone and its cyano-derivative (ng/ml) after the last oral administration of furazolidone to swine (n=10, after t=2 h n=8). Furazolidone: ●——●, the cyano-derivative: ■—■—■
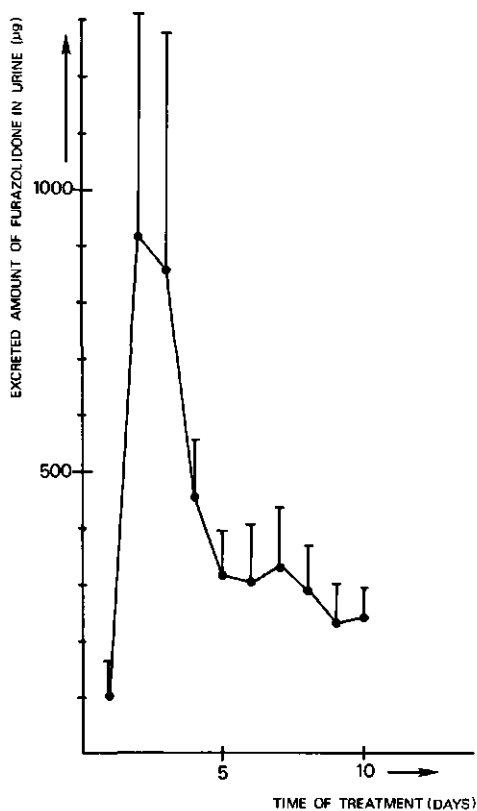

Figure 4: Mean total daily amounts of furazolidone (μg) excreted via the urine (n=10) during the medication period.

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nistration no more furazolidone could be detected in urine (< 25 ng/ml)
(data not shown).

Muscle, liver and kidney. None of the animals showed abnormalities at
autopsy. Furazolidone could not be detected in muscle, liver and kid-
ney of adult swine (< 2 ng/g tissue) 2 h after the last administration
of furazolidone on day 10. The cyano-derivative could only be detected
in muscle of swine, killed 2 h after the last drug administration:
mean level (n=2): 17 µg/kg muscle.

Mutagenicity test with Salmonella typhimurium.

Furazolidone was positive with TA 100 at all tested concentrations,
while a dose-effect relation was observed. The addition of 50 µl
S_9-mix did not influence the results (data not shown). The cyano-
derivative was negative at doses up to 0.1 mg per plate for both TA 98
and 100, with and without metabolic activation.

DISCUSSION

In earlier studies half lives of furazolidone in plasma after oral
treatment varied from 27 min for pigs weighing 30-40 kg (1000 mg fura-
zolidone per kg feed) (Yamamoto et al., 1978) to 60 min for piglets
weighing approximately 10 kg (300 mg furazolidone per kg feed) (Vroomen
et al., 1986 a). Differences in half life may be explained by the
fact that the biotransformation capacity and the renal clearance func-
tion is in general less developed in younger animals. Short et al.
(1972) reported that the rate of nitroreduction of p-nitrobenzoic acid
in liver of swine increased from 655 nmol per g of tissue for a 6 week
old piglet to 905 nmol per g of tissue for an adult swine.
Furazolidone inhibits the growth of Gram-negative and Gram-positive
pathogenic bacteria in a minimal concentration range of 1-10 µg per ml;

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however, for _Proteus_ species and _Pseudomonas aeruginose_ minimal con-
centrations up to 300 µg per ml are necessary (Van Esch et al., 1983).
Since the concentration of furazolidone reached maximum plasma levels
in adult swine of 60-120 ng/ml after oral administration through feed
in a concentration of 300 mg/kg, it may not to be effective against
other than gastrointestinal infections, as also has been observed for
the piglet (Vroomen et al., 1986a).

No furazolidone could be detected in liver, kidney and muscle 2 h
after stopping the medication. Comparable results have also been
reported in studies with piglets (Vroomen et al., 1986a), swine and
poultry (Winterlin et al., 1984).

In earlier studies it has been shown that urine is the main excretion
pathway; after oral administration of ^{14}C-furazolidone to the piglet,
barrow or rat respectively 61%, 70.5% and 81.5% of total dosed
radioactivity was recovered in urine (Vroomen et al., 1986a; Tennent
and Ray, 1971). The percentage of unchanged furazolidone excreted via
the urine was not determined in these studies.

From this study it appears that only a small fraction of the total
administered dose was excreted via the urine as unchanged furazolidone
(0.01-0.26%), apparently due to effective biotransformation.
After 2 days of medication a decrease in the concentration of the
parent compound excreted via the urine was observed, indicating that
specific metabolizing enzymes may have been induced. Detailed infor-
mation concerning the identity, depletion kinetics and toxicity of
formed metabolites is necessary for the evaluation of the risk of
residues of drugs used in food-producing animals for human.
Reduction of the nitro group is a major metabolic pathway of nitro-
furans (Swaminathan and Lower, 1978). The open-chain cyano-endproduct

of reductive metabolism of furazolidone, 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone, appeared to be an important metabolite in rats (Tatsumi et al., 1984), rat liver fractions (Tatsumi et al., 1981; Abraham et al., 1984; Vroomen et al., 1987), eels (Nakabeppu and Tatsumi, 1984) and Escherichia coli (Abraham et al., 1984). The cyano-derivative of furazolidone could also be detected in the urine of rabbits after oral treatment (Tatsumi et al., 1978).

From this study it appears that the cyano-derivative is a minor metabolite of furazolidone in swine after oral administration. This may be the result of different species specific metabolic pathways of furazolidone in swine. Several other pathways have been postulated for nitrofurans: hydroxylation of the furan ring in the 4- position or oxidation of the side chain (Swaminathan and Lower, 1978), hydrolysis of the azomethine binding, (Schmid, 1985) and for methylfurans epoxidation of the furan ring (Ravindranath et al., 1984). Another explanation may be a very fast elimination of the cyano-derivative via the urine. Also effective trapping of reactive intermediates of furazolidone such as nitroso and N-hydroxylamino-derivatives by biological macromolecules or agents like glutathione may occur (Olive, 1978; Swaminathan and Lower, 1978). Covalent binding of furazolidone with protein and DNA in vivo has been reported in piglets (Vroomen et al., 1986a). A glutathione dependant covalent binding of furazolidone to rat liver microsomal protein in vitro has been reported (Vroomen et al., 1987). Mutagenicity of furazolidone is probably also due to reductive activation to nitroso or hydroxylamino derivatives which may react with DNA rather than to the activity of the unmetabolized compound. Once reduced to stable endproducts, mutagenic activity becomes unlikely (Rosenkranz and Speck, 1976; Klemencic and Wang, 1978;

McCalla, 1983). This is supported by results of studies performed by Tatsumi et al. (1978) and our laboratory showing that the reductive endproduct, the open-chain cyano-derivative, was not mutagenic in the Ames test both with or without metabolic activation.

In conclusion these studies underline the importance of detailed metabolism studies of drugs in the proper target animals. Furazolidone is rapidly degraded in swine and not detectable in edible tissues after oral administration. The cyano-derivative which is a major metabolite in several animals, is only a minor metabolite in plasma and tissues of swine. Furthermore from studies with piglets dosed with ^{14}C-furazolidone it is known that radioactivity in the order of μg-equivalents per gram of tissue could be detected. Therefore extensive biotransformation of furazolidone must take place in swine. No further information concerning the identity, depletion kinetics and toxicity is available on metabolites present in edible tissues of swine after oral treatment with furazolidone. Further studies are in progress which may serve as a basis for a proper risk estimation of the application of furazolidone to swine in relation to human health.

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CHAPTER 5: Quantitative studies on the metabolism of furazolidone by
rat liver microsomes
Toxicology in Vitro, 1 (1987) 97-104.

QUANTITATIVE STUDIES OF THE METABOLISM OF FURAZOLIDONE BY RAT LIVER MICROSOMES

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Abstract—The nitrofuran, furazolidone, is metabolized by rat liver microsomes under both aerobic and anaerobic conditions, the rates for microsomes from 3-methylcholanthrene-induced male rats being 3.52 and 4.26 nmol/mg protein/min, respectively. The two major metabolites formed are the well-known 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone, and 2,3-dihydro-3-cyanomethyl-2-hydroxy-5-nitro-1α,2-di-(2-oxo-oxazolidin-3-yl)iminomethylfuro[2,3-b]furan (accounting for 16.6 and 16.4% of total extractable radioactivity, respectively). Cytochrome *P*-450 is not involved in the conversion of furazolidone, which was converted by rat liver microsomes to products identical to those obtained upon incubation with purified NADPH-cytochrome *P*-450 reductase, which is a microsomal reductase. During metabolism, a considerable amount of material (2–3% of total metabolites) became covalently bound to microsomal protein. This covalent binding could be inhibited by addition of glutathione, which also resulted in an almost complete shift from non-polar to water-soluble metabolites. No interaction of furazolidone with added calf thymus DNA was detected.

INTRODUCTION

Furazolidone (*N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone) is an antimicrobial veterinary drug, widely used for the therapy and prevention of intestinal infections in pigs, poultry and cattle. It is mutagenic (Klemencic & Wang, 1978; McCalla, 1983) and appears to be carcinogenic (Food & Drug Administration, 1976 & 1984).

After oral administration of furazolidone to pigs the concentration of the drug in edible tissues was below the limit of detection (0.5 μg/kg tissue) within 4 hr of the last administration (Winterlin *et al.* 1984). However, radioactive material corresponding to approximately 2 ppm-equivalents of parent compound could be detected in the tissues 14 days after treatment of piglets with [¹⁴C]furazolidone was stopped (Vroomen *et al.* 1985). A major part of the residual radioactivity was non-extractable (Vroomen *et al.* 1986).

Information on the toxicity of residual metabolites of furazolidone in animal products with regard to human health is limited, and covalently bound residues are of particular interest in this respect. Therefore, a quantitative and qualitative study of the process of covalent binding of furazolidone to biological macromolecules seemed indicated.

Reduction of the nitro group is a major metabolic pathway of nitrofurans (Swaminathan & Lower, 1978). Previous studies have identified several metabolites of reductive metabolism both *in vitro* and *in vivo*. The cyano derivative 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone appeared to be the major end product of reductive metabolism of furazolidone (Abraham *et al.* 1984; Nakabeppu & Tatsumi, 1984; Tatsumi *et al.* 1978, 1981 & 1984). Reduction of the nitro group may result in the formation of reactive intermediates, which may covalently bind to biological macromolecules such as protein and DNA (Swaminathan & Lower, 1978).

As a possible model for *in vivo* metabolism, we describe in this paper the *in vitro* aerobic and anaerobic metabolism of furazolidone by rat liver microsomes and the compound's interaction with microsomal protein and added calf thymus DNA,

MATERIALS AND METHODS

Chemicals. [¹⁴C]Furazolidone was a gift from Norwich Eaton Pharmaceuticals Inc., Norwich, NY, USA. It was labelled in the methylene groups of the oxazolidone moiety (specific activity, 2.69 μCi/mg; radiochemical purity >99% by TLC (toluene–acetic acid–acetone–water, 600:240:80:25, by vol.) and HPLC). The counting fluids used were Minisolve (Zinsser Analytic (UK) Ltd, Maidenhead, Berks.) and Dimilume-30® (Packard Instrument BV, Delft) for aqueous and non-aqueous samples, respectively. NADPH, glucose-6-phosphate and glucose-6-

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Abbreviations: GSH = glutathione; HPLC = high-pressure liquid chromatography; 3MC = 3-methylcholanthrene; PB = phenobarbital; TLC = thin-layer chromatography.

phosphate dehydrogenase were obtained from Boehringer Mannheim GmbH, Mannheim, FRG. DNA (calf thymus, type 1) and reduced glutathione (GSH) were purchased from Sigma Chemical Co., St Louis, MO. NADPH–cytochrome-P-450 reductase (purification and measurement of activity carried out according to Yasukochi *et al.* 1979) was kindly supplied by Dr P. Bonants of the Department of Biochemistry of the Agricultural University, Wageningen. All the other chemicals used were of reagent grade.

Preparation of microsomes. Male Wistar rats were pretreated either with 3-methylcholanthrene (3MC), administered as three daily ip injections each of 30 mg/kg body weight, or with phenobarbital (PB) given over 7 days at a level of 0.1% in the drinking-water. An untreated group served as the control. Livers were perfused with a 20 mM-Tris HCl, 250 mM-sucrose buffer (pH 7.4), homogenized in a teflon Potter–Elvehjem homogenizer and centrifuged for 20 min at 9000 g. The supernatant (S-9 fraction) was centrifuged for 90 min at 105,000 g. After the supernatant (cytosolic fraction) had been decanted, the microsomal pellet was washed in 150 mM-KCl and then resuspended in 100 mM-potassium phosphate buffer, pH 7.4. The S-9 fraction, cytosol and microsomes were all stored at −25°C. Protein concentration was determined by the method of Lowry *et al.* (1951).

