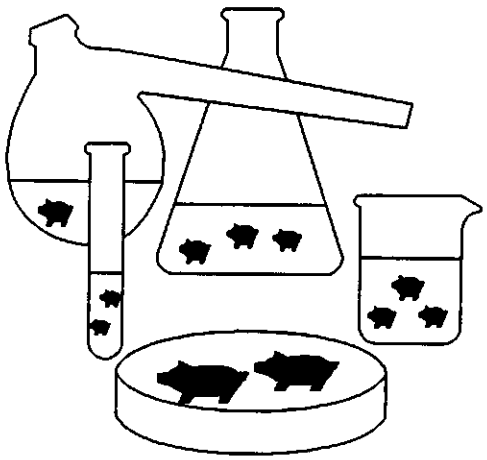
THE USE OF PIG HEPATOCYTES FOR BIOTRANSFORMATION AND TOXICITY STUDIES



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THE USE OF PIG HEPATOCYTES FOR BIOTRANSFORMATION AND TOXICITY STUDIES

Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op woensdag 10 april 1991
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

The investigations described in this thesis were carried out at the State Institute for Quality Control of Agricultural Products (RIKILT) in Wageningen, The Netherlands. The RIKILT is one of the research institutes of the Agricultural Research Department (DLO) of the Ministry of Agriculture, Nature Management and Fisheries.

STELLINGEN

1. Celcultures geïsoleerd uit slachtmateriaal van grote landbouwhuisdieren kunnen een belangrijke bijdrage leveren aan het verkrijgen van essentieel inzicht in de aard en eigenschappen van voor de consument relevante metabolieten van diergeneesmiddelen.

   -dit proefschrift

2. Bij het vaststellen van residutoleranties voor sulfonamides moet worden uitgegaan van de som van de moederstof en de N^4-acetyl-metaboliet.

   -Vree et al. (1985) in Antibiotics and chemotherapy, pharmacokinetics of sulfonamides revisited, eds Schonfeld & Hahn, Karger, Basel, pp 5-65
   -dit proefschrift

3. Er zijn sterke aanwijzingen dat de bijwerkingen van nitrofuranen bij zowel mens als dier grotendeels veroorzaakt worden door de remming van het enzymcomplex pyruvaat dehydrogenase.

   -Martindale, The extra pharmacopoeia (1982), The pharmaceutical press, London
   -Staley et al. (1978), Am. J. Path. 91: 531-541
   -dit proefschrift

4. Eiwitgebonden residuen van diergeneesmiddelen kunnen niet per definitie als onschadelijk worden beschouwd, aangezien ook hier het venijn in de staart kan zitten.

   -dit proefschrift

5. Er is onvoldoende bewijs voor de genotoxiciteit van furazolidon bij zoogdiercellen.

   -IARC monographs (1983) vol 31, pp 141-151

6. Bij de ontwikkeling en toepassing van analysemethoden gebaseerd op immunochemische technieken, kan inzicht in de biotransformatie van stoffen in belangrijke mate bijdragen aan het voorkomen van zowel vals-positieve als vals-negatieve resultaten.

7. In hun onderzoek naar het lot van bolestatine, een toxine afkomstig uit de satansboleet (*Boletus Satanas*), leveren Kretz *et al.* onvoldoende bewijs voor de absorptie van het intakte eiwit in het maagdarmkanaal.

   -Kretz et al. (1991), Xenobiotica 21: 65-73

8. Het toelatingsbeleid van pesticiden wordt door Ames *et al.* ten onrechte gebagatelliseerd.

   -Ames et al. (1990), Proc. Natl. Acad. Sci. 87: 7777-7781

9. Bij het vaststellen van normen voor nitraat in bladgroenten moet niet worden uitgegaan van het N.E.L./A.D.I. principe, maar van streefwaarden die gebaseerd zijn op natuurlijke achtergrondswaarden.

10. Zolang het gebruik van kunstmest in de landbouw niet afneemt, blijven alle pogingen om de schade aan het milieu als gevolg van het uitrijden van mest te verminderen, ongeloofwaardig.

11. De rustgevende eigenschappen van waterbedden worden veelal teniet gedaan door het sterk opgedreven stroomverbruik.

    -Consumentengids, januari 1991

12. ATB (All Terrain Bike) fietsers op bospaden bedreigen vooral de rust van de loslopende hond en zijn baas, en beschermen daardoor indirect die van het wild.

Stellingen behorend bij het proefschrift: The use of pig hepatocytes for biotransformation and toxicity studies

Laurentius A.P. Hoogenboom, 10 april 1991

CONTENTS

VOORWOORD

Zo, dit varkentje is gewassen en middels dit voorwoord wil ik nog even terugkijken op de wijze waarop dit is gebeurd. Daarbij wil ik de stelling verdedigen dat promoveren is als het met de fiets beklimmen van bergpassen.

Wanneer je voor de eerste keer aan zo'n beklimming begint, weet je niet goed waaraan je begint. Je gaat af op verhalen van anderen en je wilt de uitdaging graag aannemen. Je weet nog niet veel over het te gebruiken verzet, laat staan over het aan te houden tempo. Voordat je op weg gaat verzorg je je fiets, bestudeer je het bergprofiel en maak je een plan over de wijze waarop je je krachten denkt te verdelen. Je kiest voor jezelf een aantal mijlpalen uit, aan de hand waarvan je je gemaakte vorderingen wilt afmeten. Vervolgens ga je rustig aan de slag en al trappend leg je de eerste meters af. Na wat startproblemen gaat het steeds beter; je zit in het ritme en hebt dan nog alle tijd. Gaandeweg komen de eerste moeilijkheden die je nog vrij eenvoudig weet op te lossen, vooral als je daarbij ondersteund wordt door anderen. Bovendien bereik je je mijlpalen, hetgeen je motiveert om door te gaan, zeker wanneer je ook nog wordt aangemoedigd door andere fietsers of eventuele voorbijgangers. Wanneer je echter zo vlak onder de top zit, nemen de problemen toe, je komt er wat door te zitten. Hoe laag of hoe hoog die berg ook is, die laatste meters zijn altijd weer zwaar en soms vraag je je dan wel eens af waarvoor je het allemaal doet. De vermoeidheid is echter snel vergeten wanneer je de laatste bocht omgaat en sprintend het hoogste punt bereikt. En dan, onder het genot van een drankje, lijkt het al snel alsof het toch allemaal erg eenvoudig was en besef je dat het de moeite waard was.

Promoveren doe je niet alleen. Daarom wil ik hierbij eenieder bedanken die op enigerlei wijze heeft bijgedragen aan het tot stand komen van dit proefschrift. Allereerst zijn dat mijn ouders die mij hebben leren fietsen en nog steeds mijn trouwste supporters zijn. Harry Kuiper, Louis Vroomen en het RIKILT wil ik bedanken voor de hulp bij het uitkiezen van een voor mij interessante bergpas en het aanleveren van het benodigde materiaal. Margot Huveneers-Oorsprong, Marcel Berghmans, Melle van Kammen en Saskia Hesse wil ik bedanken voor hun deelname in het avontuur en de benodigde ondersteuning. Francis Pastoor, Welmoed Clous, Antoinette van Milaan, Trinette van Vliet en Oreana Tomassini wil ik bedanken voor hun bijdrage aan bepaalde deelaspecten van het hier gepresenteerde onderzoek. Evenals Ton Ermens, Marcel Mengelers, Monique Ploum, Sjoerdje de Boer, Maryvon Noordam, Folke Dorgelo, Maike te Riele, Paulien Oosterveld, Stefania Olmo, Nicoline Fransen, Gijs Kleter, Olga Melis en Gera Marsman wil ik hen ook bedanken voor hun bijdrage aan de prettige sfeer binnen de afdeling. Francis, jouw taart staat mij nog vers in het geheugen.

Bep van Veldhuizen, Bas Blaauboer, Wim Traag, Henk Keukens, Theo Polman, Robert Schilt, Piet Berende, Carel Funke, Walter Hazeleger en de werknemers van slachterij Hallers & Zonen te Ede wil ik bedanken voor de door hun ingebrachte deskundigheid. Professor Koeman wil ik bedanken voor zijn hulp bij het afleggen van de laatste meters, zowel de meters vlak voor het bereiken van de mijlpalen, de publicaties, als die vlak voor de top, het uiteindelijke proefschrift. Ik denk dat het verhaal daardoor een stuk boeiender is geworden. Tenslotte wil ik nog even stil staan bij de bijdrage van die mensen, die zo vaak op de achtergrond blijven maar die achter de schermen zorgen voor het reilen en zeilen van het instituut. Zonder een goede infrastructuur valt er nou eenmaal weinig te fietsen.

Karin, jou wil ik speciaal bedanken voor het begrip en het geduld dat je steeds weer weet op te brengen voor mijn hobbies. Zonder jouw actieve deelname en hulp zouden er na de laatste bocht nog vele laatste bochten gevolgd zijn. Zo, nu voorzichtig afdalen en op naar de volgende berg.

CHAPTER 1

INTRODUCTION

INTRODUCTION

The intensification in the breeding of food-producing animals during the last decades, has been accompanied by an increased use of veterinary drugs and growth-promoting agents. However, treatment of animals with drugs may result in residues of both the parent compound and its metabolites in edible products, which may imply a possible health risk for the consumer. Therefore, it is essential to obtain accurate information not only on the toxic properties of a drug, but also on its biotransformation, especially since there are numerous examples where metabolites have been shown to be responsible for the adverse effects of a compound. Since the kind of metabolites are often specific for a certain animal species, such information should preferentially be obtained from studies with target animals. However, experiments with large domestic animals are in general difficult to perform for reasons of costs, duration, housing facilities, and ethics, especially when the use of radiolabelled compounds is required in order to obtain the relevant information. *In vitro* models with hepatocytes isolated from livers of food-producing animals may offer a good alternative for at least part of the animal studies.

In vitro models with isolated hepatocytes

In recent years, there has been a large increase in the development and use of *in vitro* models, not only because of the demand of animal well-fare groups to reduce the use of laboratory animals, but also because of a number of scientific and financial advantages. This has *e.g.* resulted in the development of a large number of cell-lines, which can be used in short-term tests for screening compounds on their cytotoxic and especially genotoxic properties. A major disadvantage of these cell-lines is however the very limited biotransformation potential, and as a result the insensitivity of the cells for compounds that need to be activated in order to exert their toxic action. A possible though limited solution for this problem is the inclusion of liver homogenates and the required cofactors in the tests.

For studying the biotransformation of compounds, purified enzymes and isolated

fractions of liver cells, like microsomes, used to be the preferred *in vitro* model. However, the disruption of the cellular structure has been shown to seriously limit the value of the results with respect to the *in vivo* situation. As pointed out below, this may explain the popularity of primary cultures of hepatocytes, especially since the techniques for their successful isolation have become available.

Most methods used for the isolation of viable cultures of hepatocytes are based on the work by Howard *et al.* (1967), who used an enzymatic treatment of liver slices for the dispersion of the cells. The efficiency of the method has subsequently been improved by the introduction of an *in situ* perfusion, that is commonly used in the case of small laboratory animals (Berry & Friend 1969), and the perfusion of the liver with a buffer containing a Ca^{2+}-chelator, like EGTA, prior to the enzymatic treatment with collagenase (Seglen 1976). More recently, these methods were adapted for the isolation of hepatocytes from liver samples obtained from larger animal species, including man (Reese & Byard 1981, Strom *et al.* 1982, Guillouzo *et al.* 1985).

Advantages and disadvantages of hepatocyte cultures

The applicability of hepatocytes for various uses has been reviewed by Fry & Bridges (1979), Moldéus *et al.* (1983), Guillouzo (1986) and Chenery (1988). In general they come to the major conclusions regarding the advantages and disadvantages of the model:

-An important advantage of freshly isolated hepatocytes in comparison with cellular fractions like microsomes, is the presence of a complete set of biotransformation enzymes in the original cellular configuration. As a result compounds may be transformed by a number of subsequent metabolic steps, very sSimilar to *in vivo*. In addition, the use of expensive cofactors is superfluous, due to the intact metabolic functioning of the cells.

-The intact cellular structure offers the opportunity to use hepatocytes for studying (toxic) effects of compounds on the functioning and viability of the cells, possibly in relation to the biotransformation of a compound.

-An important advantage of hepatocytes both over the intact animal, and perfused organ, is that cells isolated from one liver or liver sample can be divided over a large number of experimental units. This is very useful for studying *e.g.* dose- and time-relationships in the biotransformation or toxic effects of a compound, or for comparing the effects of different compounds, or for studying the interactions between a number of compounds. The fact that the model is easy to handle and small in volume, makes it very suitable for the use of radiolabelled or rare and expensive compounds.

-One of the major problems encountered with rat hepatocytes, is the fact that the prolonged use of monolayer cultures is limited by a change in the activity of certain biotransformation enzymes during the ageing of the cells (for review see Paine 1990). Initially, this was shown for the very important group of cytochrome P-450 related enzyme activities, which often decrease to about 50% within 24 h. However, subsequent studies also reported changes, both increases and decreases, for other enzymes and enzymatic activities (Grant *et al.* 1985, Croci & Williams 1985, Grant & Hawksworth 1986, Vandenberghe 1988). Furthermore, the problem is not restricted to hepatocytes from rats, although in the case of human hepatocytes, the decrease in cytochrome P-450 levels appears to be much slower (Guillouzo *et al.* 1985, Mennes *et al.* 1988).

A possible solution for this problem, appeared to be the inclusion of certain hormones in the medium (Decad *et al.* 1977). Similar results were obtained by using medium containing nicotinamide or metyrapone, or medium free of cystine and cysteine, but supplemented with aminoleavulinic acid (Paine 1990). However, the application of such media has been a limited success since the apparently stable levels of cytochrome P-450 were accompanied by a selective change in the pattern of the different isoenzymes. A more promising approach appears to be the long-term cocultivation of hepatocytes with a rat epithelial cell-line, resulting in steady state levels of cytochrome P-450 after an initial decline (Bégué *et al.* 1984, Guillouzo 1986). However, the results of a recent study by Rogiers *et al.* (1990), indicate that this technique does not offer a satisfying solution for the selective shift in the cytochrome P-450 isoenzyme patterns.

Although this feature seriously limits the prolonged use of hepatocytes, it should be reminded that in general, a decline in enzyme activities will have consequences for the rate of conversion of a compound, but does not necessarily have to result in a change in metabolite patterns.

-Due to the complexity of the hepatocyte model, cellular fractions are sometimes more suitable for studying a specific biotransformation step or for the detection of reactive intermediates. In addition, often larger quantities of a specific metabolite can be prepared, due to the possibility to speed up the rate of conversion by using high levels of not only the cofactors, but also the compound under investigation, levels that may be cytotoxic for intact cells.

Toxicity end-points in hepatocyte cultures

For studying the toxicity of compounds, a wide variety of end-points can be chosen, since in theory each function of the liver cell can be monitored like genetic mechanisms, morphological changes, metabolic functions, intracellular communication but also the wide variety of different enzymes involved in the biotransformation. However, a few end-points have gained a more general popularity.

Viable hepatocytes have been shown to contain enzymes capable of recognizing and removing adducts between xenobiotics and DNA. This so-called DNA-repair mechanism, has been used to develop a test for the screening of xenobiotics on their genotoxic properties (Williams 1977, Probst *et al.* 1981, Williams *et al.* 1989).

A large number of so-called cytotoxicity tests has been developed to detect damage indicative of the death of the cells. Many references can be cited, using the leakage of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium, a parameter that has been shown to correlate very well with the diffusion of the dye trypan-blue into cells with a damaged membrane (Jauregui *et al.* 1981). More recently there has been a tendency to select end-points that are more suitable for large scale screening on cytotoxicity. This underlies the success of the so-called MTT- and NR-tests (Borenfreund *et al.* 1988), that were originally introduced for cell-lines and subsequently adapted for the use with hepatocytes. The MTT-test determines the reduction of a tetrazolium-salt by the mitochondrial enzyme succinate dehydrogenase, the NR-test on the uptake of the vital dye neutral red by the lysozymes in the cell.

An effect that in many cases may precede the death of the cell is the depletion of intracellular glutathione (GSH). This tripeptide is present at high concentrations in the cell and is involved in the detoxification of both reactive intermediates of compounds, as well as hydrogen peroxide, formed in the detoxification of superoxide (Babson *et al.* 1981, Reed & Farris 1984, Eklow-Låstbom *et al.* 1986, Jones *et al.* 1986). Therefore a decrease in GSH-levels as a result of the exposure to a certain compound may either be an indication for the increased formation of reactive oxygen species (oxidative stress), or for the formation of metabolites with nucleophilic properties, which might result in their binding to DNA and/or proteins.

The type of toxic effects that can be detected, may be specific to the liver (hepatotoxicity), but also be of a more general cytotoxic nature. *In vivo*, a specific organotoxic effect might be largely due to toxicokinetic parameters such as absorption, distribution, and excretion (Ekwall & Ekwall 1988).

The use of hepatocytes to study species-differences

Both quantitative and qualitative differences have been reported in the biotransformation of xenobiotics by different animal species (Williams 1974, Kato 1979). Initially, indications for this phenomenon came primarily from toxicity studies with small laboratory animals, showing species-differences in the sensitivity towards the toxic effects of a compound. More recently, species related differences in the biotransformation of compounds were shown in studies with isolated cellular fractions from livers of different animal species, including farmanimals (Patterson & Roberts 1970, Souhaili-El-Amri *et*

al. 1986, Watkins & Klaassen 1986, Dalvi *et al.* 1987, Short *et al.* 1988). The development of procedures for the isolation of hepatocytes from livers of different species, including man, allowed the use of this model for comparative studies on the species related biotransformation and toxicity of xenobiotics (Table 1.1.).

In vivo studies have revealed a large difference between rats and mice in their sensitivity to the hepatotoxic and carcinogenic properties of aflatoxin B1 (Croy *et al.* 1983). *In vitro* studies showed that rat hepatocytes were more sensitive than those from mice, as became apparent by the death of the cells at much lower concentrations, the much higher levels of binding of metabolites of aflatoxin B1 to TCA-precipitable cell-material (Decad *et al.* 1979, Hanigan and Laishes 1984), and the much higher levels of unscheduled DNA-synthesis (Steinmetz *et al.* 1988). Furthermore, the mycotoxin caused segregation of nucleolar components in rat hepatocytes, but not in cells from mice (Cole *et al.* 1986).

In the DNA-repair study by Steinmetz *et al.* (1988) both monkey and mouse hepatocytes gave negative test-results after exposure to 2-acetyl-aminofluorene and benzidine, in contrast to cells from rat, hamster and man. In the case of benzo(a)-pyrene, cells from mouse, hamster and especially the rat were shown to be relatively insensitive in comparison to those from the cynamolgus monkey and man. The overall differences in the DNA-repair response to the various test compounds as observed in this study between cells from monkey and man, were consistent with those obtained by Neis *et al.* (1986), using hepatocytes as activating system in the Ames test.

With a quite different set of end-points, Blaauboer *et al.* (1990) demonstrated a marked species-difference in the effect of beclofibric and clofibric acid on peroxisomal ß-oxidation and peroxisome proliferation, effects that could only be observed in hepatocyte cultures from the rat and not in those of cynamolgus monkey and man.

Several research groups have demonstrated that species differences in the biotransformation of drugs with diverse but known *in vivo* metabolite patterns, could be reproduced with hepatocytes. Gee *et al.* (1984) reported a good correlation between the results from an *in vitro* study and the *in vivo* metabolism of tolbutamide. Rat hepatocytes showed the highest activity but were unable to transform the sulfonylurea side-chain, which was the most important step performed by dog hepatocytes. In studies with amphetamine (Green *et al.* 1986), rabbit hepatocytes were shown to be much more active than cells from the rat, dog, monkey and man, and primarily N-hydroxylated the compound, whereas for rat hepatocytes the hydroxylation of the aromatic ring appeared to be the more important step. N-demethylation of ketitofen was shown to be the major metabolic route for rat and rabbit hepatocytes, in the latter case followed by sulfatation (Le Bigot 1987). The end-products of these steps were only found in traces with human hepatocytes, where the N-glucuronide and reduced ketitofen were the major metabolites. A similar difference was observed with diazepam (Seddon *et al.* 1989), where the 4-hydroxy metabolite was observed with hepatocytes from rats, but not with those from monkey, dog or man, where instead N-demethylation and, with the

Table 1.1 List of studies using hepatocytes of different animal species to compare the biotransformation and/or toxicity of compounds.

Species used	Effect studied	Compounds used	Reference
rat, mouse	biotransformation	aflatoxin B1	Decad *et al.* 1979
rat, mouse	biotransformation, cytotoxicity	aflatoxin B1	Hanigan & Laishes 1984
rat, mouse, man	toxicity	aflatoxin B1	Cole *et al.* 1986
rat, mouse, hamster, guinea pig	Ames test, DNA-repair	2-(acetyl)-amino-fluorene	Holme & Søderlund 1985
rat, mouse, hamster, guinea pig	biotransformation	2-acetyl-aminofluorene	Holme *et al.* 1986
rat, mouse, hamster, rabbit	DNA-repair	various compounds	McQueen & Williams 1987
rat, mouse, hamster monkey, man	DNA-repair	various compounds	Steinmetz *et al.* 1988
monkey, man	Metabolic activation Ames test	various compounds	Neis *et al.* 1986
rat, hamster, guinea pig	Metabolic activation Ames test	benzidine, 4-amino-biphenyl	Neis *et al.* 1984
rat, monkey, man	peroxisome proliferation	(be)clofibric acid	Blaauboer *et al.* 1990
rat, hamster, rabbit, dog	cytotoxicity, biotransformation	acetaminophen	Green *et al.* 1984
rat, rabbit, dog, monkey, man	biotransformation	amphetamine	Green *et al.* 1986
rat, rabbit, dog, monkey	biotransformation	tolbutamide	Gee *et al.* 1984
rat, rabbit, man	biotransformation	ketitofen	Le Bigot *et al.* 1987
rat, rabbit, dog, guinea pig, man	biotransformation	diazepam	Chenery *et al.* 1987
rat, dog, monkey, man	biotransformation	diazepam	Seddon *et al.* 1989
rat, dog, monkey, man	biotransformation	temelastine	Oldham *et al.* 1990

exception of the dog, 3-hydroxylation were the important steps.

The major conclusion that can be drawn from these and other comparative studies listed in Table 1.1, is that in general no two animal species appear to be comparable in the biotransformation and sensitivity towards a toxic effect of a compound. Therefore, extrapolation of data obtained with cellular fractions, hepatocytes or intact animals from one species to another species is not possible, and stresses the need for the use of target-animals for risk-assessment purposes.

Isolation and use of hepatocytes from large domestic animals

The possible isolation and use of hepatocytes from large domestic animals has been subject of a number of studies. Suspension cultures of hepatocytes, isolated from liver samples of sheep (Démigne *et al.* 1986, Chow *et al.* 1990), goats (Aiello & Armentano 1987a, 1987b, Aiello *et al.* 1989), and cows (Aiello *et al.* 1989), were *e.g.* used to study the gluconeogenesis from propionate and butyrate, a process that is relatively specific for ruminants. Monolayer cultures of pig hepatocytes, isolated by an *in situ* perfusion from livers of neonatal animals, were used to study the catabolism of lipoproteins (Pangburn *et al.* 1981, Bachorik *et al.* 1982), regarding the good similarity between pigs and men with respect to the importance of the LDL particle. In the same field of interest, Kwekkeboom *et al.* (1988, 1989) used monolayer cultures of pig hepatocytes, isolated from liver samples of young animals (up to 7 weeks), to investigate the feedback regulation of bile acid synthesis, being an important factor in the elimination of cholesterol from the body. Finally, suspension cultures of hepatocytes, isolated by an *in situ* perfusion from 2-15 days old piglets were used by Pegorier *et al.* (1982), to study the effects of fasting on the gluconeogenesis.