Microsomal incubation. Unless stated otherwise, a standard incubation mixture was used containing 1 mM-NADPH, 3 mM-MgCl₂, 0.1 M-potassium phosphate buffer, pH 7.4, and 1.46–2.95 mg protein. Incubations were started by the addition of 50 μl [¹⁴C]furazolidone dissolved in acetone (final concentration, 36.7 μM). The final volume was 2 ml. After incubation for 5 min at 37°C the reaction was terminated by the addition of 1 ml acetone. Non-polar metabolites were extracted with 4 × 2 ml ethyl acetate. After evaporation of the ethyl acetate under nitrogen, the residue was dissolved in 300 μl acetonitrile–water, 30:70, v/v (I; representing the ethyl acetate fraction) and used for HPLC or radioactivity measurements. After extraction with ethyl acetate, the protein in the aqueous phase was precipitated by the addition of ice-cold methanol. After centrifugation for 15 min at 1500 g, the aqueous/methanolic supernatant was decanted and evaporated under nitrogen. The polar metabolites were dissolved in 300 μl acetonitrile–water, 30:70, v/v (II; representing the aqueous fraction) and were used for radioactivity measurements or HPLC. The precipitated protein was washed three times with methanol, twice with ethanol and twice with ether. The resulting pellet (III; representing the protein fraction) was dissolved in 1 ml Soluene 350 (Packard Instrument BV), incubated for at least 4 hr at room temperature and used for measurement of the radioactivity (the amount of radioactivity defined as originating from covalently bound residues). After the protein was washed with methanol, no more radioactivity could be extracted. Anaerobic conditions were achieved by shaking the incubation mixture for 3 min under a constant flow of argon, after which the incubation bottles were sealed. For incubations longer than 5 min, an NADPH-regenerating system—10 mM-glucose-6-phosphate and 10 U glucose-6-phosphate

dehydrogenase—was added to the medium. Calf thymus DNA, added at 1 mg/ml of incubation mixture, was isolated as described elsewhere (Jeffreys & Flavell, 1977), dissolved in 10 mM-Tris HCl, pH 7.5, and prepared for radioactivity measurements. In studies on the effect of GSH on microsomal metabolism of furazolidone, GSH was added to the incubation mixture at levels of 0.5–10 mM. Incubations with microsomes inactivated by heating or without NADPH served as controls. Incubations and all other procedures before HPLC analysis were performed in the dark because of the photosensitivity of furazolidone in solution (Kalim, 1985).

HPLC analysis. HPLC analyses were performed with a Perkin–Elmer Series 4 HPLC system. Separation of the non-polar metabolites of furazolidone was achieved on a Chrompack Hypersil 5 ODS column (250 × 4.6 mm). After injection of 100 μl of an incubation sample, all radioactivity (>99%) was eluted with a linear 22-min gradient of 2–100% acetonitrile in water–1.0 M-acetate buffer pH 5.0–acetonitrile (900:75:25, by vol.) at a flow rate of 1 ml/min. The eluate was monitored at 254 nm and for radioactivity measurements 0.2 ml fractions were collected. After addition of 3 ml Minisolve, the amount of radioactivity was determined in a Philips liquid scintillation counter (type PW 4700). Counting efficiency was calculated by means of an external standard counting procedure. The data presented in this paper are representative of at least three experiments. The experimental variation was within 10%.

Identification of non-polar metabolites of furazolidone. For identification of the non-polar metabolites of furazolidone, aliquots of fraction I (see earlier) were chromatographed as described above. Ultraviolet (UV) absorption spectra were obtained with a Hewlett–Packard photodiode array detection system (detector 1040 A, diskdrive 9121, computer 85B and plotter 7470A). Infrared (IR) spectra of the various compounds in 5 mg KBr were recorded on a Bruker IFS-85 instrument with diffuse reflectance techniques. Proton nuclear magnetic resonance (NMR) spectra of furazolidone or its metabolites dissolved in CDCl₃ were obtained with a Bruker CXP 300. Mass spectra were obtained with an AEI MS 902 mass spectrometer coupled to a VG-ZAB console.

RESULTS

Analytical data

Chromatographic analysis of fraction I (the ethyl acetate extract of the product of microsomal metabolism of furazolidone) revealed four major peaks— A, B, V and furazolidone (Fig. 1). When the same reversed-phase HPLC method was used for samples of fraction II, most (>85%) of the radioactivity appeared at the solvent front, indicating the high polarity of the compounds in that fraction. With fraction I, peak A appeared with the solvent front. There are indications of at least three different radioactive substances in that peak but they have not yet been clearly identified.

Characteristics of metabolites B and C are shown in Table 1. The double doublet at δ 4.83 in the NMR spectrum of metabolite C (Fig. 2) coupled with the doublet at δ 5.85 and a proton in the multiplet at δ
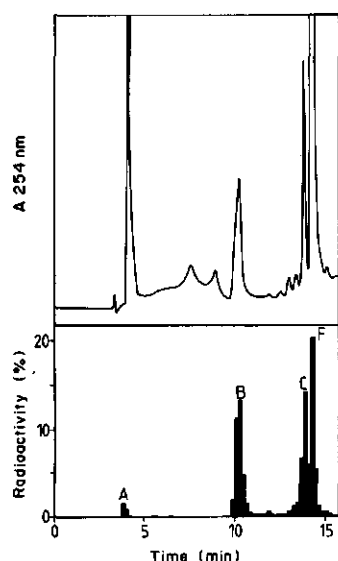
Fig. 1. HPLC analysis of metabolites (A, B and C) of [^{14}C]furazolidone (F) present in the ethyl acetate extract after microsomal incubation under aerobic conditions.

3.89. This proton at δ 3.86 also coupled with the doublet at δ 2.35. Coupling was proved by using spin decoupling methods.

In vitro *metabolism of furazolidone*

The *in vitro* aerobic metabolism of furazolidone by liver microsomes from 3MC-induced male rats is characterized by rapid conversion of the parent compound. After a 5-min incubation period, 36.1% of furazolidone was converted, of which 44.2% was located in the ethyl acetate extract and 52.9% in the aqueous phase, while 2.9% was associated with protein (Table 2). Two major metabolites (B and C) were isolated from the ethyl acetate extract (Fig. 1). Characteristics similar to those found for metabolite B (Table 1) have been described earlier for a furazolidone metabolite which was identified as

3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone (Fig. 3), the open-chain cyano derivative (Abraham *et al.* 1984; Tatsumi *et al.* 1978). The UV, IR and NMR spectra of metabolite C showed great similarity to the spectra of a product of furazolidone obtained after *in vitro* metabolism using milk xanthine oxidase and rat liver 9000-g supernatant (Tatsumi *et al.* 1981). This reduction product was tentatively identified by the authors as 2,3-dihydro-3-cyanomethyl-2-hydroxy-5-nitro-α,2-di-(2-oxo-oxazolidin-3-yl)imino-methylfuro[2,3-b]furan (Fig. 3). The doublet at δ 2.35 (2H), which can be attributed to two methylene protons, is even more likely than a signal overlapped by a signal due to water at δ 3.34, as assumed by Tatsumi *et al.* (1981).

The characteristics of the metabolism of furazolidone by rat liver microsomes are shown in Table 2. Conversion of furazolidone at a rate of 3.52 nmol/mg protein/min was measured under aerobic conditions. The amount of material converted was approximately 20% higher under anaerobic than under aerobic conditions. Microsomes of untreated male rats metabolized furazolidone at a slower rate than microsomes of 3MC-treated male rats (2.29 and 3.52 nmol/mg protein/min, respectively). There was no significant difference in conversion rates between microsomes of PB-induced and 3MC-induced male rats, but small differences in the relative amounts of the metabolites formed could be observed. An increase in the relative amount of metabolite B and a decrease in the relative amount of metabolite C were observed on increasing the incubation time from 5 to 15 min. No further changes in the percentages of individual metabolites in the ethyl acetate extract were observed on increasing the incubation time from 15 to 60 min (Table 2).

The conversion of furazolidone is an enzymatic NADPH-dependent process, which is located in the microsomal fraction but is independent of cytochrome P-450. Thus, no conversion of furazolidone was observed when the microsomes were inactivated by heating, or when NADPH was absent from the incubation mixture, indicating that NADPH-dependent enzymes are involved in the metabolism of furazolidone by rat liver microsomes. Furthermore, the fact that no influence on the metabolism of furazolidone could be detected on saturation of the incubation mixture with carbon monoxide (Table 3)

Table 1. Characteristics of metabolites B and C formed during microsomal metabolism of furazolidone

| Parameter	Metabolite B	Metabolite C
UV spectrum, λ_{max} (nm)	226, 268	226, 275
IR spectrum in KBr (cm^{-1})	2250: cyano group	3207: hydroxyl group (Fig. 2)
	1778: oxazolidone, C = 0	2247: cyano group
	1688: α, β-unsaturated C = 0 stretching	1769: oxazolidone, C = 0
		1561: nitro group
		1358: nitro group
H-NMR in CDCl, (ppm)	7.07, singlet (1H)	7.43, singlet (1H) (Fig. 2)
	4.64, triplet (2H)	7.38, singlet (1H)
	3.87, triplet (2H)	5.85, doublet (1H)
	3.33, triplet (2H)	4.83, double doublet (1H)
	2.67, triplet (2H)	4.58, multiplet (4H)
		3.89, multiplet (5H)
		2.35, doublet (2H)
Mass spectrum (m/z)		
—field desorption	195 (molecular ion)	
—electron impact	113, 82 (fragment ions)	
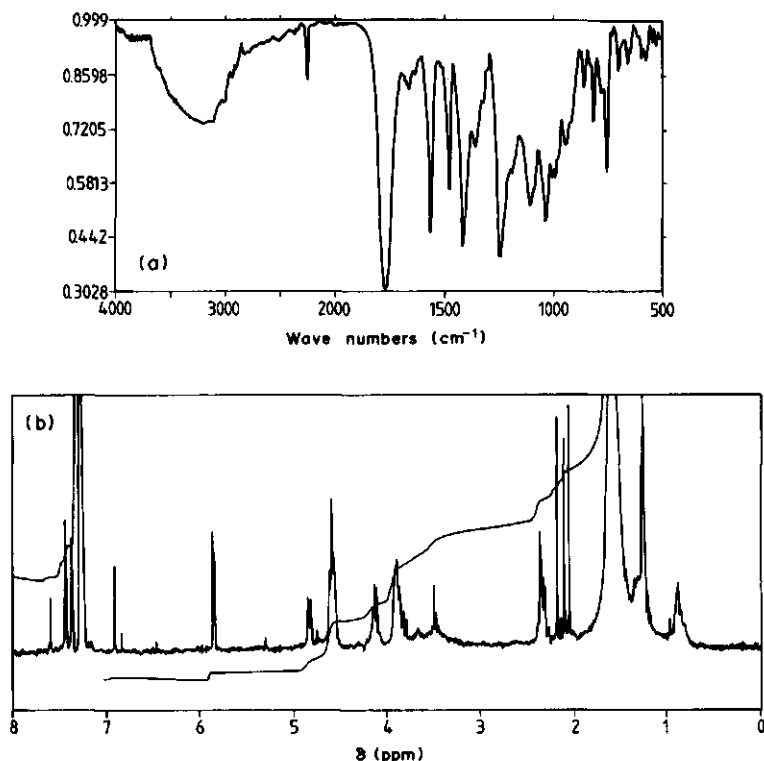
Fig. 2. (a) IR and (b) NMR spectra of metabolite C of furazolidone.

indicated that cytochrome *P*-450 is not involved in the conversion of furazolidone by rat liver microsomes *in vitro*. Furazolidone was metabolized to some extent under aerobic conditions by the 9000-g rat liver fraction, but at a much lower rate (0.67 nmol/mg protein/min) than by the microsomal fraction, while the cytosolic fraction of rat liver hardly converted furazolidone at all (Table 3).

A well known NADPH-dependent microsomal reductase is NADPH–cytochrome *P*-450 reductase (cytochrome *c* reductase). Purified cytochrome *P*-450 reductase converted furazolidone under anaerobic conditions into the same metabolites as did the microsomes (Table 4). The conversion rate for the purified enzyme was 14.5 nmol/min/unit of reductase compared with a conversion rate for the microsomes (0.2 units NADPH-dependent cytochrome *P*-450 reductase/mg protein) of 21.3 nmol/min/unit of reductase (Table 2). No conversion by the purified enzyme could be measured under aerobic conditions (data not shown) or in the absence of NADPH (Table 4).

Covalent binding of furazolidone to protein and DNA and the effect of glutathione

Under standard aerobic incubation conditions, detected covalent binding of furazolidone to protein amounted to 0.52 nmol/mg protein (2.9% of total metabolites). The amount of furazolidone associated with protein increased under anaerobic conditions and also with an increase in the amount of microsomal protein in the incubation mixture or with an increase in the incubation time (Table 2). No protein binding could be detected when the microsomes were inactivated by heating or when NADPH was absent from the incubation mixture (Table 2). Addition of calf thymus DNA (1 mg/ml) to the incubation mixture did not influence the *in vitro* conversion of furazolidone by microsomes of male rats treated with 3MC (Tables 2 & 5). No radioactivity was detected bound to DNA even after an incubation time of 60 min.

When GSH was added to the incubation mixture in a concentration of 2 mM or more, the metabolites B and C were not detected, but an increase in polar metabolites and a decrease in covalent binding to protein was observed (Table 5). Under anaerobic conditions the rate of total conversion was independent of the addition of GSH, but under aerobic conditions a decrease in rate of up to 60% was observed.

DISCUSSION

Reduction of the nitro group is an important metabolic pathway for nitrofurans (Swaminathan &

Table 2. Metabolism of furazolidone by rat liver microsomes

Treatment of male rats	Incubation time (min)	Microsomal protein (mg/2 ml*)	Specific conditions	Main EtAc-soluble metabolites (% of total metabolites in fraction I)			Metabolites (% of total) in fraction:			Covalent binding (nmol/mg protein)	Total conversion (%)†°
				A	B	C	I (EtAc)	II (water)	III (protein)		
3MC	5	1.48	Standard	5.5	37.5	37.1	44.2	52.9	2.9	0.52	36.1 (3.52)
	15	1.48	NADPH R/S‡	4.4	50.9	30.8	52.4	45.3	2.3	0.89	78.3 (2.59)
	30	1.48	NADPH R/S‡	5.4	50.9	29.8	53.0	44.7	2.3	1.02	89.0
	60	1.48	NADPH R/S‡	6.9	51.0	26.9	57.2	40.8	2.0	0.90	90.3
	5	2.95	Standard	4.4	47.3	34.8	45.7	48.7	5.7	0.81	56.9 (2.83)
	5	1.48	Anaerobic	11.2	40.1	26.9	48.5	47.1	4.4	0.94	42.9 (4.26)
	5	2.27		8.7	51.3	20.4	40.9	52.5	6.6	1.28	59.4 (3.85)
		1.48	Boiled microsomes	ND	ND	ND	ND	ND	ND	ND	ND (—)
			Without NADPH	ND	ND	ND	ND	ND	ND	ND	ND (—)
PB	5	1.48	Standard	8.0	27.4	36.6	36.5	59.8	3.7	0.63	34.5 (3.43)
	5	2.57	Standard	10.0	36.5	31.4	46.6	47.1	6.5	1.13	61.2 (3.50)
Nil	5	1.93	Standard	15.6	35.1	27.2	46.8	47.2	6.1	0.69	30.0 (2.29)

*2 ml of incubation mixture.
†Numbers in brackets are rates of metabolism expressed as nmol substrate metabolized/mg protein/min.
‡NADPH regenerating system: 10 mM-glucose-6-phosphate and 10 U glucose-6-phosphate dehydrogenase.