Only recently, investigators have become aware of the opportunities to use hepatocytes from large domestic animals for studying the possibly species-specific biotransformation and toxicity of compounds used in veterinary practice. Suolinna & Winberg (1985) studied the deethylation of 7-ethoxycoumarin and the conjugation of methylumbelliferone by suspension cultures of bovine hepatocytes, isolated from liver samples obtained at the slaughterhouse from fetal or adult (1.5-2 years) animals. Shull *et al.* (1986), introduced the surgical removal of the caudate lobe of the bovine liver, and its subsequent use for the isolation of hepatocytes. Subsequently the biotransformation of 7-ethoxycoumarin and the pesticide aldrin was studied in monolayer cultures. Mennes *et al.* (1988) included sheep hepatocytes in a study with rat, hamster, dog, monkey and human hepatocytes, in order to compare the decline of cytochrome P-450 levels in monolayer cultures. Recently Pool *et al.* (1990) used suspension cultures of pig hepatocytes obtained from 12 months old animals, to study the effect of a number of nitros-

amines on the frequency of single strand breaks in the DNA.

These studies clearly show that it is possible to isolate viable hepatocytes from large domestic animals. However, the possible use of these cells for biotransformation and toxicity studies of compounds used in veterinary practice, has not been thoroughly investigated.

Scope of the study

The main aim of the present study was to develop a method for the isolation of viable hepatocytes from liver samples of adult pigs, secondly, to investigate whether these cells can be used for biotransformation and toxicity studies, and thirdly, to demonstrate the value of the model, more in particular for studies on the identity and properties of metabolites of veterinary drugs, that might be present as residues in edible products. The opportunities of the model for studying the mechanisms underlying adverse effects in target-animals was therefore beyond the scope of this study.

The procedure for the isolation of pig hepatocytes (Chapter 2), as well as the use of the cells for biotransformation (Chapters 3, 4) and toxicity studies (Chapter 5) was investigated in the first part of the study. For this purpose, the antibacterial drugs sulfadimidine and a number of nitrofurans, as well as the steroid hormone ß-nortestosterone were used as model compounds.

In the second part (Chapters 6, 7, 8), the identity and properties of metabolites of furazolidone were studied in more detail, as a follow-up of the studies performed in our laboratory by Dr Vroomen (1987). Previous *in vivo* studies with ^{14}C-labelled furazolidone, revealed that this drug is extensively biotransformed in the pig, partly resulting in the presence of unextractable radiolabelled compounds in the protein fraction. Regarding the persistence of this, possibly drug-like, material, as compared to the parent compound and other metabolites, special attention was paid to the identity and possible toxic properties of these type of metabolites. The issue of bound-residues of furazolidone and other drugs has since long been one of most controversial and difficult problems in the field of residue toxicology.

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CHAPTER 2

ISOLATION AND CULTURING OF PIG HEPATOCYTES

Materials and Methods

ISOLATION AND CULTURING OF PIG HEPATOCYTES

Materials and Methods

Introduction

Hepatocytes were isolated from a piece of pig liver based on a method described by Seglen (1976) for the isolation of rat hepatocytes, modified for liver biopsies as described by Strom *et al.* (1982). Cells are dispersed by the subsequent treatment with the Ca^{2+}-chelator EGTA and the bacterial enzyme collagenase, and purified by filtration and centrifugation. This chapter describes in more detail the methods used for the isolation, culturing and use of the cells, as well as some of our experiences with the methods, that might be of use to other research groups.

Materials and methods

Chemicals

Williams' medium E powder, Hanks' balanced salts (HBS), fetal calf serum (FCS), penicillin/streptomycin, Trypan Blue solution (0.4%), and HEPES buffer were purchased from Flow Laboratories (Rickmansworth, U.K.), bovine insulin, bovine serum albumin (RIA-grade) and collagenase types I and IV from Sigma (St.Louis, U.S.A.), collagenase type H from Boehringer (Mannheim, F.R.G.), phenol red from Serva (Heidelberg, F.R.G.), and NaCl, KCl, NaOH, $CaCl_2.2H_2O$, $NaHCO_3$ and EGTA from Merck (Darmstadt, F.R.G.). Water was purified using a water purification system (Millipore Q UF, Etten-Leur, The Netherlands). Tissue culture dishes were obtained from Costar (Badhoevedorp, The Netherlands). An oxygenator as described by Moldéus *et al.* (1978) was made at the RIKILT by M.L. Karruppannan.

Animals

Livers were obtained from sows, both non-pregnant and pregnant (3-12 days), and castrated males (Dutch Landrace x Yorkshire), with an age varying between 5 and 15

months. Animals were kept at the Department of Animal Husbandry of the Agricultural University in Wageningen, and had not been treated recently with veterinary drugs.

Preparation

Buffer solutions and media were in general prepared one or two days before the perfusion. For the isolation of the cells five HEPES buffered solutions were prepared. Buffer I contained 8.3 g NaCl, 0.5 g KCl, 2.4 g HEPES and 0.19 g EGTA per litre, Buffer II 8.3 g NaCl, 0.5 g KCl and 2.4 g HEPES per litre, buffer III 3.9 g NaCl, 0.5 g KCl, 24.0 g HEPES and 0.7 g $CaCl_2.2H_2O$ per litre, buffer IV 2 g BSA, 9.91 g HBSS and 2.4 g HEPES per litre and buffer V 9.91 g HBSS and 4.8 g HEPES. All buffers were brought with 5 N NaOH to pH 7.65 (at room-temperature), sterilized by filtration over a 0.2 μM Sterivex filter (Millipore) and stored at 4 °C. Occasionally, buffers were enriched with 10 mg phenol red per litre. A sixth solution containing 9 g NaCl per litre, was autoclaved at 120 °C for 20 min. All glassware and steel equipment was sterilized at 180 °C for 4 h, plastic material was autoclaved at 120 °C for 20 min.

A solution of collagenase in buffer III was prepared 2 h before the perfusion (200 mg/100 ml), filtersterilized (0.45 μM), stored at 4 °C and diluted with 300 ml prewarmed buffer III shortly before use. Buffers I and II were preincubated for 2 h at 40 °C.

Isolation and purification of hepatocytes

Pigs were transported to a slaughterhouse in the town of Ede and killed by electrocution followed by exsanguination. Subsequently a caudal lobe of the liver was obtained (about 150 g) and washed with 0.5 litre of icy-cold NaCl-solution via the veins and arteries using a 50 ml syringe. The sample was then transported to the laboratory in 1 litre of icy-cold NaCl. At the laboratory the weight of the sample was reduced to about 100 g, and the sample was placed in a large Büchner funnel covered by a large watchglass (Figs 2.1 and 2.2A). Small pipettips (20-200 μl) were used to connect four of the larger bloodvessels of the liver sample with the oxygenator (Fig. 2.2B). The oxygenator was furthermore connected to a peristaltic pump (run at 120 ml/min) and a gascylinder with O_2/CO_2 95/5 (about 2 bubbles/sec), and was placed in a waterbath set at 40 °C. During the perfusion the pressure within the system was kept between 40 and 60 cm water pressure, as controlled with the open manometer. The liver sample was perfused with respectively 1 litre of buffer I, 1 litre of buffer II, and finally 400 ml of the collagenase solution for 30 min under recirculation. During this period the liver swell and the surrounding connective tissue started to leak.

Subsequently the sample was disconnected from the system and placed in a beaker. All further handlings were performed under sterile conditions in a laminar flow cabinet (Clean Air, Woerden, The Netherlands). The sample was cut into small pieces using a pair of scissors and the suspension filtered over respectively a Büchner funnel, to remove the larger parts, and a 250 μm nylon filter. The filters were washed with icy-

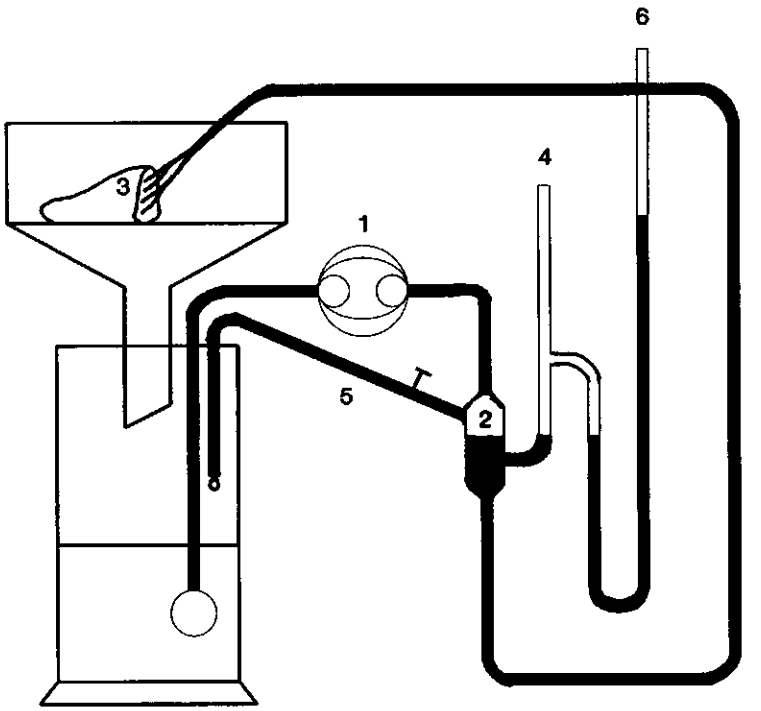

Fig. 2.1 Schematic presentation of the perfusion during the recirculation step with collagenase. The buffer is pumped by a peristaltic pump (1) via the oxygenator (2) to the liver sample (3), which is placed in a Büchner funnel. Oxygen is added via the oxygenator at (4) and escapes via the overload (5). The pressure is monitored with the manometer (6). All vessels containing buffer solutions, the oxygenator and the tubing are placed in a waterbath (40 °C).

cold buffer IV. The filtrate (about 200 ml) was poured into four 50 ml centrifuge tubes (Costar), cooled on ice for 5 min, and centrifuged at 100 g for 4 min at 4 °C using a Sorvall RC-3B centrifuge. The supernatant was removed and the pellet resuspended in buffer V, using a glass pipet. The cells were collected again by centrifugation and washed twice with buffer V. Eventually the pellets were resuspended in 100 ml Williams' medium E and filtered through a 250 μm nylon filter. A sample of the filtrate was diluted 1/1 with a solution of trypan blue, and after 5 min used to determine both the concentration of cells and the fraction of trypan blue excluding cells (viability) in a Bürker counting chamber.

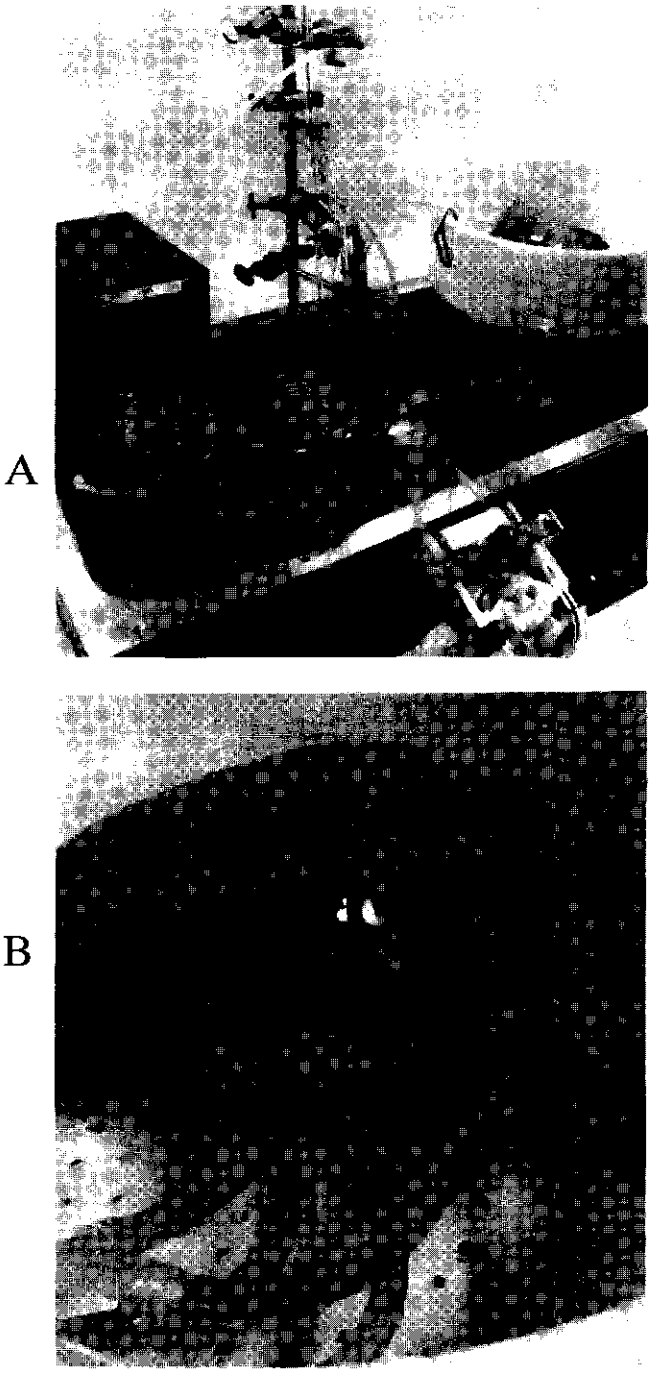

Fig. 2.2 Equipment used for the perfusion of a sample of pig liver (A) as well as the connection of the sample with the oxygenator (B).

Cell culturing

Unless indicated, cell suspensions were diluted to a density of 1.25-1.5 million viable cells per ml of Williams' medium E, supplemented with 5% FCS, 0.5 μg insulin per ml, 50 IU penicillin per ml and 50 μg streptomycin per ml. Cells were seeded in either 60 mm dishes (2.5 ml/dish), or in 24-multiwell dishes (0.5 ml/well of a suspension containing 0.6-0.75 million viable cells per ml), and incubated in an incubator at 38 °C, 5% CO_2 and high humidity. After 4 h the medium was replaced by new medium.

Appraisal

The method described above has been successfully applied on a large number of liver samples. About 2.5-8 million viable cells could be isolated per gram of liver, with 93 ± 3 % of the cells being impermeable to trypan blue, as calculated over the last 40 samples. In the majority of the cases, isolated cells attached themselves to uncoated

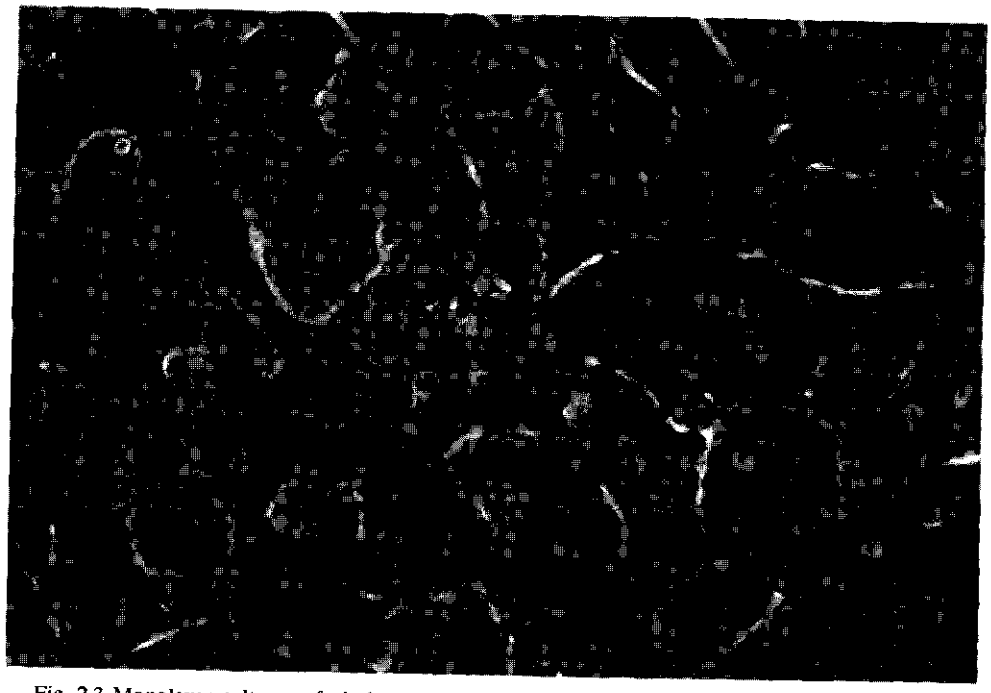

Fig. 2.3 Monolayer cultures of pig hepatocytes, 48 h after seeding.

tissue culture dishes and stretched within 24 h, resulting in nice monolayer cultures (Fig. 2.3). However, occasionally cultures were obtained with a high fraction of trypan blue excluding cells, but with low plating efficiency, for inapparent reasons. At this point age, sex and early pregnancy can apparently be ruled out as factors which prevent a successful isolation of the cells. On the other hand it appears that the type of collagenase is crucial. With collagenase type IV (Sigma), a large amount of cells, however with low viability, were obtained, unable of forming monolayer cultures. No improvement was obtained by reducing the duration of the collagenase treatment. For a while collagenase type H from Boehringer was successfully applied, than with a new shipment of the same batch the isolation failed completely, resulting in a large number of ruptured cells and large losses during the washing of the cells. With collagenase type I (Sigma), which had been used successfully at the start of the project, the problems disappeared immediately. When occasionally other batches of the collagenase obtained from Boehringer were used, the problems returned. Although no systematic comparison of different types of collagenase at the same time was performed, it seems that this is an important factor, which might however be different for livers of other animal species.

Despite these occasional problems, it is evident that viable cells can be isolated from liver samples of pigs on a regular base and that quantities of cells are large enough for large scale experiments.

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CHAPTER 3

THE USE OF PIG HEPATOCYTES FOR BIOTRANSFORMATION STUDIES OF VETERINARY DRUGS

This chapter is based mainly on the following publication:

Hoogenboom L.A.P., Pastoor F.J.H., Clous W.E., Hesse S.E. and Kuiper H.A. (1989). The use of porcine hepatocytes for biotransformation studies of veterinary drugs. *Xenobiotica* 19: 1207-1219.

completed with some data from:

Hoogenboom L.A.P., Milaan A.J. van, Hesse S.P., Vroomen L.H.M. and Kuiper H.A. The use of porcine hepatocytes for biotransformation and toxicity studies of veterinary drugs. In: Proceedings of the 4th congress of the European Association for Veterinary Pharmacology and Toxicology, Budapest, Hungary 1988 (in press).

THE USE OF PIG HEPATOCYTES FOR BIOTRANSFORMATION STUDIES OF VETERINARY DRUGS

Summary

Cultures of porcine hepatocytes with high viability were isolated from a liver sample by a simple procedure. In aging monolayer cultures the cytochrome P-450 content and 7-ethoxy-coumarin-O-deethylase activity decreased gradually while glutathione levels increased.

The nitrofuran, furazolidone, was rapidly transformed by suspension cultures, partly resulting in the formation of 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidinone. Acetylating and deacetylating activities towards sulphadimidine and its N^4-acetyl metabolite were present in suspension cultures of porcine hepatocytes. Relative and absolute levels of these activities varied in different batches of hepatocytes.

In aging monolayer cultures the biotransformation of furazolidone was relatively low during the first 24 h as compared to the next 72 h. The acetylation of sulfadimidine tended to increase with culture age whereas the deacetylation of the N^4-acetyl metabolite decreased.

No differences were seen in a number of enzyme activities measured in cytosolic and microsomal fractions isolated from different lobes of one liver. Differences between livers from different animals were marked.

Abbreviations used: ECOD= ethoxy-coumarin-O-deethylase; LDH= lactate-dehydrogenase; GSH= reduced glutathione; DMSO= dimethyl-sulfoxide

Introduction

Most foreign compounds are intensively metabolized upon administration to animals, and the outcome of the biotransformation process, *e.g.* the kind of metabolites formed, may vary from species to species and may also be influenced by factors like sex, age and nutrition (Smith *et al.* 1984, Souhaili-El-Amri *et al.* 1986, Watkins & Klaassen 1986). Therefore, the toxicity and biotransformation of veterinary drugs should preferably be studied in target animals. Yet, food-producing animals are rarely used for this purpose since such studies are expensive, time-consuming, and require special technical facilities. This is particularly true in the case of experiments with radiolabelled compounds, often necessary for identification of unknown metabolites.

The use of *in vitro* models such as cell fractions and cell cultures isolated from organs of food-producing animals may help to overcome some of these problems as pointed out by Shull *et al.* (1987). Specific biotransformation steps of a drug may be elucidated as well as the influence of other compounds or metabolic processes which occur *in vivo* (Vroomen *et al.* 1987a). Furthermore, the use of intact cells offers the opportunity to integrate biotransformation and toxicity studies. As described in Chapter 1, the isolation and application of hepatocytes from food-producing animals has been reported for larger animal species, like cattle (Shull *et al.* 1986; Suolinna and Winberg 1985), and sheep (Demigne *et al.* 1986). Furthermore, hepatocytes have been isolated from newborn or foetal pigs by an *in situ* perfusion technique and subsequently been used for studying fat and carbohydrate metabolism (Bachorik *et al.* 1982, Pangburn *et al.* 1981). As described in Chapter 2, we have now developed a method to isolate hepatocytes from liver samples of adult pigs.

Aim of the present study was to investigate the value and properties of pig hepatocytes, both in suspension and monolayer cultures, using a number of different parameters. The cytochrome P-450 content, as well as the deethylation of 7-ethoxy-coumarin was studied both in suspension and monolayer cultures, regarding the important role of cytochrome P-450 isozymes in biotransformation processes, and the reported decline of P-450 related enzymatic activities in monolayer cultures of hepatocytes from other animal species (Chapter 1). Intracellular glutathione (GSH) concentrations were measured, regarding its possible role in both the biotransformation of foreign compounds and in the protection against toxic effects.

It should be stressed that studies with isolated models should always be validated by results from *in vivo* studies. Therefore, the biotransformation of two widely used veterinary drugs, sulphadimidine and furazolidone, were studied. *In vivo* studies with pigs have revealed that sulphadimidine is excreted mainly as its N^4-acetyl metabolite (50%) (Hoogenboom *et al.* 1988, Vree *et al.* 1985). Furazolidone is thought to be metabolized by reduction of the nitro-group, partly resulting in the formation of an open chain cyano metabolite (Vroomen *et al.* 1987b). In addition to studies with

suspension cultures, special attention was paid to the effect of ageing of monolayer cultures on the biotransformation of these compounds.

Major causes of variation in metabolic potential between hepatocytes isolated from different livers are most likely due to individual differences between animals. Furthermore, in the case of rat livers it is well known that there are differences in enzyme activities between peri-venous and peri-portal hepatocytes originating from the same liver (Kera *et al.* 1987, Quistorff *et al.* 1986). Whether such differences are also reflected in hepatocyte cultures prepared from different lobes is unclear. We therefore studied several enzyme activities in cell fractions prepared from the four major lobes of a pig liver and, in addition, we compared the enzyme activities in cell fractions isolated from livers of six different animals.

Materials & Methods

Chemicals and solvents

Williams' medium E, Hanks' balanced salts, HEPES buffer, foetal calf serum, penicillin/streptomycin, glutamine and trypan blue were purchased from Flow Laboratories (Rickmansworth, UK), Ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and Folin reagens from Merck (Darmstadt, FRG), insulin, cytochrome C, sulphatase, collagenase type I, bovine serum albumin, diethyl 4-nitrophenol phosphate (paraoxon), fluoro-dinitrobenzene and sulphadimidine from Sigma (St Louis, MO, USA), resorufin, 1-chloro-2,4-dinitrobenzene and hydroxy-coumarin from Aldrich (Brussels, Belgium), NADPH, GSH, 7-ethoxycoumarin from Boehringer (Mannheim, FRG) and 7-ethoxyresorufin from Pierce Chemicals (Oud Beijerland, The Netherlands). Tissue culture dishes were purchased from Costar (Badhoevedorp, The Netherlands). Furazolidone was a gift from Orphahell B.V. (Mijdrecht, The Netherlands) and N-acetyl sulphadimidine from ILOB-TNO (Wageningen, The Netherlands). The open-chain cyano-metabolite of furazolidone was synthesized as described previously (Vroomen *et al.* 1987b).