EtAc = Ethyl acetate 3MC = 3-Methylcholanthrene ND = Not detectable PB = Phenobarbital
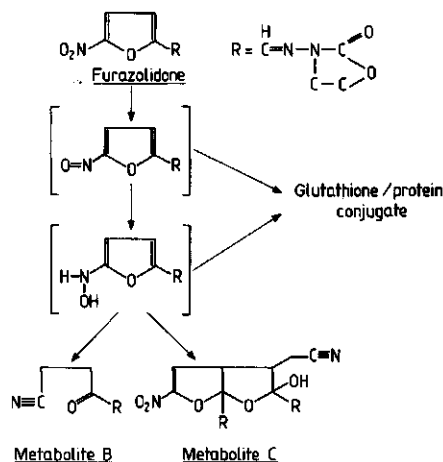
Fig. 3. Proposed metabolic pathway of furazolidone.

Lower, 1978). Several metabolites of *in vivo* or *in vitro* reductive metabolism of furazolidone have already been identified (Abraham *et al.* 1984; Nakabeppu & Tatsumi, 1984; Tatsumi *et al.* 1978, 1981 & 1984). This report presents data that support the reductive metabolism of furazolidone *in vitro:*

(1) The well-known 3-(4-cyano-2-oxobutyl-ideneamino)-2-oxazolidone (metabolite B), the open-chain cyano derivative that has also been found in other biological systems such as rabbits (Tatsumi *et al.* 1978), eels (Nakabeppu & Tatsumi, 1984), rats (Tatsumi *et al.* 1984), rat liver fractions (Abraham *et al.* 1984; Tatsumi *et al.* 1981) and *Escherichia coli* (Abraham *et al.* 1984), has been identified as an end product of the reductive metabolism of furazolidone.

(2) In contrast to another study on the metabolism of furazolidone by rat liver microsomes (Abraham *et al.* 1984), we found a second non-polar metabolite (C). The characteristics of this metabolite show great similarity to the characteristics of a reduction product of furazolidone obtained after *in vitro* metabolism using milk xanthine oxidase and rat liver 9000-g supernatant (Tatsumi *et al.* 1981). The presence of a cyano group in the IR spectrum indicates that this metabolite is the result of a process of furazolidone reduction. When metabolite C was separated from metabolite B and then redissolved in water, no metabolite B was detected by HPLC analysis, and similarly no metabolite C was detected in an aqueous solution of isolated metabolite B, indicating that no equilibrium exists between these two non-polar metabolites. Furthermore the concentration ratio of the two non-polar metabolites did not change on prolonged incubation (> 15 min), indicating that a common intermediate may precede the formation of both metabolites.

(3) The nitro reduction may be catalysed by nitro reductases such as the microsomal enzyme

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Table 3. Aerobic metabolism of furazolidone by several liver fractions from 3-methylcholanthrene-induced male rats after incubation for 5 min

Liver fraction	Protein (mg/2 ml*)	Specific conditions	Main EtAc-soluble metabolites (% of total metabolites in fraction)			Metabolites (% of total) in fraction:			Covalent binding (nmol/mg protein)	Total conversion (%)†
			A	B	C	I (EtAc)	II (water)	III (protein)		
Cytosol	1.48	Standard	ND	ND	ND	ND	100.0	ND	ND	0.6 (0.06)
S-9 fraction	1.47	Standard	13.9	29.1	33.8	25.2	62.4	12.5	0.42	6.7 (0.67)
Microsomes	1.48	+CO	6.0	29.7	41.8	49.8	47.3	2.9	0.52	35.8 (3.56)
Microsomes		Standard	5.5	37.5	37.1	44.2	52.9	2.9	0.52	36.1 (3.52)

EtAc = Ethyl acetate   ND = Not detectable

*2 ml of incubation mixture.
†Numbers in brackets are rates of metabolism expressed as nmol substrate metabolized/mg protein/min.

NADPH–cytochrome P-450 reductase (Gillette, 1971). This is supported by the observation that the conversion of furazolidone under anaerobic conditions by purified NADPH–cytochrome P-450 reductase resulted in the same end products as were obtained after anaerobic conversion by rat liver microsomes. Furthermore, the characteristics of this microsomal conversion of furazolidone are similar to the NADPH–cytochrome P-450 reductase activities demonstrated for the metabolism of several other nitrofurans (Boyd et al. 1979; Mattammal et al. 1985; Swaminathan & Lower, 1978; Wang et al. 1974; Zenser et al. 1981). Under aerobic conditions, furazolidone is also converted by rat liver microsomes. Nitro reduction under aerobic conditions has been reported elsewhere for rat liver microsomes (Abraham et al. 1984) and isolated perfused rat liver (Bond et al. 1984). However no conversion under aerobic conditions could be measured in studies with purified NADPH–cytochrome P-450 reductase. The reason for this difference is not known, but may be found in the re-oxidation of the initial product by oxygen (Gillette, 1971), a reaction that may occur more readily with the purified enzyme than when the enzyme is present in liver microsomes.

Although PB, in contrast to 3MC, is an inducer of NADPH–cytochrome P-450 reductase (Gillette, 1971; Wang et al. 1974) no difference in furazolidone conversion under aerobic conditions was observed between microsomes from 3MC- and from PB-induced rats, indicating the possible involvement of other NADPH-dependent enzymes that may be induced by 3MC-pretreatment of rats. This is supported by the observation that under anaerobic conditions the conversion rate for microsomes (nmol/min/unit of NADPH-dependent cytochrome P-450 reductase) was some 50% higher than the conversion rate for the purified enzyme.

Reduction of the nitro group in nitrofurans is a multi-step process with two important intermediates—the nitroso derivative and subsequently the hydroxylamine derivative (Swaminathan & Lower, 1978; Fig. 3). These reactive intermediates are believed to be responsible for the inactivation of susceptible bacterial strains (Asnis et al. 1952; Paul et al. 1952) and for the mutagenic and carcinogenic activities of some nitrofurans (Cohen,

Table 4. In vitro anaerobic conversion of furazolidone (36.7 μM) by purified NADPH-dependent cytochrome P-450 reductase

Incubation time (min)	Specific conditions*	Main EtAc-soluble metabolites (% of total metabolites in fraction I)			Metabolites (% of total) in fraction:		Total conversion (%)
		A	B	C	I (EtAc)	II (water)	
0	Standard	ND	ND	ND	ND	ND	ND
2	Standard	12.6	26.2	32.7	15.9	84.1	79.0
5	Standard	12.4	27.4	35.0	19.4	80.6	88.7
30	Standard	13.7	29.5	33.6	24.8	75.2	88.1
30†	†	13.4	33.2	37.5	25.3	74.7	84.5
30	Without NADPH	ND	ND	ND	ND	ND	ND

EtAc = Ethyl acetate   ND = Not detectable
*Cytochrome P-450 reductase was added throughout at 2 U/2 ml incubation mixture, 1 U being the amount of reductase that reduces 1 μmol cytochrome c/min at 25°C.
†Incubation for 15 min under anaerobic conditions followed by 15 min in an aerobic atmosphere.

Table 5. Influence of addition of glutathione (GSH) or DNA on the aerobic and anaerobic metabolism of furazolidone by liver microsomes from 3-methylcholanthrene-induced male rats after incubation for 5 min

GSH (mM)	DNA (mg/2 ml*)	Conditions†	Main EtAc-soluble metabolites (% of total metabolites in fraction I)			Metabolites (% of total) in fraction:			Covalent binding (nmol/mg protein)	Total conversion (%)‡
			A	B	C	I (EtAc)	II (water)	III (protein)		
0	—	Aerobic	5.5	37.5	37.1	44.2	52.9	2.9	0.52	36.1 (3.52)
0.5	—	Aerobic	27.7	19.6	15.7	11.5	85.5	2.9	0.45	31.1 (3.09)
1	—	Aerobic	28.5	17.0	13.6	7.3	90.7	2.0	0.29	28.7 (2.85)
2	—	Aerobic	24.3	ND	ND	5.7	92.6	1.7	0.13	14.9 (1.48)
5	—	Aerobic	43.0	ND	ND	5.7	93.4	1.0	0.09	17.9 (1.78)
10	—	Aerobic	37.7	ND	ND	8.7	90.4	0.9	0.06	12.6 (1.25)
0	—	Anaerobic	11.2	40.1	26.9	48.5	47.1	4.4	0.94	42.9 (4.26)
2	—	Anaerobic	38.2	ND	ND	7.8	90.9	1.3	0.30	46.7 (4.63)
5	—	Anaerobic	50.3	ND	ND	6.5	92.8	0.7	0.15	42.2 (4.19)
10	—	Anaerobic	62.6	ND	ND	6.1	93.5	0.4	0.08	38.4 (3.82)
—	1	Aerobic	5.0	47.1	39.8	48.0	52.0	—§	—§	38.2 (3.79)
—	2	Anaerobic	10.2	35.6	28.7	49.4	50.6	—§	—§	44.8 (4.45)

EtAc = Ethyl acetate   ND = Not detectable

*2 ml of incubation mixture.
†Each incubation mixture contained 1.48 mg protein/2 ml.
‡Numbers in brackets are rates of metabolism expressed as nmol substrate metabolized/mg protein/min.
§DNA was purified and separated from the protein by an enzymatic splitting of the protein, so the amount of radioactivity in the protein could not be measured.

1978; Klemencic & Wang, 1978). Reduction of aromatic nitro compounds to arylamines has been shown to occur via the same reactive intermediates *in vivo* and *in vitro* (Kriek & Westra, 1979). These nitroso and hydroxylamine derivatives of aromatic compounds easily react with GSH (Dölle *et al.* 1980; Eyer, 1979; Eyer & Kampffmeyer, 1982; Eyer & Schneller, 1983; Mulder *et al.* 1982 & 1984). In the present study, it was found that the non-polar metabolites could not be detected upon addition of GSH, in concentrations at or above 2 mM, to the incubation mixture. Such an effect has also been described in *E. coli* systems (Abraham *et al.* 1984). In addition, the covalent binding of [¹⁴C]furazolidone to protein decreased upon addition of GSH. These findings are probably the result of an interaction of the nitroso or hydroxylamine derivative of furazolidone with GSH. GSH conjugation has already been described for the nitrofuran N-(4-(5-nitro-2-furyl)-2-thiazolyl)acetamide (Mattammal *et al.* 1985). Studies aimed at the elucidation of the structure of the protein adduct(s) *in vitro* and *in vivo* are being undertaken.

The N-hydroxy derivatives are the intermediates responsible for interaction with DNA after activation of aromatic amines and amides by N-oxidation (Dölle *et al.* 1980; Eyer, 1979; Eyer & Kampffmeyer, 1982; Eyer & Schneller, 1983; Kriek & Westra, 1979; Mulder *et al.* 1982 & 1984). In the study reported here, no interaction with calf thymus DNA was observed *in vitro*, in contrast to findings *in vivo* (Vroomen *et al.* 1986). Perhaps a difference exists between the *in vitro* and *in vivo* situation in the availability of hydroxylamine derivatives for reaction with DNA.

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CHAPTER 6: Identification of a reactive intermediate of furazolidone
formed by swine liver microsomes
Chemico-Biological Interactions, in press.

IDENTIFICATION OF A REACTIVE INTERMEDIATE OF FURAZOLIDONE FORMED BY SWINE LIVER MICROSOMES

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SUMMARY

Furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone) is
metabolized by swine liver microsomes under aerobic and anaerobic
conditions (rate: 2.55 and 3.25 nmol per mg protein per minute respec-
tively). Covalent binding to microsomal protein amounted aerobically
to 0.29 nmol per mg protein per minute. Of all amino acids tested,
only addition of cysteine to the incubation mixture decreased micro-
somal protein binding of furazolidone, indicating that covalent
binding may occur at protein thiol groups. Two known metabolites of
furazolidone, 3-(4-cyano-2-oxobutylidene-amino)-2-oxazolidone and 2,3
dihydro-3-cyanomethyl-2-hydroxyl-5-nitro-1 α, 2-di(2-oxo-oxazolidin3-yl)
iminomethyl-furo[2,3-b]furan, were minor metabolites. At least 50% of
total metabolites is formed by swine liver microsomes via a reductive
process of furazolidone as indicated by the formation of a furazoli-
done-mercaptoethanol conjugate after the addition of mercapto-ethanol
to the incubation mixture. The conjugate was identified as 3-(4cyano-
3-β-hydroxyethylmercapto-2-oxobutylidene amino)-2-oxazolidone, indi-
cating that the open-chain acrylonitrile-derivative is the reactive
intermediate of furazolidone which also may be responsible for inter-
action with protein.

INTRODUCTION

Furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone) is a
nitrofuran widely used as an antimicrobial agent in veterinary practice.
Because of its mutagenic and carcinogenic properties (1,2) residues
should be avoided in edible tissues of animals treated with the drug.
In the case of treatment of swine with furazolidone, no furazolidone
could be detected 2-4 hours after terminating a prolonged treatment
(3,4). However, using $[^{14}C]$-furazolidone it was shown that radio-
activity at μg-equivalents of parent compound per gram of tissue could
be detected in tissues, even 14 days after medication withdrawal. A
major part of this residual radioactivity was non-extractable, presu-
mably covalently bound to cellular macromolecules (4). No information
is available concerning the identity and toxicity of these residual
metabolites, which hampers a proper evaluation of the potential risk
for humans of edible tissues of animals treated with furazolidone.
In general reduction of the nitro-group is a major metabolic pathway
of nitrofurans, resulting in the formation of nitroso-and hydroxyl-
amino-derivatives as reactive intermediates responsible for inter-
action with biological macromolecules, and ultimately in a cyano-
derivative as stable endproduct of reductive metabolism (5).
The cyano-derivative 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone
of furazolidone has been described as an important metabolite _in vivo_
in rats, rabbits and eels (6,7,8) and in model systems using rat liver
fractions and E. coli (9,10,11). In swine however, formation of this
cyano-derivative occurred to a minor extent indicating a species
difference in metabolism of furazolidone (12).

We have studied into more detail the metabolic conversion of furazo-
lidone in swine liver microsomes in order to elucidate the biotrans-
formation pathway in the target animal and to identify intermediate(s)
which may react with cellular protein. Furthermore possible reaction
sites for the interaction between furazolidone and microsomal proteins
are suggested.

MATERIAL AND METHODS

Chemicals. [^{14}C]-furazolidone was a gift of Norwich Eaton Pharmaceuticals
Inc. Norwich, New York; it was labeled in the methylene groups of the
oxazolidone moiety (specific activity: 605 µCi/mmol, radiochemical
purity > 99% by TLC-toluene/acetic acid/acetone/water = 600:240:80:25
by vol. and HPLC-conditions see below).
Counting fluids: Minisolve (Zinsser Analytical (UK) LTD, Maidenhead)
and Dimilume-30^R (Packard Instrument BV, Delft) for aqueous and non-
aqueous samples, respectively. NAD(P)H, glucose-6-phosphate and
glucose-6-phosphate dehydrogenase were obtained from Boehringer
Mannheim GmbH, Mannheim, FRG.
The deoxyribonucleosides and reduced glutathione (GSH) were purchased
from Sigma Chemical Co. St. Louis, MO. The amino acids (Standard Kit
22) were purchased from Pierce Chemical Company, Rockford Illinois,
USA. All other chemicals used were of reagent grade.

Preparation of microsomes. A liver of a 90 kg weighing female swine,
with a known history without drug treatment, was perfused with a 1.15%
KCl-solution, homogenized in a teflon Potter-Elvehjem homogenizer and
centrifuged for 20 min at 9000 g.
The supernatant (S9-fraction) was centrifuged for 90 min at 105,000 g.
After the supernatant (cytosolic fraction) had been decanted, the
microsomal pellet was washed in 1.15% KCl and then resuspended in
100 mM-potassium phosphate buffer, pH 7.4.
The S9-fraction, cytosol and microsomes were all stored in liquid
nitrogen. Protein concentration was determined by the method of
Lowry et al. (13).

Microsomal incubation. Unless stated otherwise, a standard incubation
mixture was used containing 1 mM-NADPH, 3 mM-MgCl$_2$, 0.1 M-potassium
phosphate buffer, pH 7.4, 2.61 mg protein, 10 mM-glucose-6-phosphate
and 10 U glucose-6-phosphate dehydrogenase.
After a preincubation of 2 minutes, incubations were started by the
addition of [^{14}C]-furazolidone dissolved in 50 µl of acetone (final
concentration, 36.7 µM; corresponding to 44.4 x 10^{-3} µCi per 2 ml
incubation mixture). The final volume was 2 ml. After standard
incubation for 6 min at 37°C the reaction was terminated by the addi-
tion of 1 ml of acetone. Non-polar metabolites were extracted with
4 x 2 ml of ethyl acetate. After evaporation of the ethyl acetate in
a stream of nitrogen, the residue was dissolved in 300 µl of acetoni-
trile-water, 30:70, v/v (I; representing the ethyl acetate fraction)

and used for HPLC or radioactivity measurements. The HPLC-recovery
of the ethyl acetate fraction was > 95%. After extraction with ethyl
acetate, the protein in the aqueous phase was precipitated by the
addition of ice-cold methanol. After centrifugation for 15 min at
1500 g, the aqueous/methanolic supernatant was decanted and evaporated
in a stream of nitrogen. The polar metabolites were dissolved in 300 µl
of acetonitrile-water, 30:70, v/v (II; representing the aqueous frac-
tion) and were used for radioactivity measurements. The precipitated
protein was washed three times with methanol, twice with ethanol and
twice with diethyl ether. The resulting pellet (III; representing the
protein fraction) was dissolved in 1 ml of soluene 350 (Packard
Instrument BV) and used for measurement of the radioactivity (the
amount of radioactivity defined as originating from covalently bound
residues). After the protein was washed with methanol, no more
radioactivity could be extracted.
Anaerobic conditions were achieved by shaking the incubation mixture
for 3 min under a constant flow of argon, after which the incubation
bottles were sealed.
In studies on the effect of GSH, mercapto-ethanol, desoxyribonucleosi-
des and amino acids on the microsomal metabolism of furazolidone,
these reagents were added to the incubation mixture at levels of
5 mM or 10 mM.
Incubations with microsomes inactivated by boiling for 3 min or
without NADPH served as controls. Incubations and all other procedures
before HPLC analysis were performed in the dark because of the photo-
sensitivity of furazolidone in solution (14).

HPLC analysis. HPLC analyses were performed with a Waters 6000 A
HPLC system. Separation of the non-polar metabolites of furazolidone
and the mercapto-ethanol conjugate was achieved on a Chrompack Hyper-
sil 5 ODS column (250 x 4.6 mm). After injection of 100 µl of an incu-
bation sample, all radioactivity was eluted with a linear 22-min
gradient of 2-100% acetonitrile in a mixture of water-1.0 M-sodium-
acetate buffer pH 5.0-acetonitrile (900:75:25, by vol.) at a flow rate
of 1 ml/min. The eluent was monitored at 254 nm and for radioactivity
measurements 0.2 ml fractions were collected.
After addition of 3 ml of Minisolve, the amount of radioactivity was
determined in a Philips liquid scintillation counter (type PW 4700).
Counting efficiency was calculated by means of an external standard
counting procedure. Data presented in this paper are mean values of
at least three experiments. The experimental variation was within
10 percent.
HPLC-analysis of the aqueous phase after incubation in the absence or
presence of glutathione was performed on a Hamilton PRP-1 column using
a 22 min gradiënt of 2-99% acetonitrile in a 5 mM ammonia formate
buffer, pH 3.6, at a flow rate of 1 ml/min (curve 7). The protein was
separated by ultrafiltration (Amicon Centrifree Micropartition System).
The eluent was monitored at 254 nm and for radioactivity measurements
0.2 ml fractions were collected.

Identification of non-polar metabolites and a mercapto-ethanol con-
jugate. For identification of a mercapto-ethanol conjugate of fura-
zolidone, aliquots of fraction I (see earlier) were chromatographed on
a semi-preparative Hibar prepacked column 250 mm x 10 mm Lichrosorb
RP-18 (7 µm) using a linear 22-min gradient of 2-100% acetonitrile in

a mixture of water – 1.0 M sodium acetate buffer pH 5.0 – acetonitrile (900:75:25, by vol.) at a flow rate of 4 ml/min. The eluent was monitored at 254 nm.
The fractions containing a mercapto-ethanol conjugate were collected and after evaporation to dryness under vacuum and redissolving in acetonitrile/water (20/80) the conjugate was chromatographed isocratically on the same column (acetonitrile/water:20/80). Fractions containing the mercapto-ethanol conjugate of furazolidone were collected and after gentle evaporation of the solvent under nitrogen used for identification.
Ultraviolet (UV) absorption spectra were obtained with a Hewlett-Packard photodiode array detection system (detector 1040 A, diskdrive 9121, computer 85B and plotter 7470A). Infrared (IR) spectra of the various compounds in 5 mg KBr were recorded on a Bruker IFS-85 instrument with diffuse reflectance techniques. Proton nuclear magnetic resonance (NMR) spectra of metabolites dissolved in $CDCl_3$ were obtained with a Bruker CXP-300. Mass spectra were obtained with an AEI MS 902 mass spectrometer coupled to a VG-ZAB console.

RESULTS

In vitro metabolism of furazolidone

Conversion of furazolidone by swine liver microsomes was linear for

at least 8 min under the standard conditions used.

After a 6-min aerobic incubation, 53.6% of the furazolidone was con-

verted (2.55 nmol/mg protein/min) of which 27.4% was extractable in

ethyl acetate and 61.1% in water, while 11.6% was covalently bound to

protein (Table 1). Under anaerobic conditions the amount of converted

furazolidone was approximately 25% higher (rate: 3.25 nmol/mg

protein/min). No conversion of furazolidone was observed when the

microsomes were inactivated by heating, when NADPH was absent from the

incubation mixture or when NADH was used instead of NADPH, indicating

that NADPH-dependent enzymes are involved in the metabolism of

furazolidone by swine liver microsomes. Furthermore cytochrome P-450

is not involved in this conversion of furazolidone as indicated by

the fact that carbon monoxide caused only a slight decrease in total

conversion on saturation of the incubation mixture. Similar results

have been described for rat liver microsomes (10). The cytosolic

Table 1: Metabolism of furazolidone by liver microsomes of pigs and rats

	Incubation time (min)	Microsomal protein (mg/2 ml*)	n	Specific conditions	Main EtAc-soluble metabolites (% of total metabolites in fraction I)			Metabolites (% of total) in fraction			Covalent binding (nmol/mg protein/min)	Total conversion % +	
					A	B	C	I (EtAc)	II (water)	III (protein)			
pig	6	2.61	5	aerobic	18.9	38.4	23.3	27.4	61.1	11.6	0.29	53.6	(2.55 ± 0.18)
pig	6	5.22	3	aerobic	20.9	31.1	19.6	17.4	60.8	21.8	0.38	74.0	(1.76 ± 0.08)
pig	6	2.61	3	+CO	24.1	21.9	19.2	15.2	71.3	13.2	0.30	49.1	(2.33 ± 0.12)
pig	6	2.61	6	anaerobic	17.3	35.6	26.0	26.1	62.0	12.0	0.28	68.5	(3.25 ± 0.19)
rat**	5	1.93	3	aerobic	15.6	35.1	27.2	46.8	47.2	6.1	0.14	30.0	(2.29)

EtAc = Ethyl acetate

* 2 ml of incubation mixture; in initial experiments it was established that the conversion was linear with time for at least 8 min at a protein concentration of 2.61 mg/2 ml under aerobic conditions.

** Data taken from ref. 10

+ Number in brackets are rates of metabolism expressed as nmol substrate metabolized/mg protein/min after correction for recovery (mean ± S.D.).

fraction of swine liver gives a very low conversion of furazolidone
under aerobic conditions in the presence of NADPH (rate: 0.11 nmol/mg
protein/min). HPLC-analysis of the ethyl acetate extracts revealed
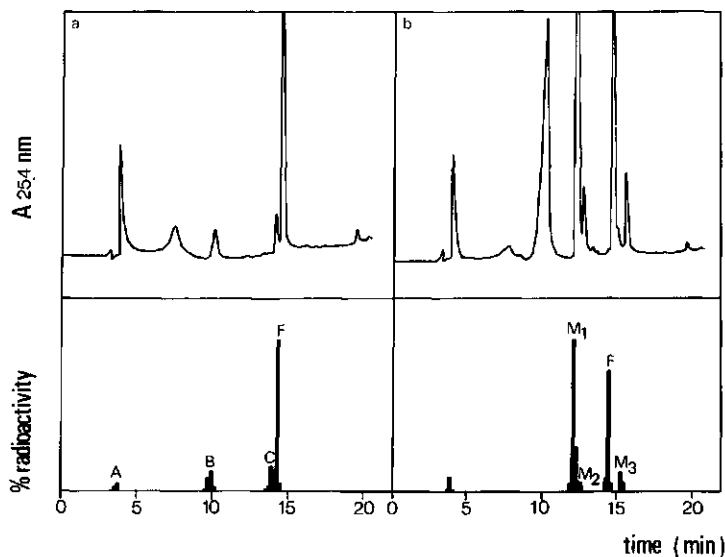three major peaks (A, B and C) (figure 1).

Figure 1: HPLC-analysis of the ethyl acetate extract after microsomal
metabolism of $[^{14}C]$-furazolidone under aerobic conditions
without (a) and with (b) the addition of 5 mM mercapto-
ethanol to the incubation mixture. For structures of meta-
bolites B, C and M_1 see figure 4. F = furazolidone.

The metabolites B and C (10.5% and 6.4% of total metabolites formed
respectively) were identified as 3-(4-cyano-2-oxobutylidene amino)-2-
oxazolidone and 2,3-dihydro-3-cyanomethyl-2-hydroxy-5-nitro-1α,2-di-
(2-oxo-oxazolidin-3-yl)imino- methylfuro[2,3-b]furan, respectively
which have also been identified previously (9,10,11).
Peak A appeared with the solvent front indicating high polarity and
was not further identified.

Covalent binding of furazolidone to protein and the effect of gluta-
thione, mercapto-ethanol, deoxyribonucleosides and amino acids.
Under standard aerobic conditions, conversion-dependent covalent

binding of furazolidone to microsomal protein amounted to 0.29 nmol/mg

protein/min (11.6% of total metabolites). Covalent binding increased

under anaerobic conditions (0.38 nmol/mg protein/min). Covalent

binding of furazolidone to microsomal protein is higher after aerobic

incubations with swine liver microsomes (0.29-0.38 nmol/mg protein/min)

compared with rat liver microsomes (0.14 nmol/mg protein/min) (10).

(Table 1).

Figure 2: Effect of 10 mM amino acids, glutathione, mercapto-ethanol
and deoxyribonucleosides on protein binding of $[^{14}C]$-fura-
zolidone during microsomal metabolism. For glutathione,
guanosine and histidine the protein binding was corrected
for a decrease of the total conversion (54.2%, 66.7% and
68.8% respectively compared with the control). In all other
experiments the total conversion was not influenced. The
experimental variation was within 10 percent.

The influence of the addition of 10 mM glutathione (GSH), mercapto-ethanol, amino acids and deoxyribonucleosides to the incubation mixture on the covalent binding of furazolidone to microsomal protein is shown in figure 2. Substantial decreases were observed only in the case of S-H-group containing agents cysteine, GSH and mercapto-ethanol, but not for the other compounds. In the case of methionine an increase could be observed.