Animals

Liver samples were obtained from seven to nine month old sows in early pregnancy (7-10 days), used in programmes of the Department of Animal Husbandry of the Agricultural University at Wageningen. Animals were not treated with veterinary drugs during a considerable period prior to slaughtering. The presented data are based on studies with hepatocytes isolated from livers of seventeen different animals. The results of each experiment were checked for reproducibility in at least one similar experiment using hepatocytes isolated from another liver.

Hepatocyte isolation

Hepatocytes were isolated from a liver sample as described in Chapter 2, with one difference; following the treatment with collagenase, the sample was minced and the homogenate was diluted with 100 ml of buffer containing fresh collagenase (0.05%), and incubated for another 10 min at 37 °C, followed by filtration.

Culture conditions

For monolayer cultures, 2.5 million viable cells in 2.5 ml of medium were seeded in 60 mm dishes. Routinely Williams E medium supplemented with 5% foetal calf serum, insulin (0.5 μg/ml), glutamine (2 mM), penicillin (50 IU/ml) and streptomycin (50 μg/ml) was used. After four hours cells were attached and the medium was renewed.

For suspension cultures, cells were washed and diluted to a density of 2.5 million viable cells per ml in Krebs/Henseleit buffer (Moldéus et al. 1978) containing 20 mM HEPES. Cells were preincubated for 15 minutes at 37 °C prior to the addition of the substrates.

Cell-monitoring

At various time intervals, cells in monolayer cultures were washed and removed from the dishes in 1 ml of either phosphate buffered saline or a specially defined P-450 buffer, as described by Topp and van Bladeren (1986) (0.1 M sodium phosphate buffer pH 7.4, 0.5% (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM EDTA and 1 mM dithiothreitol). Cells collected in phosphate buffered saline were immediately frozen and stored in liquid nitrogen. For determination of the cytochrome P-450 content, cell suspensions were thawed on ice, homogenized and an aliquot of 0.4 ml was diluted with 0.2 ml of the special P-450 buffer. The remaining cell suspension (0.6 ml) was used for determination of intracellular GSH. Cells collected in the special P-450 buffer were homogenized using a teflon pestle, and subsequently centrifuged at 14,000 g. The supernatant was stored in liquid nitrogen. Cytochrome P-450 levels were determined by the CO-difference spectrum as described by Omura and Sato (1964). No differences in cytochrome P-450 levels were observed due to the two methods of cell collection and preparation.

Protein in cell homogenates or cellular fractions (see below) was determined by the method of Lowry as modified by Peterson (1977). Glutathione (GSH) was determined by HPLC as described by Reed *et al.* (1980), using an amino spheri-5 column (4.6 x 220 mm; Brownlee, Santa Clara, USA). A 0.5 M citrate-buffer (pH 4.6) was used instead of the regular acetate buffer. Possible deterioration of the analytical column by the remaining fluoro-dinitrobenzene and its breakdown products was prevented by using a column switch technique in combination with an amino guard column (4.6 x 30 mm, Brownlee). Intracellular GSH levels were not influenced by storage of the cells in liquid nitrogen.

7-Ethoxycoumarin O-deethylase (ECOD) activity

Cells in suspension or monolayer culture were incubated in medium containing different concentrations of 7-ethoxycoumarin. A concentration of 100 μM proved to be useful for monitoring monolayer cultures. Small aliquots were taken after different time intervals, snap-frozen and stored at -20 °C. For analysis, samples were thawed, treated with sulphatase/ß-glucuronidase for 1 h and diluted in glycine/NaOH buffer (pH 10.3) as described by Aitio (1978). Fluorescence was measured within a few minutes because of the instability of 7-hydroxy-coumarin at pH 10.3. All measurements were done on a MPF-2A fluoro-spectrophotometer (Perkin Elmer) (λex 370 nm, λem 457 nm). The efficiency of the method was checked by measuring the concentrations of 7-ethoxy-coumarin under the same conditions (λex 330 nm; λem 390 nm).

Biotransformation of sulphadimidine, its N^4-acetyl metabolite and furazolidone

Cell suspensions (2.7 ml), containing 2.5 million viable cells per ml, were preincubated for 15 min at 37 °C in Williams' E supplemented with 20 mM HEPES. Unless indicated, furazolidone, sulphadimidine or N^4-acetyl sulphadimidine were added in DMSO (final concentration 1%). After different incubation times (0, 20, 40 and 80 min) aliquots of 0.2 ml were taken and snap-frozen. For analysis, samples were thawed, diluted with 1 ml sodium acetate buffer (0.1 M, pH 5) and extracted three times with 2 ml ethyl acetate. The extracts were analyzed by HPLC coupled to diode array equipment (Hewlett Packard) as described previously (Hoogenboom *et al.* 1987). This technique allowed confirmation of the identity and purity of the metabolites both by their chromatographic behaviour and their UV spectra.

In the case of monolayer cultures, the incubation medium was replaced by 3 ml of Williams' E supplemented with furazolidone, sulfadimidine or the N^4-acetyl metabolite. At t=0 and t=24 h samples of 0.5 ml were taken, mixed with 0.5 ml sodium acetate buffer (0.1 M, pH 5) and 2 ml EtAc, and stored at -20 °C until analysis, as described above. The amount of drug transformed was calculated by comparing the concentrations before and after the incubation.

Preparation of cellular fractions

Whole livers or parts of livers were obtained as described and transported on ice to the laboratory. Livers were divided by separation of the four lobes. Lobes were perfused with 0.9% NaCl and homogenised under nitrogen in twice their volume of a 1.15% KCl-solution using a Braun blender mixer. Homogenates were centrifuged for 20 min at 9000 g (4 °C). The supernatant was centrifuged for 90 min at 105,000 g (4 °C), the cytosolic fraction was aspirated and the pellet was resuspended in 1.15% KCl. Following centrifugation, the microsomes were resuspended in 0.1 M phosphate buffer (pH 7.4). Both microsomal and cytosolic fractions were stored in liquid nitrogen.

Enzyme activities in cellular fractions

Microsomal fraction

Cytochrome P-450 levels were determined as described by Omura & Sato (1964). Ethoxyresorufin- and ethoxycoumarin-O-deethylase activities were determined using a direct fluorescence technique (Prough *et al.* 1978). Substrate concentrations were 0.5 μM and 100 μM for 7-ethoxyresorufin and 7-ethoxycoumarin, respectively. NADPH-dependent cytochrome-C-reductase activity was measured by the method of Strobel and Dignam (1978). UDP-glucuronyl transferase activity was determined as described by Vessey & Zakim (1972) using p-nitrophenol as substrate; optimal activity was obtained by preincubating the microsomes in 12.5 mM Tris/HCl buffer (pH 8.0) containing 0.6% Triton X-100 for 10 min at 20 °C.

Cytosolic fraction

Glutathione-S-transferase activity was measured as described by Habig and Jakoby (1981) using 1-chloro-2,4-dinitrobenzene as substrate. Acetyl-CoA-acyltransferase activity was determined by incubating cytosolic fractions in 0.1 M phosphate buffer (pH 7.4) containing 0.16 mM sulphadimidine at 37 °C. At regular time intervals samples were taken and analyzed by HPLC for the N^4-acetyl metabolite as described previously (Hoogenboom *et al.* 1987). GSH concentrations were determined after storage for one year in liquid nitrogen. GSH levels determined in three freshly homogenized and fractionated livers gave results in the same range as observed in stored samples.

Results

Using a biopsy technique, hepatocytes were isolated from livers of adult sows with a yield varying between 2.5 and 10 million cells per gram wet weight. The fraction of cells excluding trypan blue varied between 90 and 95%. Usually, cells readily attached themselves to uncoated culture dishes resulting in typical monolayer cultures. However, in three out of seventeen cases, cultures were obtained with poor plating efficiency despite an initial high fraction of trypan blue excluding cells.

There was a large variation in intracellular glutathione (GSH) levels in freshly isolated hepatocytes (Fig. 3.1). In all cultures GSH-levels increased markedly during the first 48 h. Cellular levels of cytochrome P-450 decreased to about 70 and 40% within respectively 24 and 48 h (Fig. 3.2A). Similar results were obtained for the ECOD-activity in aging monolayer cultures (Fig. 3.2B). ECOD-activities in suspension cultures at t=0 h at the same cell density of 1 million/ml, were markedly lower, *i.e.* 0.33 ± 0.01 and 0.19 ± 0.00 nmol/min per mg protein (mean ± SEM; n=3) for the two batches

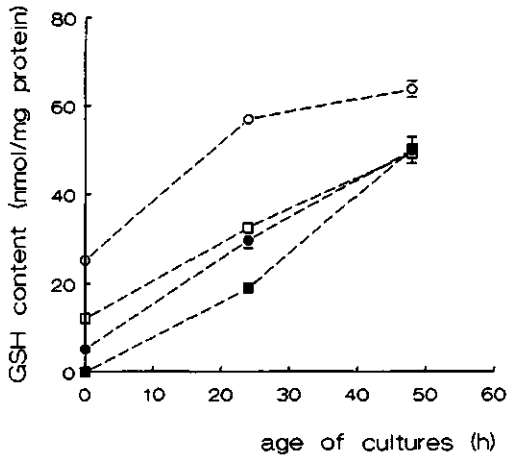

Fig. 3.1 Intracellular GSH levels in aging monolayer cultures of pig hepatocytes, isolated from livers of 4 different animals. Results are expressed per mg cellular protein (mean ± SEM; n=3).

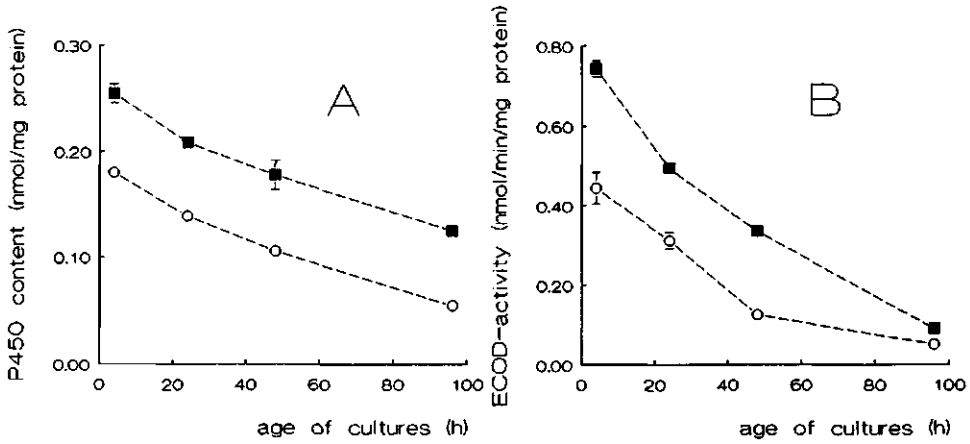

Fig. 3.2 Cytochrome P-450 levels (A) and ECOD-activity (B) in aging monolayer cultures of hepatocytes isolated from livers of two different pigs. Cultures were started at t=0 h; medium was renewed at t=4 h. Results are expressed per mg total cellular protein (mean ± SEM; n=3).