Identification of a mercapto-ethanol conjugate of furazolidone.

From the above results it is clear that thiol groups may react with an intermediate formed from furazolidone. When GSH was added to the incubation mixture the total conversion decreased, a yet inexplicable observation also made in other experiments (10); an increase of the relative amount of radioactivity could be detected in the aqueous phase while the percentages radioactivity in the ethyl acetate extract and covalently bound to protein decreased (Table 2). Using [^{3}H]-GSH and [^{14}C]-furazolidone, HPLC-analysis of the aqueous phase yielded a double labeled [^{3}H]/[^{14}C] fraction in a ratio of 1:1 (retention time: 13.4-14.5 min).

However, since under the conditions used the fractionated material was unstable, identification was not possible. When 5 mM mercapto-ethanol was added to the incubation mixture, a decrease in the amount of radioactivity in the aqueous phase was found, as well as a decrease of the radioactivity covalently bound to protein (Table 2). This radioactivity was accounted for by the increase in the ethyl acetate fraction. HPLC-analysis of this fraction showed the disappearance of metabolite B and C, and revealed a new peak (M$_1$), stable for at least 48 hours at room temperature and representing 45-51% of total metabolites formed. The formation of two minor new peaks M$_2$ and M$_3$ could

Table 2: Effect of the addition of 5 mM mercapto-ethanol (MSH) and 10 mM glutathione (GSH) on the aerobic or anaerobic metabolism of [14C]-furazolidone by swine liver microsomes (2.61 mg protein per 2 ml incubation mixture)*.

t (min)	Conditions	Main EtAc-soluble metabolites (% of total metabolites in fraction I)						Metabolites (% of total) in fraction			Covalent binding (nmol/mg protein/min)	Total conversion (%)**
		A	B	M1	M2	M3	C	I (EtAc)	II (water)	III (protein)		
6	aerobic	18.9	38.4	N.D.	N.D.	N.D.	23.3	27.4	61.1	11.6	0.29	53.6 (2.55 ± 0.18)
8	aerobic +10 mM GSH	-	N.D.	N.D.	N.D.	N.D.	N.D.	20.5	77.6	2.0	0.02	27.0 (0.96 ± 0.05)
8	aerobic +5 mM MSH	5.9	N.D.	74.8	6.7	12.6	N.D.	59.6	38.7	1.6	0.04	73.5 (2.62 ± 0.07)
8	anaerobic +5 mM MSH	7.3	N.D.	69.3	8.9	14.5	N.D.	74.3	24.5	1.1	0.03	72.9 (2.60 ± 0.09)

EtAc = Ethyl acetate   N.D. = not detectable

* Values are means of three experiments
** Number in the brackets are rates of metabolism expressed as nmol substrate metabolized/mg protein/min (mean ± S.D.)
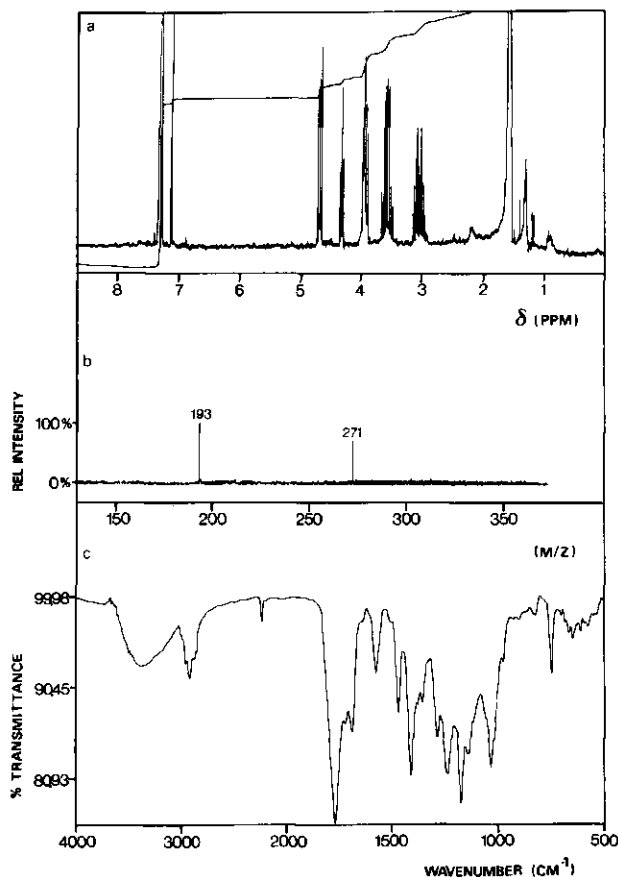
Figure 3: NMR (a), MS (b) and IR (c) spectra of the mercapto-ethanol
conjugate of furazolidone.

IR spectrum in KBr (cm^{-1}): 3399: hydroxyl-group, 2927: CH$_2$-stretching,
2240: cyano-group, 1772: oxazolidone, C = 0, 1691: α,β-unsaturated,
C = 0 stretching;

H-NMR spectrum in CDCl$_3$ (ppm): 7.08: singlet (1 H): CH imine group;
4.64: triplet (2 H): CH$_2$ oxazolidone ring; 4.27: triplet (1 H):
CH open furan ring; 3.92: multiplet (4 H): CH$_2$ oxazolidone ring,
CH$_2$ β-hydroxyethylmercaptogroup; 3.56: multiplet (2 H): CH$_2$ open
furan ring; 3.02: multiplet (2 H): CH$_2$ hydroxyethylmercaptogroup.
The triplet at δ4.27 (1 H) is coupled with the multiplet at δ3.56
(2 H). The multiplet at δ3.92 (two CH$_2$-groups) coupled with the
multiplet at δ3.02 (2 H) and with the triplet at δ4.64 (2 H).
These couplings were proved by using homo-decoupling experiments;

Mass spectrum (m/z):
- field desorption: 271 (molecular ion), 193 (fragment ion: M$^+$-78
(= mercapto-ethanol)

UV spectrum, λ_{max} (nm) 272;

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also be observed accounting for 4 and 8% of total metabolites formed respectively. Metabolites B and C had disappeared completely (Figure 1; Table 2). The compound M_1 was isolated by preparative HPLC and its spectral characteristics are shown in Figure 3.

On the basis of these spectral characteristics the product was tentatively identified as 3-(4-cyano-3(or 4)-β-hydroxyethylmercapto-2-oxobutylidene amino)-2-oxazolidone, a mercapto-ethanol conjugate of furazolidone.

DISCUSSION

Microsomal incubation of furazolidone results in the formation of two identified ethyl acetate extractable metabolites, in an appreciable amount of covalent binding and in the formation of yet unknown water-soluble metabolites. Performing the incubations in the presence of 5 mM mercapto-ethanol results in the disappearence of these two metabolites, in the disappearence of an appreciable amount of water-soluble unidentified metabolites and in a decrease of covalently bound material. Addition of 10 mM mercapto-ethanol caused an almost complete inhibition of the covalent binding. All radioactivity could be accounted for by an increase in the ethyl acetate fraction. HPLC-analysis revealed a new peak M_1, representing approximately 50% of total metabolites formed. This metabolite was shown to be a mercapto-ethanol adduct of the open-chain acrylonitrile derivative of furazolidone (I, figure 4), which is therefore the reactive intermediate from which the other products (B, C, M_1 and at least part of the polar metabolites, see figure 4) are formed and which may be responsible for the interaction with microsomal protein. The NMR-spectrum did not give conclusive evidence on the position of the mercapto-ethanol moiety, but from the fact that metabolite C, the adduct with furazolidone
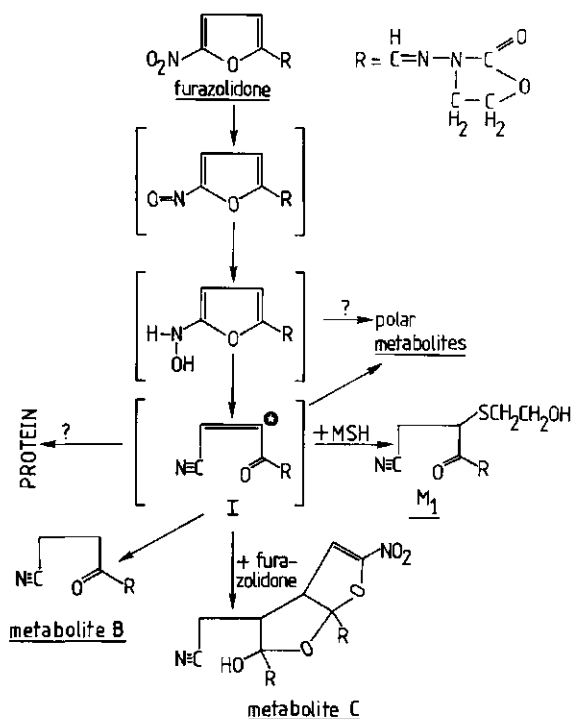
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Figure 4: Proposed metabolic pathway of furazolidone by swine liver microsomes (MSH = mercapto-ethanol).

itself, also results in reaction at the 3 position, it can be inferred that the thiol will attack the same carbon atom (see figure 4). It should be stressed that, if the 3-position is the point of attack, the reactive intermediate in the metabolism of furazolidone is the open-chain acrylonitrile derivative and not the hydroxylamino derivative, since the latter would give adducts at the 2- or 4 positions (15-17). In earlier studies with the nitrofuran N-[4-(5-nitro-2-furyl)-2-thiazo-lyl]-acetamide (18) and with aromatic amines and amides using GSH as trapping agent (19-25) it has been reported that the N-hydroxy or nitroso-derivatives are responsible for the interaction. Apparently this is not the case for furazolidone, using mercapto-ethanol as

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trapping agent. However, since an appreciable amount of radioactivity in the aqueous phase and the identity of M_2 and M_3 are still unknown, it cannot be excluded that adducts and/or metabolites are also formed from the N-hydroxy or nitroso-derivatives.

The site of alkylation on microsomal protein appears to be the thiol group of cysteine, because this is the only amino acid that causes inhibition of furazolidone protein binding. Such an effect has also been reported for the 5-nitro imidazole ronidazole (26). An increase of covalent binding to microsomal protein after addition of methionine to the incubation mixture has also been reported for acetaminophen (27). Studies aimed at the elucidation of the process of covalent binding of furazolidone to protein with special attention for the role of its open-chain acrylonitrile-derivative, are presently undertaken.

Acknowledgements

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CHAPTER 7: Reversible interaction of a reactive intermediate derived
          from furazolidone with glutathione and protein
          Submitted.

REVERSIBLE INTERACTION OF A REACTIVE INTERMEDIATE DERIVED FROM FURAZOLIDONE WITH GLUTATHIONE AND PROTEIN.

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ABSTRACT

Swine liver microsomes convert the nitrofuran furazolidone into
N-(4-cyano-2-oxo-3-butenylidene)-3-amino-2-oxazolidone, a reactive
open-chain acrylonitrile derivative. This derivative may be trapped
with such thiol-group containing agents as glutathione and mercapto-
ethanol. However, this reaction is reversible: e.g. adding an excess
of mercapto-ethanol to an aqueous solution (pH 7.4) of the glutathione-
conjugate results in conversion of 43% of this compound into the
mercapto-ethanol conjugate. In addition, when microsomal protein is
added to the glutathione-conjugate or the mercapto-ethanol conjugate,
36% and 44% respectively becomes covalently bound to the protein. The
amount of this covalently bound radioactivity decreases again on
prolonged incubation at 37°C (42% disappearance within 24 hours),
suggesting that the acrylonitrile derivative also reacts reversibly
with thiol groups of microsomal protein. Indeed an excess of mercapto-
ethanol could remove covalently bound radioactivity from microsomal
protein resulting in the formation of the mercapto-ethanol conjugate.
The reversibility of the reaction is dependent on pH, as is demonstrated
for the mercapto-ethanol conjugate. Below pH 2 this conjugate is
stable; optimal exchange to microsomal protein is found between pH 7
and 10. At very high pH (> 11) no binding to protein is found,
although the conjugate disappears rapidly. The mercapto-ethanol
conjugate exhibits mutagenic activity in the Salmonella/microsome-test
indicating that the acrylonitrile derivative of furazolidone also
interacts with DNA.

INTRODUCTION

Furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone), is a
drug widely used in veterinary practice as a therapeutic agent against
gastro-intestinal infections (Brander and Pugh, 1971; Rossoff, 1974).
Because of its mutagenic and carcinogenic properties, possible residues
in meat have become a matter of interest (Food and Drug Administra-
tion, 1976; McCalla, 1983). When piglets were dosed orally with [^{14}C]
furazolidone, radioactivity at µg-equivalents of parent compound per
gram of tissue could be detected two weeks after the treatment,
although furazolidone itself was not present in tissues 2 hours after
the last administration. Up to 55% of radioactivity in tissues proved
to be non-extractable (Vroomen et al., 1986), suggesting covalent
binding of reactive intermediates to tissue macromolecules.
For the risk-evaluation of the consumption of meat, information is
necessary concerning the metabolism of furazolidone in swine and the
nature of possible covalently binding metabolite(s). In several spe-
cies and model systems nitroreduction has been reported as an impor-
tant metabolic pathway, with a cyano-derivative [3-(4-cyano-2-
oxobutylideneamino)-2-oxazolidone] as major endproduct (Tatsumi et al.,
1978, 1981, 1984; Nakabeppu and Tatsumi, 1984; Abraham et al., 1984;
Vroomen et al, 1987). Although this cyano-derivative was shown to
be only a minor metabolite of furazolidone in tissues of swine in vivo
(Vroomen et al., in press b), in vitro using swine liver microsomes,
nitroreduction was responsible for at least 50% of total metabolism.
The reactive intermediate formed by nitroreduction was shown to be
an acrylonitrile derivative of furazolidone by the identification of
3-(4-cyano-3-β-hydroxyethyl-mercapto-2-oxobutylidene amino)-2-
oxazolidone, a conjugate with mercapto-ethanol which was used as
trapping agent (Vroomen et al., in press a).

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Using [^{3}H] glutathione and [^{14}C] furazolidone, evidence was collected

for the formation of a glutathione-conjugate of furazolidone.

Both mercapto-ethanol and glutathione could inhibit the covalent

binding of furazolidone to microsomal protein, which is therefore pre-

sumably mediated by the same reactive acrylonitrile-derivative. In

the present study the stability of the conjugates is studied and the

conjugation is found to be a reversible process. The structural

identity of the glutathione conjugate is established and the covalent

binding to protein is shown to be governed by the same rules as con-

jugate formation.

MATERIALS AND METHODS

Chemicals. [^{14}C] Furazolidone was a gift from Norwich Eaton Pharmaceu-
ticals Inc., Norwich, N.Y., U.S.A. It was labelled in the methylene
groups of the oxazolidone moiety (specific activity 2.69 µCi/mg;
radiochemical purity > 99% by TLC (toluene-acetic acid-acetone-water,
600:240:80:25, by vol.) and HPLC. Glutathione [glycine-2-^{3}H]-, was
obtained from Du Pont (specific activity at time of use: 21.0 µC/mmol);
radiochemical purity > 99% by TLC (n-butanol-acetic acid-water, 25:4:10
by vol.) and HPLC (on a Bondapak C_{18} column using the mobile phase
0.25 % acetic acid).
Scintillation liquids: Minisolve (Zinsser Analytical (UK) LTD, Maidenhead)
and Dimilume-30^R (Packard Instrument BV, Delft) for aqueous and non-
aqueous samples, respectively. NADPH was obtained from Boehringer
Mannheim GmbH, Mannheim, FRG. Glutathione (GSH) was purchased from
Sigma Chemical Co. St. Louis, MO. All the other chemicals used were of
reagent grade.

Microsomal incubation. the preparation of swine liver microsomes has
been described elsewhere (Vroomen et al., in press a). A standard
incubation mixture was used containing 1 mM NADPH, 3 mM $MgCl_2$, 0.1 M
potassium phosphate buffer (KPi) pH 7.4 and 2.61 mg protein. After a
preincubation of 2 minutes, incubations were started by the addition
of [^{14}C]-furazolidone dissolved in 50 µl acetone (final concentration:
36.7 µM). The final volume was 2 ml. After incubation of 6-8 minutes
at 37°C, non-polar metabolites were extracted with ice-cold ethyl ace-
tate (3 x 5 ml). The protein fraction of the aqueous phase was
separated by ultrafiltration (Amicon Centrifree Micropartition System)
at 4°C during 15 minutes at 6000 g.
The ultrafiltrate was directly used for HPLC. In studies on the effect
of mercapto-ethanol and GSH on the metabolism of furazolidone these
reagents were added to the incubation mixture at a final concentration
of 5 mM. Incubations and all other procedures before HPLC analysis
were performed in the dark because of the photosensitivity of furazo-
lidone in solution (Kalim, 1985).

HPLC-analysis. HPLC analyses were performed with a Waters Associates 6000A HPLC system. Separation of the polar metabolites of furazolidone and the glutathione-conjugate was achieved on a Hamilton PRP-1 column (250 x 4.6 mm int.diam.) using a 22 min.-gradient of 2-99% acetonitrile in a 5 mM ammonium formate buffer, pH 3.6 at a flow rate of 1 ml/min (concave gradient curve 7).

After extraction with ethyl acetate, evaporation to dryness under nitrogen and redissolving in acetonitrile/water (30/70 by vol.), the separation of the mercapto-ethanol conjugate of furazolidone was achieved on a Chrompack Hypersil 5 ODS column (250 x 4.6 mm int.diam.) using a linear 22 min.-gradient of 2-100% acetonitrile in a mixture of water - 1.0 M sodium acetate/acetic acid buffer pH 5.0 - acetonitrile (900:75:25, by vol.) at a flow rate of 1 ml/min. The eluents were monitored at 254 nm and for radioactivity measurement 0.2 ml fractions were collected. When the mercapto-ethanol conjugate was extracted from the eluent with ethyl acetate, evaporated to dryness under nitrogen and stored in the dark at -40°C, the compound was stable for at least 1 month.

Exchange studies. For studies on the exchange of the glutathione or mercapto-ethanol conjugates of furazolidone, reaction mixtures were worked up and chromatographed as described above.

The fractions containing the radioactive glutathione- or mercapto-ethanol conjugate were collected in a final 0.1 M KPi buffer pH 7.4 containing 10 mM mercapto-ethanol or 10 μM - 10 mM glutathione respectively (at room temperature). The initial conjugate concentrations were approximately 10 μM.

For studies on the exchange to microsomal protein from the gluta-thione- and mercapto-ethanol conjugate, 5.2-5.6 mg microsomal protein was added to 6.3 and 8.1 nmol of the respective conjugates in a volume of 2 ml 0.1 M KPi buffer pH 7.4.

For studies on the effect of pH on the exchange of the reactive inter-mediate from the mercapto-ethanol conjugate to microsomal protein 5.6 mg microsomal protein were added to 5.7 nmol of the conjugate in a volume of 2 ml 0.1 M KPi buffer. Covalent binding was measured after 1-50 hr at room temperature. Studies on the pH or time dependent stability of the mercapto-ethanol conjugate were also performed at room temperature.

Covalent binding to protein. The protein was precipitated by the addi-tion of ice-cold methanol. After centrifugation for 15 min at 1500 g at 4°C, decanting the aqueous/methanolic supernatant and redissolving the protein in 0.1 M KPi buffer pH 7.4, the protein was again precipi-tated by the addition of ice-cold methanol. This procedure was repeated three times. Subsequently the precipitated protein was washed three times with methanol, twice with ethanol and twice with ether. The resulting pellet was dissolved in 1 ml of Soluene 350 (Packard Instrument B.V., Delft) and used for radioactivity-measurement (the amount of radioactivity defined as originating from covalently bound residues).

U.V. spectra. Ultraviolet (UV) absorption spectra of GSH/mercapto-ethanol-conjugates of furazolidone were obtained with a Hewlett-Packard photodiode array detection system (detector 1040A, diskdrive 9121, computer 85B and plotter 7470A).

Radioactivity measurements. Radioactivity was measured in a Philips
liquid scintillation counter (type PW 4700). Counting efficiency was
calculated by means of an external standard-counting procedure.

Salmonella/microsome-test. The Salmonella/microsome test was carried
out as described by Maron and Ames (1983) using strain TA 100.
Furazolidone and the mercapto-ethanol conjugate were tested dissolved
in DMSO in the absence of S9-mix, using a preincubation of 1 hour.

RESULTS

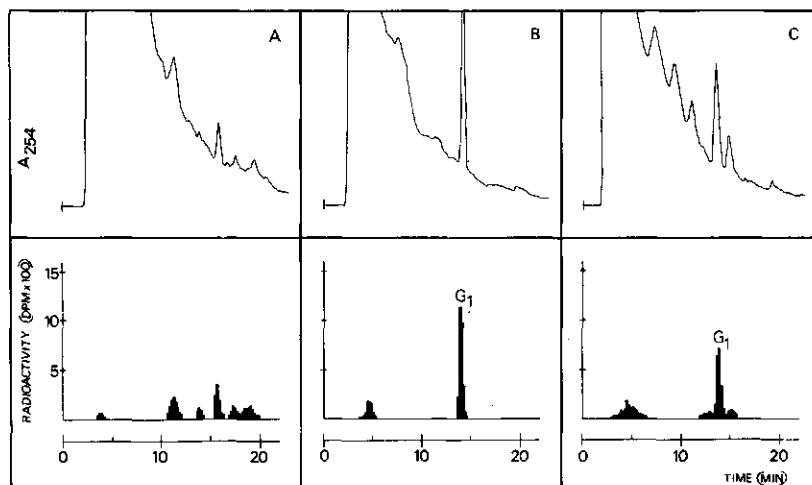1. Formation of a glutathione-conjugate derived from furazolidone

Figure 1: HPLC-analysis of the ultrafiltered aqueous phase after
         microsomal incubation of [^{14}C]-furazolidone
         A) without the addition of glutathione (inj.vol.: 200 µl)
         B) with the addition of 5 mM glutathione, immediately after
            stopping the incubation (inj.vol.: 100 µl)
         C) with the addition of 5 mM glutathione, 2 hours after
            stopping the incubation (inj.vol.: 100 µl).

Upon conversion of [^{14}C]furazolidone by swine liver microsomes under

aerobic conditions, HPLC-analysis of the ultrafiltered aqueous phase

revealed at least five peaks (Figure 1a). When 5 mM glutathione was

added to the incubation mixture, HPLC-analysis of the ultrafiltered

aqueous phase immediately after stopping the incubation showed that these peaks could not be detected and a new peak (G_1) was observed: retention time: 13.5–14.5 min (Figure 1b). This peak accounted for 64.2% of total radioactivity in the ultrafiltered aqueous phase. Ultrafiltration of the aqueous phase at 4°C immediately after extraction with ice-cold ethyl acetate, proved to be an essential step for the stability of peak G_1 (no change for at least 24 hours at 4°C). Previously, instability was reported when this procedure was omitted (Vroomen et al., in press a). The ultraviolet absorption maximum of peak G_1 was 270 nm, indicating that the nitrofuran-ring was converted into an open-chain moiety (λ_{max} furazolidone: 366 nm, λ_{max} open-chain cyano-derivative: 268 nm). When the ultrafiltrate (pH 7.4) was incubated at 37°C for several hours before HPLC-analysis, peak G_1 disappeared slowly and a number of new peaks could be observed (Figure 1c). Using [^{3}H]glutathione and [^{14}C]furazolidone, it was shown that peak G_1 corresponded to a double labeled [^{3}H]/[^{14}C] fraction (ratio 1:1) indicating the formation of a labile glutathione-conjugate from furazolidone (results not shown; Vroomen et al., in press a).

2. Exchange of furazolidone residues between thiol groups at pH 7.4

A. Exchange from glutathione to mercapto-ethanol. After addition of the glutathione-conjugate to 0.1 M KPi buffer pH 7.4 containing 10 mM mercapto-ethanol and the usual workup procedures (within 24 hours), HPLC-analysis (see materials and methods) revealed a peak with a retention time of 12.4–12.8 min, accounting for 43% of initial radioactivity and co-eluting with the mercapto-ethanol conjugate formed from the open-chain acrylonitrile derivative of furazolidone (M_1) of which the structure has been elucidated previously (Vroomen et al., in press a).

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After collection of this peak, evaporation to dryness under nitro-
gen and redissolving in acetonitrile/water (20/80, by vol.), the frac-
tion was chromatographed isocratically on the same column (acetoni-
trile/water: 20/80, by vol.) with a retention time of 5.5-6.3 min,
again co-eluting with M_1. Furthermore, the ultraviolet absorption
spectra was superimposable with M_1 (λ_{max}= 272 nm). The mercapto-
ethanol conjugate M_1 can thus be formed from the glutathione-conjugate
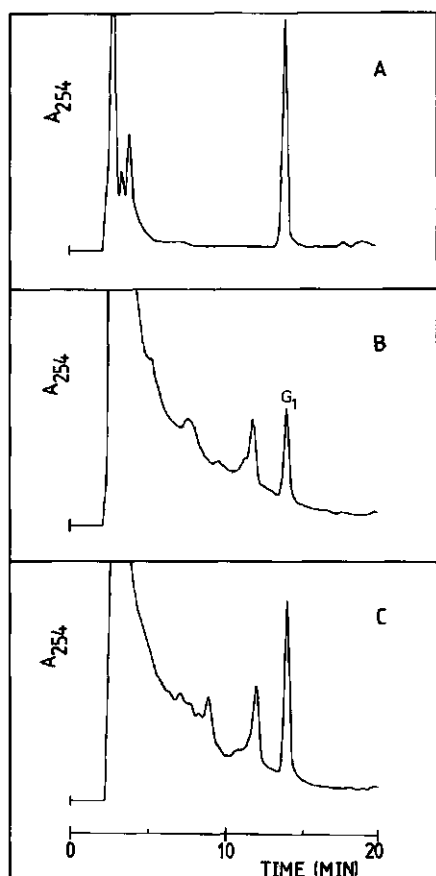derived from furazolidone.

Figure 2: HPLC-analysis of a compound formed from M_1 after addition of
glutathione, inj.vol.: 200 µl (A); G_1 obtained from microso-
mal incubation in the presence of glutathione, inj.vol.:
100 µl (B) and a combination of both compounds, inj.vol.:
200 µl = 100 µl (A) combined with 100 µl (B)(C).
For structures of M_1 and G_1 see Figure 5.

B. Exchange from mercapto-ethanol to glutathione. One hour after the
addition of 10 mM GSH to the radiolabeled mercapto-ethanol conjugate
M_1 at pH 7.4, HPLC-analysis revealed a $[^{14}C]$-labeled peak which co-
eluted with G_1 (Figures 2a, 2b, 2c) and accounted for 37.5% of total
initial radioactivity. Residual unreacted M_1 (retention time:
17.7-19.0 min) was removed by ethyl acetate extraction. The dependency
of the formation of G_1 from M_1 on the glutathione concentration after
an incubation period of 1 hour at room temperature and pH 7.4 is shown
in Figure 3. Maximum exchange (approximately 35%) was observed at con-
centrations > 100 µM GSH. Approximately 50% of the mercapto-ethanol
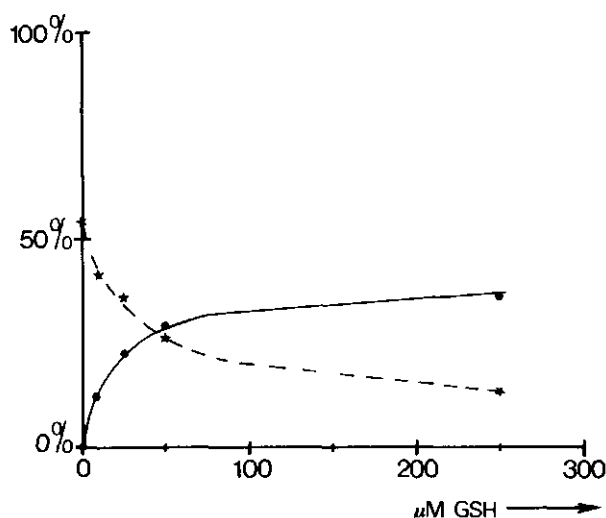conjugate is converted in yet unknown substances.