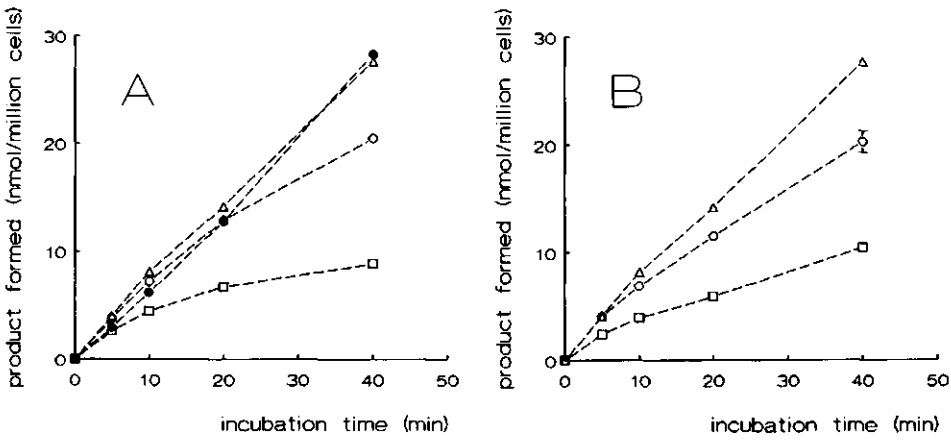

Fig. 3.3 ECOD-activity of suspension cultures of one batch of pig hepatocytes at different (A) substrate concentrations and (B) cell densities. A: cell density was 0.5 million/ml; concentrations of 7-ethoxycoumarin were: 10 □ , 30 ○ ,100 △ , and 300 ● μM. B: substrate concentration was 100 μM; cell densities were: 0.5 △ , 1.0 ○ , and 2.5 □ million/ml. Results are expressed as mean ± SEM (n=3).

of cells. This was not reflected in the cytochrome P-450 levels, being 0.26 ± 0.01 and 0.22 ± 0.02 nmol/mg protein.

Fig. 3.3 shows the ECOD-activity of freshly isolated cells in suspension at different concentrations of 7-ethoxycoumarin (A) and at different cell densities (B). In a similar experiment where cells were incubated for 30 min with 100 μM 7-ethoxy-coumarin at a density of 0.75, 1.50 and 3.00 million cells per ml, ECOD-activities of respectively 0.33 ± 0.01, 0.23 ± 0.01 and 0.15 ± 0.01 nmol/min per million cells were measured. Addition of 95% oxygen (carbogen) to the cultures at a cell-density of 1.5 million per ml resulted in an activity of 0.37 ± 0.01 nmol/min per million cells.

Furazolidone (50 μM) was rapidly metabolized in suspension cultures, partly resulting in the formation of the open chain cyano metabolite, 3-(4-cyano-2-oxobutylidene-amino)-2-oxazolidinone (Fig. 3.4). In a second experiment, where cells, isolated from another liver, were incubated for 80 min with 20 μM furazolidone either without further additions or in the presence of 1% DMSO or 2% BSA, respectively 38.7 ± 2.7, 37.4 ± 0.4 and 36.3 ± 0.5 % of the drug was metabolized.

Addition of sulphadimidine to suspension cultures resulted in the formation of low levels of N^4-acetyl sulphadimidine. The reversed reaction was observed upon addition of N^4-acetyl sulphadimidine to the cells. In the dose range studied, both activities increased proportionally with the concentration of the substrate (Fig. 3.5). Incubation of cells (culture 2, Fig. 3.6) with either sulphadimidine or its N^4-acetyl metabolite in the

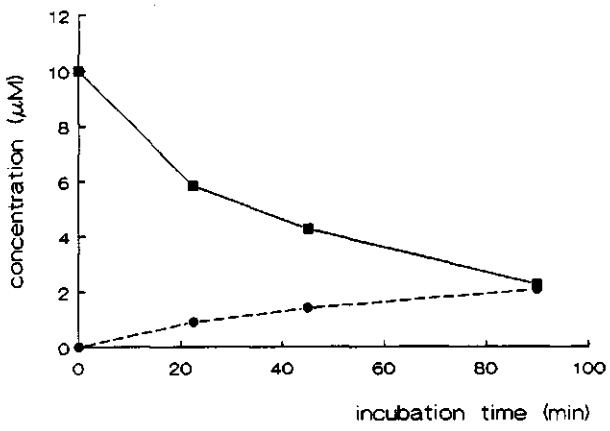

Fig. 3.4 Biotransformation of furazolidone (■—■) and formation of the open-chain cyano metabolite (●--●) by suspension cultures (mean ± SEM; n=3). Substrate concentration 10 μM.

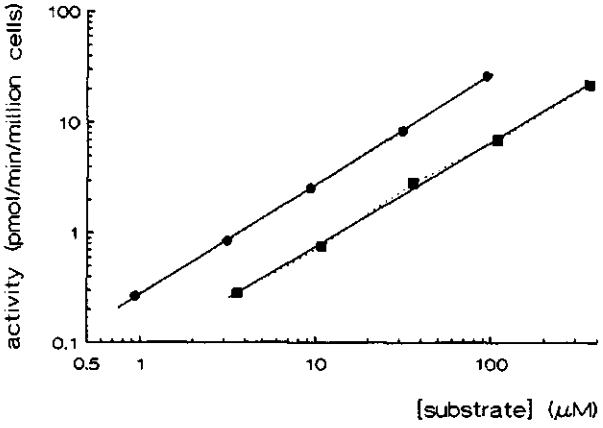

Fig. 3.5 Acetylation of sulphadimidine (■) and deacetylation of N^4-acetyl sulfadimidine (●) by suspension cultures at different substrate concentrations (mean ± SEM; n=3). Cells from culture no.1 (Table 3.1) were used for this experiment.

presence of paraoxon (2.5 μg/ml), resulted in an almost complete inhibition of the deacetylation reaction and a 1.3-fold increase in the amount of acetylated drug (Table 3.1). No increase in LDH-release could be observed due to treatment with paraoxon (data not shown). Both the amounts of acetylated and deacetylated products were lower when 2% bovine serum albumine was added to the medium (Table 3.1). The presence of 1% DMSO resulted in a clear reduction of the deacetylation rate while the acetylation rate tended to increase. There was a marked variation in the acetylation and deacetylation activities between batches of hepatocytes prepared from different livers (Fig. 3.6, Table 3.1). As a result there was a clear difference in the relative activities of the two enzymes as shown by the deacetylation/acetylation ratio (Table 3.1).

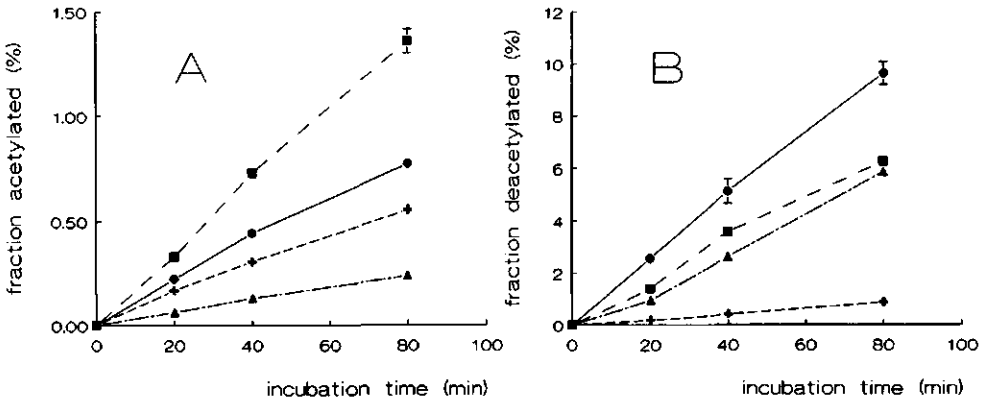

Fig. 3.6 Acetylation (A) and deacetylation (B) by four different batches of porcine hepatocytes (Culture no. 1 ■, 2 ●, 3 +, 4 ▲). Substrate concentrations: 360 μM sulphadimidine and 94 μM N^4-acetyl sulphadimidine. Results are expressed as mean ± SEM (n=3).

Table 3.1 Biotransformation of sulfadimidine and its N^4-acetyl metabolite by hepatocytes isolated from livers of six different pigs, and the influence of paraoxon, BSA and DMSO on the (de)acetylation rate. Activities are expressed as the fraction of drug transformed after 1 h. (mean ± SEM). Substrate concentrations were 360 μM (sulfadimidine) and 94 μM (N^4-acetyl sulfadimidine), except for culture 6 where a concentration of 20 μM for both compounds was used. DMSO, paraoxon and BSA were used at concentrations of 1% (v/v), 2.5 μg/ml and 20 mg/ml respectively. Cell density was 2.5 million viable cells/ml. ND= not determined.

Culture no.[1]	Addition	Fraction of drug metabolized in 1 h (%) Acetylation (A)	Deacetylation (D)	Ratio (D/A)
1	DMSO	1.01 ± 0.05	4.70 ± 0.13	4.6
2	DMSO	0.58 ± 0.02	7.21 ± 0.32	12.5
2	DMSO/Paraoxon	0.80 ± 0.00	0.40 ± 0.06	0.5
2	DMSO/BSA	0.39 ± 0.01	ND	
3	DMSO	0.41 ± 0.00	0.66 ± 0.01	1.6
4	DMSO	0.18 ± 0.01	4.50 ± 0.10	25.0
5	DMSO	0.38 ± 0.03	6.39 ± 0.08	16.7
5	DMSO/BSA	0.23 ± 0.03	3.08 ± 0.08	13.7
5		0.35 ± 0.03	8.99 ± 0.17	25.5
5	BSA	0.17 ± 0.01	3.93 ± 0.11	23.8
6	DMSO	0.41 ± 0.08	6.88 ± 0.08	16.7
6	DMSO/BSA	0.27 ± 0.02	4.48 ± 0.11	16.6
6		0.35 ± 0.04	9.77 ± 0.16	27.7

[1] rates for cultures 1 to 4 were calculated from the data presented in Fig. 3.6.

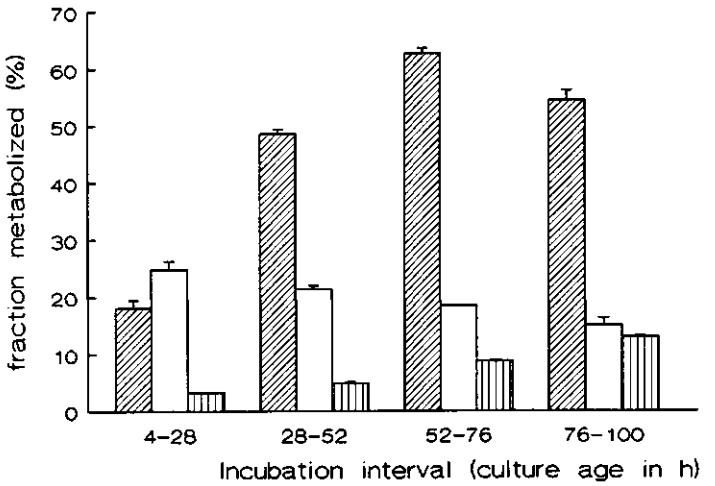

Fig. 3.7. Biotransformation of furazolidone, sulfadimidine and N^4-acetyl-sulfadimidine by monolayer cultures of different ages. Monolayer cultures were incubated in Williams' E containing 50 μM furazolidone (▨), 250 μM sulfadimidine (▥), or 250 μM N^4-acetyl-sulfadimidine (□) (0.5% DMSO) for 24 h starting 4, 28, 52 or 76 h after the seeding of the cells.

Furazolidone, sulfadimidine and its N^4-acetyl-metabolite were readily transformed by monolayer cultures of pig hepatocytes (Fig. 3.7). The transformation of the nitrofuran drug was relatively low during the first 24 h as compared to the next three periods of 24 h. The contribution of the cyano-metabolite to the total amount of metabolites was calculated to be 15.5 ± 2.7, 7.7 ± 0.8, 11.9 ± 0.6 and 15.6 ± 2.0 % at the end of respectively interval 1 to 4. The deacetylation of N^4-acetyl-sulfadimidine decreased in aging cultures, whereas the acetylation of the parent compound increased.