Figure 3: Influence of the glutathione concentration on the exchange
of the $[^{14}C]$acrylonitrile-derivative (I) from 10 µM of the
$[^{14}C]$mercapto-ethanol conjugate (M_1; -★- -★-) to glutathione
resulting in the formation of the $[^{14}C]$glutathione-conjugate
(G_1; _●__●_). Values expressed as percentages of the total
radioactivity (= 10 µM M_1 at t=0) in the mixture after an
incubation of 1 h.
For structures of I, M_1 and G_1 see figure 5.

C. Reversible exchange from glutathione or mercapto-ethanol to protein.

When microsomal protein was added to the glutathione-conjugate (pH 7.4),

0.44 nmol equivalents were covalently bound per mg of protein after an

incubation period of 2 hours at 37°C. As shown in table 1 this amount

of covalently bound material decreased after a prolonged incubation

time (42% disappearance within 24 hours).

Table 1: Percentage covalent binding of furazolidone residues to
microsomal protein after addition of the protein to the
mercapto-ethanolconjugate (M_1) or glutathione-conjugate (G_1)
formed form [14C] furazolidone.

Substrate	Protein (mg)	Incubation time* (hours)	% covalent binding** to microsomal protein
8.1 nmol M_1	–	2	0
8.1 nmol M_1	5.6	2	40.4
6.3 nmol G_1	–	2	0
6.3 nmol G_1	5.2	2	39.2
6.3 nmol G_1	5.2	8	25.4
6.3 nmol G_1	5.2	24	22.6
6.3 nmol G_1	5.2	50	21.5

* Incubations were performed in a total volume of 2 ml 0.1 M KP_i buffer
pH 7.4, 37°C. For working procedures see Methods and Materials.
** Data are the mean of 2 experiments. Experimental variation was within
10%.

In the case of the mercapto-ethanol conjugate covalent binding to

microsomal protein could similarly be detected: 0.63 nmol equivalents

per mg of protein. This reaction is reversible. After suspending 5 mg

of microsomal protein (to which 2 nmol equivalents of radioactivity

were covalently bound) in 2 ml 0.1 M KPi buffer (pH 7.4) containing

10 mM mercapto-ethanol, followed by precipitation of the protein after

1 hour at room temperature, HPLC-analysis of the supernatant showed

that 69.5% of total radioactivity had become conjugated to mercapto-

ethanol.

3. pH dependency of the exchange reaction. In Figure 4 an experiment is depicted in which 5.7 nmol of the mercapto-ethanol conjugate was incubated at room temperature with or without microsomal protein at different pH values.
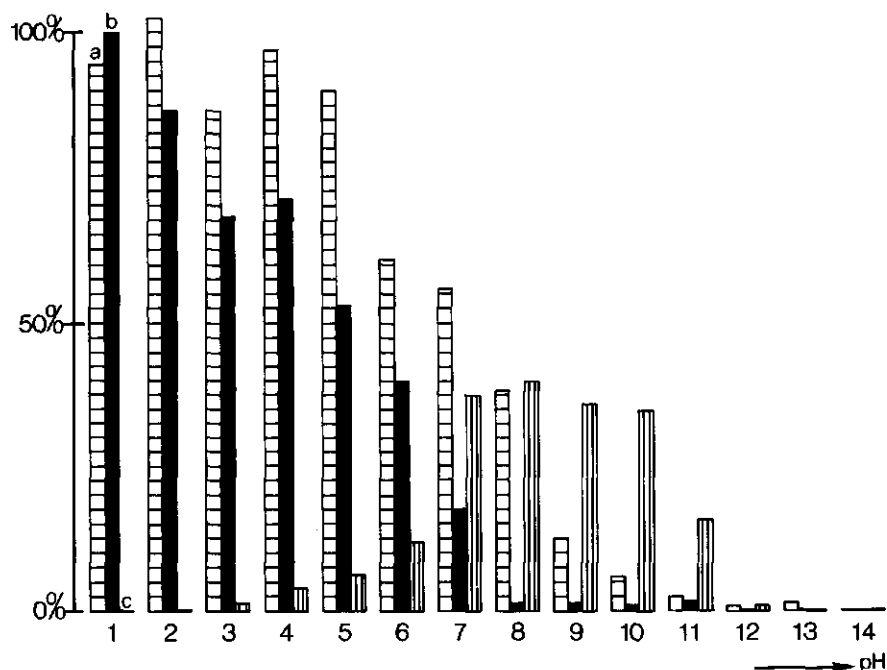

Figure 4: pH dependency of the stability of the mercapto-ethanol conjugate M_1 at room temperature
a) percentage unchanged M_1 after 1 hr
b) percentage unchanged M_1 after 24 hrs
c) percentage of M_1 covalently bound after incubation for 1 hr, in the presence of 5.6 mg microsomal protein.
For the structure of M_1 see figure 5.

At pH values lower than 2 the conjugate is stable and no protein binding occurs. At higher pH values progressively more of the conjugate M_1 disappears ($t_{1/2}$ at pH 4: 100 wk, $t_{1/2}$ at pH 7.4: 3.7 hr), with a concomitant increase in covalent binding to protein. Optimum conditions for protein binding are between pH 7 and 10. At higher pH values the amount of protein binding decreases drastically. Also the conjugate disappears rapidly at these high pH's.

4. Mutagenicity tests with Salmonella typhimurium TA 100. The mercapto-
ethanol conjugate M_1 (unlabeled) was tested in the Salmonella/microsome
assay (without any added metabolizing system) using a preincubation
period of 1 hour. A strong positive response was found with a clear
dose-response relationship (Table 2).

Table 2: Mutagenic activity of the mercapto-ethanol conjugate (M_1) of
furazolidone in Salmonella typhimurium TA 100 without any added
metabolizing system (n=3).

Compound	µg/plate	revertants/plate mean ± S.D.
DMSO		113 + 5
Mercapto-ethanol (µl)	50	111 + 7
Furazolidone	0.1	957 + 35
M_1	0.05	107 + 15
	0.5	251 + 11
	5.0	1810 + 68

DISCUSSION

In earlier studies using swine liver microsomes it was shown that the
non-polar compounds 3-(4-cyano-2-oxobutylidene-amino)-2-oxazolidone
and 2,3 dihydro-3-cyano-methyl-2-hydroxyl-5-nitro-1α,2-di(2-oxo-
oxazolidin-3-yl)iminomethyl-furo[2,3-b]furan (Figure 5, metabolites B
and C) were minor metabolites formed via the open-chain acrylonitrile-
derivative of furazolidone (Figure 5, metabolite I). Most of the meta-
bolites formed (61%) could not be extracted with ethyl acetate
(Vroomen et al., in press a). At least 5 polar metabolites were formed
by swine liver microsomes. When glutathione was added to the incuba-
tion mixture, these metabolites as well as metabolites B and C disap-
peared in favour of a glutathione-conjugate of furazolidone (G_1),
indicating that the polar metabolites were also formed via inter-
mediate I of furazolidone, which is also responsible for interaction

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with glutathione. Addition of mercapto-ethanol to the glutathione-conjugate of furazolidone resulted in the formation of 3-(4-cyano-3-β-hydroxyethylmercapto-2-oxobutylidene amino)-2-oxazolidone (metabolite M_1), a conjugate previously identified as formed from the open-chain acrylonitrile-derivative of furazolidone (Figure 5, metabolite I) with mercapto-ethanol (Vroomen et al., in press a).
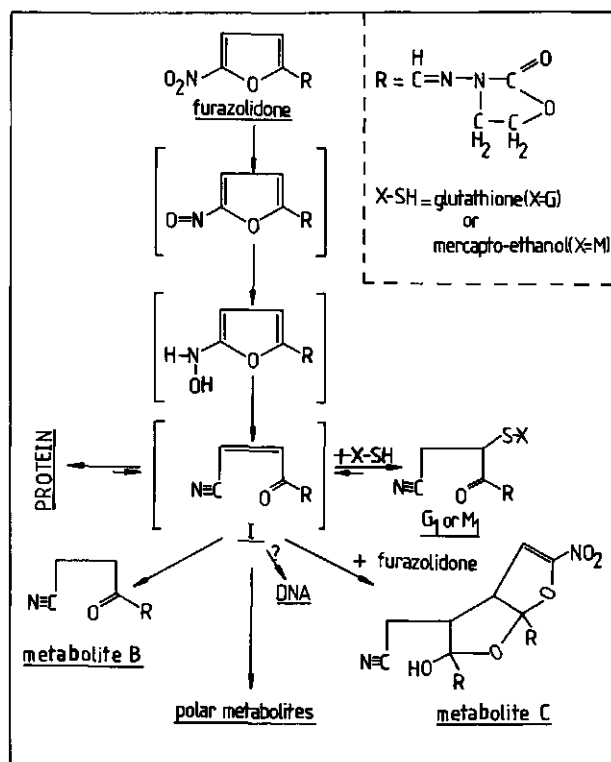

Figure 5: Proposed metabolic pathway of furazolidone by swine liver microsomes.

Furthermore such a chemical exchange could also be observed when glutathione was added to M_1, resulting in the formation of the gluta-thione conjugate G_1 or when microsomal protein was added to the con-jugates resulting in covalent binding to protein. Covalent binding can

thus be mediated by mercapto-ethanol- as well as glutathione-conjugates.
Covalent binding to protein decreased on prolonged incubation at pH
7.4, suggesting that at least part of the covalent binding of the
acrylonitrile derivative (I) to thiol groups of microsomal protein is
also reversible.

Indeed, an excess of mercapto-ethanol could remove covalently bound
radioactivity from microsomal protein resulting in the formation of
the mercapto-ethanol conjugate. The stability of the conjugate as well
as the extent of covalent binding observed are dependent on pH. In
addition to covalent binding the mercapto-ethanol conjugate of furazo-
lidone also exhibits mutagenic activity in Salmonella typhimurium TA
100, indicating that the released acrylonitrile derivative I can also
interact with DNA.

The open-chain acrylonitrile derivative (I) is clearly the central
reactive intermediate in the metabolism of furazolidone. The behaviour
of this intermediate in its reversible reaction with thiols is in
agreement with the occurrence of Michael and "retro-Michael" reactions
as found by others for the reaction between thiols and α,β-unsaturated
ketones (Onkenhout et al, 1982; 1983).

Due to the fact that conjugation can be followed by deconjugation at
pH 7.4, glutathione may act as a carrier of the reactive acrylonitrile
derivative I of furazolidone. Whether this phenomenon also occurs in
the in vivo situation is unknown, but there are some indications. For
example, dosing [^{14}C] furazolidone orally to piglets during 10 days,
results in interaction with DNA and in non-extrable radioactivity in
all tissues to approximately the same extent (Vroomen et al., 1986).
Perhaps in vivo glutathione conjugates are formed from furazolidone in
the liver, excreted in the blood, and release reactive intermediates
at other sites in the organism.

Recently an equilibrium of free reactive agents with the derived glutathione-conjugates has also been recognized in the case of allyl and benzyl isothiocyanate (Bruggeman et al., 1986). Furazolidone would thus constitute the second example where such an equilibrium plays a role in the toxic effects. Further studies are indicated to investigate the occurrence of these processes *in vivo* for furazolidone, because a possible equilibrium reaction of reactive intermediates with thiol groups of tissue-protein may have far-reaching consequences for the risk-evaluation of the consumption of meat contaminated with covalently bound furazolidone residues.

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PART III CONCLUSIONS AND SUMMARY

CHAPTER 8: Summary and concluding remarks

SUMMARY AND CONCLUDING REMARKS

The experiments described in this thesis were undertaken to get a
better understanding of the kinetics and biotransformation of the
nitrofuran furazolidone upon oral administration to swine. Such
information forms an essential prerequisite for making an appropriate
assessment of the consumer hazards of edible products originating from
animals treated with this compound. The experiments were conducted
according to two different approaches:

- kinetic studies in the target animal to determine the elimination
  kinetics of furazolidone and its metabolites from plasma and tissues
  upon oral administration of furazolidone
- in vitro biotransformation studies using swine and rat liver
  microsomes to elucidate biotransformation routes and to identify
  reactive intermediates responsible for the interaction with
  biological macromolecules.

Part I of this thesis starts with a description of some developments
in animal husbandry during the last decades. This is followed by a
review of literature data on physical/chemical and antimicrobial
characteristics, toxicity, elimination kinetics and biotransformation
of furazolidone (chapter 1).

Part II of this thesis deals with the present study. In chapter 2
a fast, sensitive method has been described for the determination
of furazolidone in swine plasma, muscle, liver, kidney, fat and urine
based on high-performance liquid chromatographic separation after
solid-phase extraction on Extrelut[R] 1. The sensitivity of the method
was 1-2 ng/ml (g) for plasma and tissues and 25 ng/ml for urine.

From the kinetic studies the conclusion can be drawn that no accumu-
lation of furazolidone occurs in blood after oral administration of
furazolidone to both piglets (chapter 3) and adult swine (chapter 4);
the half life time was respectively 45 and 60 minutes. Furazolidone
was rapidly and almost completely metabolized and urine proved to be
the major excretion pathway of formed metabolites: 61% of the radio-
active dose administered to piglets had been exreted via the urine
and 18% via faeces, while in urine of adult swine only traces of total
dosed furazolidone could be recovered. In tissues of piglets and adult
swine no residues of furazolidone could be detected at all. However,
the experiments with piglets showed that relatively high levels of
radioactivity were present in all tissues studied. After a withdrawal
period of 14 days the concentrations varied from 0.9-4.3 µg-equivalents
per gram tissue. Up to 56% of the radioactivity detected in organs and
tissues appeared to be non-extractable and was partly associated with
DNA. This may either be indicative for endogenous incorporation in
physiologically occurring compounds or for covalent binding of reac-
tive intermediate metabolites of furazolidone to biological macro-
molecules (chapter 3).

Studies with the adult swine further revealed the formation of a
cyano-derivative from furazolidone, namely 3-(4-cyano-2-oxobutylidene
amino)-2-oxazolidone. In studies in other animal species, it was
claimed that this cyano-derivative was a major metabolite in urine.
However, this compound proved to be a minor metabolite in plasma
(half life time: 4 hours) and tissues of adult swine. This may be
explained by species differences in biotransformation of furazolidone,
by a very fast elimination of the cyano-derivative via urine or by an
effective trapping of reactive intermediate metabolites of furazolidone

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by biological macromolecules or agents like glutathione (chapter 4).

Two major ethyl acetate extractable metabolites of furazolidone could be observed upon incubation in rat liver microsomes: the cyano-derivative referred to before and a reaction product of furazolidone with its open-chain acrylonitrile derivative, namely 2,3-dihydro-3-cyanomethyl-2-hydroxy-5-nitro-1α,2-di-(2-oxo-oxazolidin-3-yl)imino-methylfuro[2,3-b]furan. Approximately 2-7% of the totally formed metabolites proved to be covalently bound to microsomal protein. This covalent binding could be inhibited by addition of glutathione, which also resulted in an almost complete shift from non-polar to water-soluble metabolites. No binding to DNA was detected when calf thymus DNA was added to a microsomal incubation mixture with furazolidone in contrast to the in vivo DNA interaction observed in the piglets. This may be explained by differences between the in vitro and in vivo situation in the availability of reactive intermediate metabolites of furazolidone for reaction with DNA (chapter 5).

The above mentioned ethyl acetate extractable metabolites of furazolidone proved to be only minor conversion products of furazolidone upon incubation in swine liver microsomes, while the percentage of the protein bound metabolites formed was much higher, namely 12-22%. The site of attack on microsomal protein is probably formed by the thiol-group of cysteine as indicated by results of experiments performed with amino acids.
Using mercapto-ethanol as a trapping agent for reactive intermediates, a new metabolite could be isolated upon incubation of furazolidone in swine liver microsomes. This compound was identified as a conjugation

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product of the open-chain acrylonitrile derivative of furazolidone
with mercapto-ethanol, namely 3-(4-cyano-3-β-hydroxyethyl-mercapto-
2-oxobutylidene amino)-2-oxazolidone. This conjugate accounted for
approximately 50% of total amount of metabolites formed. In addition,
neither the above mentioned ethyl acetate extractable metabolites nor
covalent binding to microsomal protein could be observed when mercapto-
ethanol was added to the incubation mixture, indicating that the open-
chain acrylonitrile derivative plays a central role in reductive bio-
transformation of furazolidone by swine liver microsomes (chapter 6).

The open-chain acrylonitrile derivative of furazolidone binds rever-
sibly with thiolgroup containing agents such as glutathione and
mercapto-ethanol or with microsomal protein.
The reversibility of the exchange reaction is dependent on pH as is
demonstrated for the mercapto-ethanol conjugate. Below pH 2 this con-
jugate is stable; optimal exchange to microsomal protein is found
between pH 7 and 10. The mercapto-ethanol conjugate gives a distinct
direct positive response in the Salmonella/microsome test using tester
strain TA 100. This is probably due to an interaction of the acrylo-
nitrile moiety with DNA (chapter 7).

It can be concluded that furazolidone is rapidly and almost completely
metabolized upon oral administration to piglets. A major part of the
formed metabolites proved to be non-extractable from the tissues.
It can not be excluded that these non-extractable metabolites are the
result of covalent binding of a reactive intermediate metabolite of
furazolidone to biological macromolecules such as protein or DNA.
From in vitro studies using swine liver microsomes evidence has been
obtained showing that the open-chain acrylonitrile derivative plays
a central role in the biotransformation of furazolidone.

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It has been shown that this reactive intermediate can bind reversibly to microsomal protein. Whether this non-enzymatic reaction also occurs in vivo is not yet known. As the possible presence of such covalently bound residues in edible tissues could have serious consequences for the acceptibility of the drug for massmedication of food producing animals, the identity of the non-extractable radioactive material in tissue of piglets should be further investigated, together with its bioavailability and toxicity. Efforts should also be made to identify the extractable radioactivity in tissues of piglets as only a small part of this fraction as so far has been identified.

CHAPTER 9: Samenvatting en slotbeschouwingen

SAMENVATTING EN SLOTBESCHOUWINGEN

Dit onderzoek had tot doel meer inzicht te krijgen in de omzetting en verdwijning van furazolidon uit weefsels en organen van varkens na toediening via het voer. Dergelijke informatie is essentieel om op adequate wijze de risico's voor de consument te bepalen van eetbare delen afkomstig van dieren behandeld met dit geneesmiddel. Hierbij is gebruik gemaakt van twee verschillende benaderingen:

- kinetische studies in het doeldier om de verdwijning van furazolidon en metabolieten uit plasma en weefsels te bepalen na orale toediening
- in vitro studies in levermicrosomen van varkens en ratten met als doel biotransformatieroutes op te helderen en reactieve inter-mediairen te identificeren die verantwoordelijk zijn voor de interactie met biologische macromoleculen.

In deel I van dit proefschrift wordt allereerst de ontwikkeling in de intensieve veehouderij gedurende de laatste decennia beschreven. Dit wordt gevolgd door een overzicht van literatuurgegevens van furazoli-don betreffende fysisch/chemisch en antimicrobiële eigenschappen, de toxiciteit, residuen en het verdwijnen daarvan uit plasma, weefsels en organen en tenslotte de biotransformatie (hoofdstuk 1).

In deel II worden in een 6-tal hoofdstukken de resultaten van het onderzoek beschreven.

Hoofdstuk 2 beschrijft een snelle en gevoelige methode voor de bepa-ling van furazolidon in plasma, urine, spier, lever, nier of vet van varkens gebaseerd op HPLC-scheiding na voorzuivering op een Extrelut-kolom. De gevoeligheid van de methode werd bepaald op 1-2 ng/ml (g) voor plasma en weefsels en op 25 ng/ml voor urine.

Uit de resultaten van de kinetische studies kan geconcludeerd worden dat geen accumulatie plaats vindt van furazolidon in bloed van biggen (hoofdstuk 3) en volwassen varkens (hoofdstuk 4); de respectievelijke halfwaardetijden bedroegen 45 en 60 minuten. Furazolidon wordt snel en bijna volledig omgezet. De gevormde metabolieten blijken voornamelijk via de nieren te worden uitgescheiden: 61% van de totaal toegediende radioactiviteit aan jonge biggen werd uitgescheiden in de urine en 18% in de faeces, terwijl in de urine van volwassen varkens slechts sporen van furazolidon konden worden aangetoond. In weefsels van zowel biggen als volwassen varkens konden geen residuen van furazolidon gemeten worden.

De resultaten van de biggenstudie geven echter aan, dat wel radio-activiteit aantoonbaar was op relatief hoog niveau. Na een wacht-periode van 14 dagen varieerden de gehalten in de verschillende weefsels tussen 0,9-4,3 µg-equivalenten per gram. Van de achterblij-vende radioactiviteit bleek tot 56% niet extraheerbaar te zijn uit de organen en weefsels. Deze radioactiviteit was voor een gedeelte geassocieerd met DNA. Deze bevindingen kunnen een indicatie zijn voor óf endogene incorporatie in fysiologisch voorkomende bestanddelen óf covalente binding van reactieve intermediaire metabolieten aan biolo-gische marcomoleculen (hoofdstuk 3).

Studies met het volwassen varken toonden de vorming aan van een cyano-metaboliet, namelijk 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone. In de literatuur wordt gesteld dat deze cyano-metaboliet een belang-rijk omzettingsprodukt is in de urine van andere diersoorten. Deze stof blijkt echter van minder belang te zijn in plasma (half-waardetijd: 4 uur) en weefsels van volwassen varkens.

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Mogelijke verklaringen zouden kunnen zijn speciesverschillen in metabolisme van furazolidon, een zeer snelle eliminatie van de cyano-metaboliet via de nieren of het effectief wegvangen van reactieve intermediaire metabolieten van furazolidon door biologische macromoleculen of stoffen zoals glutathion (hoofdstuk 4).

Gebleken is dat microsomen uit de lever van ratten furazolidon omzetten. Twee belangrijke, ethylacetaat extraheerbare metabolieten konden worden geïdentificeerd: de reeds eerder genoemde cyano-metaboliet en een reactieprodukt van furazolidon met zijn acrylonitrilderivaat namelijk 2,3-dihydro-3-cyanomethyl-2-hydroxy-5-nitro-1α,2-di-(2-oxo-oxazolidin-3-yl) iminomethylfuro [2,3-b]furan. Van de totaal gevormde hoeveelheid metabolieten werd 2-7% covalent gebonden aan microsomaal eiwit. De toevoeging van glutathion aan het incubatiemengsel verminderde deze covalente binding en veroorzaakte tevens een bijna complete verschuiving van ethylacetaat extraheerbare metabolieten naar wateroplosbare metabolieten. In tegenstelling tot de in vivo situatie bij jonge biggen werd geen binding aan DNA waargenomen, nadat thymus DNA afkomstig uit kalveren was toegevoegd aan een microsomaal incubatiemengsel met furazolidon. Dit kan wellicht verklaard worden door verschillen tussen in vitro en in vivo betreffende de "toegankelijkheid" van het DNA voor reactieve verbindingen van furazolidon (hoofdstuk 5).

De bovengenoemde met ethylacetaat extraheerbare metabolieten bleken slechts in mindere mate gevormd te worden in varkensmicrosomen terwijl de covalente binding aan microsomaal eiwit aanzienlijk hoger was: 12-22% van totaal gevormde hoeveelheid metabolieten. De thiolgroep van cysteine is hoogstwaarschijnlijk verantwoordelijk voor de covalente binding aan microsomaal eiwit zoals blijkt uit studies uitgevoerd met aminozuren.

Na omzetting van furazolidon door varkenslever microsomen in aanwezig-
heid van mercapto-ethanol voor het wegvangen van reactieve inter-
mediairen, kon een nieuwe metaboliet worden geïsoleerd. Deze stof werd
geïdentificeerd als een conjugatieprodukt van het acrylonitrilderivaat
van furazolidon met mercapto-ethanol, namelijk 3-(4-cyano-3-β-hydroxy-
ethyl-mercapto-2-oxobutylidene amino)-2-oxazolidone. Dit conjugaat nam
ongeveer 50% van totaal gevormde metabolieten voor haar rekening.
Toevoeging van mercapto-ethanol aan het incubatiemengsel leidde er
bovendien toe dat noch de bovengenoemde ethylacetaat extraheerbare
metabolieten noch covalente binding aan microsomaal eiwit konden wor-
den waargenomen. Deze bevindingen duiden er op dat het acrylonitril
derivaat een centrale rol speelt in biotransformatie van furazolidon
door varkenslever microsomen (hoofdstuk 6).

Er kon een reversibele reactie worden waargenomen tussen het acrylo-
nitril derivaat van furazolidon enerzijds en glutathion, mercapto-
ethanol en microsomaal eiwit anderzijds. Het reversibel karakter van
deze uitwisseling blijkt afhankelijk te zijn van de pH, zoals wordt
aangetoond voor het mercapto-ethanol conjugaat. Beneden pH 2 is dit
conjugaat stabiel; een optimale uitwisseling naar microsomaal eiwit
vindt plaats tussen pH 7 en 10. Het mercapto-ethanol conjugaat ver-
oorzaakt een duidelijk direct positief effect in de Salmonella/micro-
soom test (stam TA 100), hoogstwaarschijnlijk veroorzaakt door een
interactie van het acrylonitril gedeelte met DNA (hoofdstuk 7).

Concluderend kan gesteld worden dat furazolidon na orale toediening
aan biggen snel en bijna volledig omgezet wordt. Hierbij ontstaan
produkten die voor een belangrijk deel niet extraheerbaar zijn uit
weefsels.

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Het kan niet uitgesloten worden dat deze niet extraheerbare metabo-
lieten het gevolg zijn van covalente binding van reactieve in-
termediaire omzettingsprodukten van furazolidon aan biologische
macromoleculen zoals eiwit en DNA. Studies met varkenslever microsomen
laten zien dat het acrylonitril derivaat een centrale rol speelt in de
reductieve omzetting van furazolidon. Het is aangetoond dat dit
reactief intermediair reversibel kan binden aan microsomaal eiwit. Of
deze niet-enzymatische reactie ook plaats vindt in vivo is onbekend.
Omdat de mogelijke aanwezigheid van dergelijke covalent gebonden resi-
duen in eetbare weefsels ernstige gevolgen kan hebben voor de aan-
nemelijkheid van dit geneesmiddel voor massamedicatie van voedsel-
producerende dieren, moet de identiteit van het niet-extraheerbare
materiaal in weefsels van biggen verder onderzocht worden samen met de
biologische beschikbaarheid en toxiciteit ervan. Ook moeten experimen-
ten gestart worden met als doel de extraheerbare radioactiviteit in
weefsels van biggen te identificeren omdat slechts een klein gedeelte
van deze fractie tot nu toe is opgehelderd.

CURRICULUM VITAE

Louis H.M. Vroomen werd geboren te Sittard op 30 november 1954. In
1973 behaalde hij het diploma gymnasium β aan de Scholengemeenschap
St. Michiel te Geleen. In datzelfde jaar werd begonnen met de studie
Humane Voeding aan de Landbouwhogeschool te Wageningen. In januari
1980 behaalde hij het ingenieursdiploma met als hoofdvakken Humane
Voeding (prof. dr J.G.A.J. Hautvast) en Toxicologie (prof. dr J.H.
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Na de militaire dienst vervuld te hebben als vertaler van Russische
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1 september 1983 werkt hij als wetenschappelijk ambtenaar bij de
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