Differential centrifugation of the 9000 g supernatant of liver homogenates at 105,000 g resulted in the separation of a cytosolic and a microsomal fraction containing 2/3 and 1/3 of the protein respectively. The differences in a number of enzyme activities in microsomal and cytosolic fractions, prepared from different lobes of one liver were relatively small (Table 3.2). However, with the exception of cytochrome P-450 and glutathione levels, larger variations were observed in enzyme activities in cytosolic and microsomal fractions prepared from livers of different animals.

Table 3.2 Enzyme activities in microsomal and cytosolic fractions prepared from four different lobes of one liver (A1-A4, B1-B4) or livers from six different pigs (A-F). Enzyme activities are expressed per mg cytosolic or microsomal protein (mean ± SEM for n=3, n=2 (cytochrome P-450) or n=1 (GSH)).

	Cytosolic fraction			Microsomal fraction				
Liver	GSH-S-transferase (μmol/min per mg protein)	Acetyl-CoA-acyltransferase (pmol/min per mg protein)	GSH (nmol/mg protein)	Cytochrome P-450 (nmol/mg protein)	ethoxyresorufin-O-deethylase (pmol/min per mg protein	ethoxycoumarin-O-deethylase (nmol/min per mg protein)	cytochrome-c-reductase (nmol/min per mg protein)	UDP-glucuronyl-transferase (pmol/min per mg protein
A1	1.46 ± 0.05	30 ± 0.1	50	0.75 ± 0.00	72 ± 4	1.4 ± 0.03	120 ± 2	36 ± 1
A2	1.47 ± 0.06	23 ± 0.2	47	0.70 ± 0.04	78 ± 1	1.2 ± 0.02	133 ± 2	36 ± 1
A3	1.43 ± 0.02	22 ± 0.4	46	0.70 ± 0.00	81 ± 3	1.1 ± 0.04	127 ± 2	32 ± 1
A4	1.41 ± 0.05	26 ± 0.4	42	0.76 ± 0.01	78 ± 2	1.3 ± 0.02	125 ± 1	35 ± 1
B1	1.50 ± 0.09	60 ± 0.2	44	0.90 ± 0.00	94 ± 4	1.8 ± 0.04	165 ± 5	27 ± 1
B2	1.66 ± 0.08	49 ± 0.5	48	0.87 ± 0.01	87 ± 5	1.6 ± 0.06	176 ± 6	26 ± 1
B3	1.88 ± 0.01	39 ± 0.0	49	0.81 ± 0.01	76 ± 1	1.4 ± 0.04	161 ± 4	25 ± 1
B4	1.40 ± 0.11	47 ± 0.9	47	0.81 ± 0.03	86 ± 1	1.3 ± 0.04	168 ± 4	27 ± 1
C	1.88 ± 0.06	36 ± 0.2	53	0.84 ± 0.04	166 ± 4	1.7 ± 0.04	83 ± 2	29 ± 1
D	0.58 ± 0.01	56 ± 0.3	65	0.82 ± 0.01	268 ± 15	1.8 ± 0.02	92 ± 3	38 ± 1
E	1.65 ± 0.09	53 ± 0.2	62	0.83 ± 0.02	231 ± 13	2.5 ± 0.08	212 ± 4	28 ± 1
F	1.64 ± 0.11	66 ± 0.1	52	0.99 ± 0.03	149 ± 3	2.7 ± 0.06	206 ± 2	32 ± 0

Discussion

The results presented in this chapter demonstrate the possibility of isolating viable hepatocytes from a liver sample obtained shortly after exsanguination of a pig. This was shown both by trypan blue exclusion and the ability of the cells to form monolayer cultures. However, in a small number of cases, cultures showed poor plating efficiency despite an initial high fraction of trypan blue excluding cells. This might have been due to the low levels of intracellular glutathione (GSH) present in freshly isolated hepatocytes (see Fig. 3.1). A possible relationship between low intracellular GSH levels and poor plating efficiency has been pointed out previously in the case of rat hepatocytes (Morrison *et al.* 1985). The cause of the low initial GSH levels is unclear. However, extrapolation of GSH concentrations measured in the cytosolic fractions (Table 3.2) would result in cellular concentrations of 25-40 nmol/mg protein, considering that the cytosolic protein accounts for about 2/3 of the cellular protein. Comparison of these values with GSH levels measured in hepatocytes indicates a loss of GSH during the isolation of the cells. Cytochrome P-450 levels in freshly isolated hepatocytes were similar to those in microsomes, regarding a cytosolic/microsomal protein ratio of 2/1 (Table 3.2). In aging monolayer cultures, cytochrome P-450 concentrations decreased, as has been reported for hepatocyte cultures isolated from livers of other species like rat and man (Grant *et al.* 1986, Strom *et al.* 1982). The cytochrome P-450 dependent ECOD-activity was shown to decrease even more rapidly.

Four hours after seeding, ECOD-activity in monolayer cultures was about twice as high as in suspension cultures of the same cell-density, at the time of seeding. The difference could not be explained by the small number of non-viable cells, present in suspension cultures, nor by a difference in cytochrome P-450 content. However, several other factors may influence the ECOD-activity, such as oxygen-tension, serum albumins, organic solvents and cell-density (Rogiers *et al.* 1986). In our experiments, ECOD-activity in suspension cultures was shown to decrease with increasing cell-densities (Fig. 3.3B), possibly because the diffusion of oxygen into the medium can no longer meet the oxygen demand. This theory is supported by the observation that aeration with 95 % oxygen resulted in a 1.8 fold increase in the ECOD-activity.

The nitrofuran furazolidone was readily metabolized, partly resulting in the formation of the open-chain cyano derivative (Fig. 3.4). The latter compound has been shown to be a minor metabolite in blood plasma of pigs (Vroomen *et al.* 1987b). In our experiments, the contribution of the cyano-metabolite to the overall biotransformation of the drug might even be overestimated, regarding the low initial GSH concentrations. Using rat and pig liver microsomes, Vroomen *et al.* (1987b) have demonstrated that the formation of this metabolite decreased with increasing concentrations of GSH, probably due to the formation of a conjugate between GSH and a reactive intermediate of furazolidone. Therefore, similar to the *in vivo* situation, little is known about the majority of the metabolites of furazolidone.

The acetylation of sulphadimidine to its N^4-acetyl metabolite was slow in both cytosolic fractions and hepatocytes (Table 3.2, Fig. 3.5). Deacetylation of N^4-acetyl sulphadimidine proceeded more rapidly in all batches of hepatocytes tested (Fig. 3.6). No differences were seen in the relative proportion acetylated or deacetylated product in the wide range of concentrations examined (Fig. 3.5). This indicates that in the case of sulphadimidine only part of the formed N^4-acetyl metabolite is again deacetylated to the parent compound. This hypothesis is supported by the fact that inhibition of deacetylation by non-toxic concentrations of paraoxon resulted only in a 1.3 fold increase in the proportion of acetylated drug (Table 3.1). These results clearly demonstrate the presence of an equilibrium between two opposing biotransformation pathways, as discussed by Vree *et al.* (1985). *in vivo*, the N^4-acetyl metabolite seems to be more important, as judged by the high proportion of this compound in the urine of pigs (Hoogenboom *et al.* 1988, Vree *et al.* 1985). This may be due to the fact that the acetylated compound is cleared more efficiently by the kidney. In addition the role of other internal or external factors cannot be excluded, as demonstrated by our experiments (Table 3.1). It was shown that both the amounts of acetylated and deacetylated product were lower in the presence of albumins. This might be explained by a reduction of the concentration of free drug, since it has been shown that both compounds bind to albumins by hydrophobic interactions (Hoogenboom *et al.* 1988, Vree *et al.* 1985). DMSO, which was routinely used in most experiments, appeared to inhibit the deacetylation activity, as has previously been reported in the case of cytochrome P-450 related enzyme activities like the deethylation of 7-ethoxy-coumarin (Rogiers *et al.* 1986). As a result of this inhibition, there seems to be an underestimation of the already high deacetylation/acetylation ratio. Fig. 3.6 clearly demonstrates the existence of large differences in both the absolute and relative acetylation and deacetylation capacities of sulphonamides by cell cultures isolated from livers of different animals. It is unclear, whether such differences reflect the possible existence of slow and fast acetylation phenotypes, which has been reported in the case of humans (Vree *et al.* 1985).

As shown in this study, the cells can be used both as suspension cultures and as monolayer cultures. Monolayer cultures offered the advantage of prolonged incubation intervals, and allow the cells to recover from the isolation. The latter might explain the increase in the biotransformation of furazolidone in older cultures (Fig. 3.7). In the case of ethoxy-coumarin, sulfadimidine and its N^4-acetyl metabolite, aging of the monolayer cultures clearly affected the velocity of the degradation, but it did apparently not affect the metabolite pattern. Looking at the formation of the cyano-metabolite, a similar conclusion might be drawn in the case of furazolidone, but it should be mentioned that the identity of more than 85% of the metabolites is unknown, which makes it difficult to compare metabolite patterns.

No large differences could be observed in enzyme-activities in cellfractions isolated from different lobes of one liver (Table 3.2). However, variation between livers of

different animals was marked. The intraspecies differences observed both in cellular fractions and hepatocytes stress the need to investigate the reproducibility of results obtained with material from one liver.

This is the first report on the use of adult porcine hepatocytes isolated from a liver sample for biotransformation studies. Since the known *in vivo* metabolites of furazolidone and sulfadimidine could be detected in this *in vitro* system, we are confident that the use of cell cultures prepared from pig livers and possibly other organs of food-producing animals may largely contribute to elucidating biotransformation pathways of veterinary drugs, thus lowering the need to use intact animals.

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CHAPTER 4

THE USE OF PIG HEPATOCYTES TO STUDY THE BIOTRANSFORMATION OF ß-NORTESTOSTERONE BY PIGS

Identification of glucuronidated 15α-hydroxy-norandrostenedione as an important *in vitro* and *in vivo* metabolite

This chapter is based on the following publications:

Hoogenboom L.A.P., Berghmans M.C.J. and Traag W.A. (1989). Biotransformation of ß-nortestosterone by cultured porcine hepatocytes. *J. Chromatography, Biomedical Applications* 489: 105-109.

Hoogenboom L.A.P., Berghmans M.C.J., Veldhuizen A. van, and Kuiper H.A. (1990). The use of pig hepatocytes to study the biotransformation of ß-nortestosterone by pigs: identification of glucuronidated 15α-hydroxy-norandrostenedione as an important *in vitro* and *in vivo* metabolite. *Drug Metab. Dispos.* 18: 999-1004.

THE USE OF PIG HEPATOCYTES TO STUDY THE BIOTRANSFORMATION OF ß-NORTESTOSTERONE BY PIGS

Identification of glucuronidated 15α-hydroxy-norandrostenedione as an important *in vitro* and *in vivo* metabolite

Summary

Pig hepatocytes were used to examine the biotransformation of ß-nortestosterone. Initially the major metabolite of ß-nortestosterone was norandrostenedione, which upon prolonged incubation was further transformed, primarily to the glucuronide of 15α-hydroxy-norandrostenedione. No differences were observed in this pattern between hepatocytes isolated from livers of sows or castrated male pigs. With increasing culture age the rate of formation of norandrostenedione did not change, contrary to a decrease in its further oxidation and glucuronidation. 15α-Hydroxy-norandrostenedione, primarily as its glucuronide, was identified in the urine of pigs injected with ß-nortestosterone. In addition norandrostenedione and the glucuronide of the parent compound were detected, although in much smaller amounts.

Abbreviations used are: NOR= ß-nortestosterone; NA= norandrostenedione; 15αOH-NA= 15α-hydroxy-norandrostenedione; PCB-138= 2,3,4-2',3',5'-polychlorbiphenyl; IS= internal standard; TMS= trimethyl-silyl; MOX= methoxyamine-hydrochloride; MID= multiple-ion detection; EtAc= ethyl-acetate

Introduction

Primary cultures of hepatocytes, isolated from livers of food-producing animals, have been shown to be a useful model for studying the biotransformation of growth-promoting agents and veterinary drugs (Shull *et al.* 1987). In previous chapters we described the isolation of pig hepatocytes and showed that these cells were able to transform the veterinary drugs sulfadimidine and furazolidone, resulting in the formation of compounds previously detected *in vivo*. Aim of the present study was to further demonstrate the value of this model for studying the biotransformation of xenobiotics. For this purpose, ß-nortestosterone (4-estren-17ß-ol-3-one) was used as a model compound regarding the special interest in metabolites of these type of compounds, that might be used for controlling their misuse.

In the first part of the study we investigated the biotransformation of ^{3}H-labelled ß-nortestosterone by monolayer cultures of pig hepatocytes, isolated from livers of both female and castrated male pigs. One of the main problems encountered with monolayer cultures of hepatocytes is the change in certain enzyme activities with increasing age of the cultures (Fry and Bridges 1979, Grant *et al.* 1985). In Chapter 3 we described *e.g.* the decrease in cytochrome P-450 levels and the cytochrome P-450 dependant deethylation of ethoxycoumarin (ECOD-activity) in ageing monolayer cultures of pig hepatocytes. Since the role of cytochrome P-450 isozymes in the oxidation of steroids is well established (Guengerich 1987, Waxman 1988), we investigated how this decrease affects the biotransformation of ß-nortestosterone and therefore limits the prolonged use of the model.

In the second part of the study castrated male pigs were injected with unlabelled ß-nortestosterone and the urine of these animals was investigated for the presence of the major *in vitro* metabolites.

Materials & Methods

Materials

[^{3}H]ß-nortestosterone (703 GBq/mmol) was obtained from Amersham (Amersham, UK), ß-nortestosterone, ß-glucuronidase (*Escherichia coli*), and insulin from Sigma (St. Louis, MO, USA), 2,3,4-2',3',5'-polychlor-biphenyl (PCB-138) from Promochem (Wesel, FRG), methoxylamine-hydrochloride from Chrompack (Middelburg, The Netherlands), N,O-bis-(trimethylsilyl)-trifluoroacetamide from Pierce (Rockford, Il, USA), collagenase (type H) from Boehringer (Mannheim, FRG), Williams' E, penicillin/streptomycin and fetal calf serum from Flow Labs (Rickmansworth, UK). Norandrostenedione and 15α-hydroxy-norandrostenedione were kindly supplied by Organon (Oss, The Netherlands).

Isolation and culturing of hepatocytes

Hepatocytes were isolated from livers of pigs (Dutch Landrace x Yorkshire) obtained from the Department of Animal Husbandry (Agricultural University, Wageningen, The Netherlands) as described in Chapter 2. Isolated hepatocytes were diluted to a density of 1.5 million viable cells per ml in Williams' medium E supplemented with 5% FCS, penicillin/streptomycin (50 IU and 50 μg/ml respectively) and insulin (0.5 μg/ml), and plated in 60 mm tissue culture dishes (2.5 ml/dish). Prior to the addition of ß-nortestosterone, monolayer cultures were washed twice with Krebs-Henseleit medium supplemented with 10 mM glucose and preincubated for 30 min in this medium. ß-Nortestosterone was dissolved in dimethyl sulphoxide (0.5 mM) and added to the cultures at a final concentration of 5 μM. For radioactivity studies, [^{3}H]ß-nortestosterone was diluted with unlabelled hormone to a specific activity of 1.8 GBq/mmol.

Animal study

Three pigs were injected intramuscularly with 200 mg ß-nortestosterone, dissolved in 5 ml arachid oil/benzyl alcohol (90:10 v/v). Urine samples were collected during different time intervals, namely before injection (blank), and 0-4, 4-8, 8-12, 12-24, 24-34 and 34-48 h after injection, and stored at -20 °C.

Chromatography

For *in vitro* studies with [^{3}H]ß-nortestosterone, samples were taken from the medium and injected on the HPLC-system either without further pretreatment or after incubation with ß-glucuronidase for 1 h at 37 °C. The HPLC-system was equipped with a PRP-1 column (250x4.6 mm I.D.; Hamilton, Reno, NV, USA) and coupled to a radioactivity-monitor (Berthold, Wildbad, FRG). Compounds were eluted from the column using a 0-22 min liniar gradient of 2-100 % acetonitrile in water.

For identification of the major *in vitro* metabolite after prolonged incubation, the medium was extracted three times with EtAc, treated with ß-glucuronidase for 1 h at pH 7.0 and 37 °C, and extracted again three times with EtAc. The organic phase was evaporated under nitrogen and the residue dissolved in acetonitrile/water (3:7 v/v). To facilitate the further purification by HPLC, the system was equipped with a PRP-1 semi-preparative column (305x7 mm I.D.; Hamilton) and coupled to a diode-array detector.

Aliquots of urine (5 ml) were mixed with 5 ml methanol and evaporated to dryness under nitrogen. The residue was dissolved in 0.35 ml acetonitrile/ water (3:7 v/v) and injected on the HPLC-system described above. The fraction containing the polar metabolites (retention time 9-12 min) was collected, concentrated under nitrogen, mixed with 1 ml KPi (0.1 M, pH 7.0), treated with ß-glucuronidase (1 h, 37 °C) and extracted with EtAc (3x4 ml). Following evaporation of the organic solvent under nitrogen, the residue was dissolved in 50 μl acetone and investigated by HPTLC and GC/MS. HPTLC analysis of 5 μl of this solution was performed with Silica-60 thin layer plates

(Merck, Darmstadt, FRG) which were chromatographed in two directions (eluent 1: hexane/EtAc/ dichloromethane 10:20:20 v/v, eluent 2: chloroform/ethanol/ toluene 45:3.5:5 v/v). Spots were developed by dipping the plates in ethanol/sulphuric acid (95:5 v/v) for 30 sec and heating at 95 °C for 8 min. For GC/MS analysis, samples were investigated either with or without further derivatization as described by Tuinstra *et al.* (1983).

For identification of norandrostenedione and unconjugated ß-nortestosterone, aliquots of medium or urine (5 ml) were extracted twice with EtAc (5 ml), which was subsequently evaporated. The residue was dissolved in 0.3 ml acetonitrile/water 3:7 (v/v) and 0.15 ml of this solution was injected on the HPLC-system described above. The fractions containing norandrostenedione and ß-nortestosterone were collected, evaporated to dryness and derivatized with respectively TMS or MOX, before GC/MS analysis.

Identification

^{1}H-NMR spectra were recorded in $CDCl_3$ on a Bruker CXP-300 instrument. GC/MS analysis was performed on a Hewlett-Packard GC-MS instrument (HP-MSD 5970B), equipped with a CpSil5-CB column (25 m x 0.25 mm I.D., 0.12 μm coating; Chrompack, Middelburg, The Netherlands) using a temperature-gradient of 80 °C for 3 min followed by a linear increase of 15 °C/min up to 280 °C. Before derivatization PCB-138 was added as an internal standard. For positive identification of compounds, the relative intensities of the M^+-peak and a number of important fragment-peaks were measured, putting the highest intensity at 100% (Multiple Ion Detection (MID)-analysis). Two compounds were judged identical when the variations in the intensities of all fragment-peaks were within 10%. The identity of norandrostenedione in the incubation medium was confirmed by full spectrum analysis.

Results

The biotransformation of ß-nortestosterone by pig hepatocytes could easily be studied, using a radiolabelled compound in addition to a radioactivity detector coupled on-line to the HPLC-system. The biotransformation of ß-nortestosterone by 24 h old monolayer cultures of hepatocytes isolated from the liver of a castrated male pig, resulted initially in the formation of a less hydrophilic compound, eluting after 21 min (Fig. 4.1A), which was subsequently metabolized to more hydrophilic compounds (Figs 4.1B and 4.1C). Deglucuronidation of these metabolites resulted primarily in the formation of a compound with a retention time of about 15 min (Fig. 4.1D). A similar pattern was obtained with hepatocytes isolated from the liver of a sow (Fig. 4.1E).

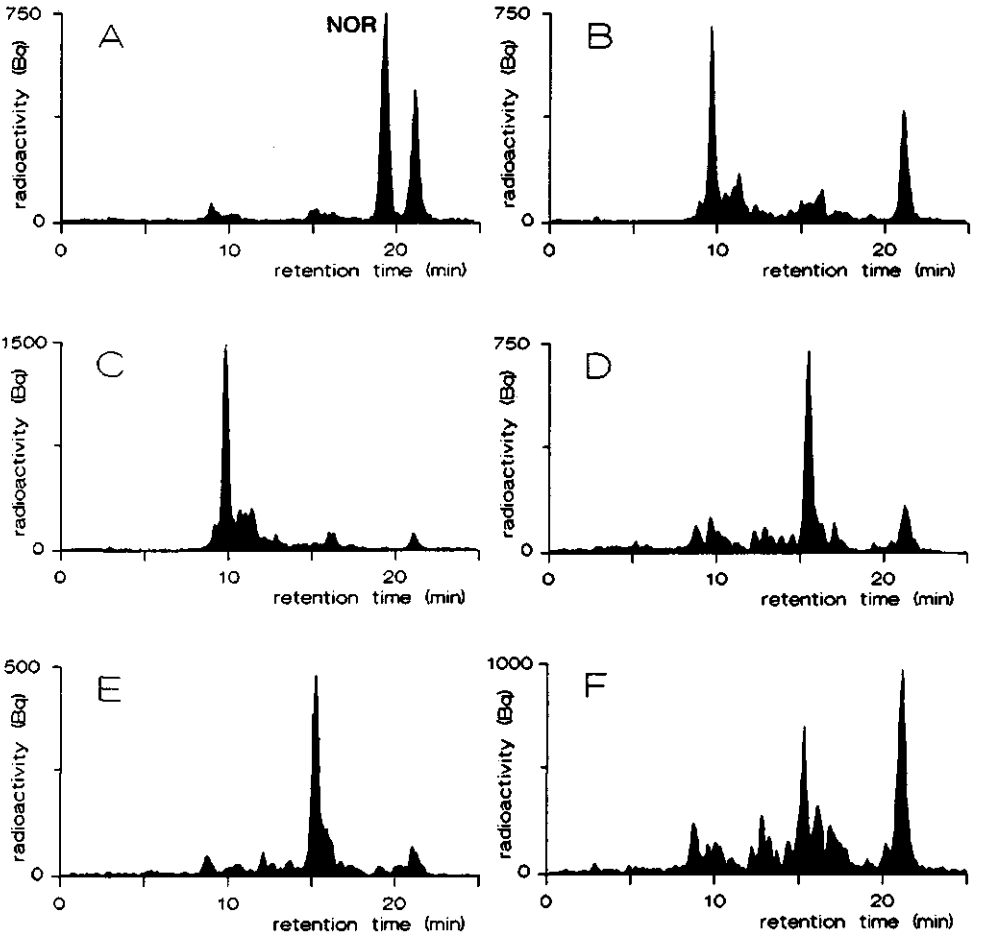

Fig. 4.1. Biotransformation of ß-nortestosterone by pig hepatocytes and the influence of sex and culture age.
Cells from a castrated male pig (A-D), and a sow (E,F) were incubated with ß-nortestosterone for 10 (A), 60 (B) or 150 (C-F) min. The medium was analyzed before (A,B,C), or after (D,E,F) treatment with ß-glucuronidase for 1 h, using an on-line radioactivity detector. The chromatograms shown were obtained with cultures aged 24 h in the case of the castrated male pig, or 4 h (E) and 72 h (F) for the sow.

Table 4.1 Influence of culture age on the biotransformation of ß-nortestosterone by pig hepatocytes. Hepatocytes from a sow, cultured for 4, 24, 48 or 72 h, were incubated with ß-nortestosterone for different time intervals. Media samples were analyzed by HPLC for the presence of ß-nortestosterone, norandrostenedione or polar metabolites (retention time 9 to 13 min (Fig. 4.1)). Part of the samples, taken after 150 min, were treated with ß-glucuronidase and analyzed for the amount of 15α-hydroxy-norandrostenedione (mean ± SEM; n=2).

culture age (h)	incubation time (min)	fraction of total radioactivity (%)			
		NOR	NA	polar metabolites	15αOHNA (+ DC)
4	20	22.2 ± 0.9	43.0 ± 1.7	11.0 ± 1.3	ND
	150	0.3 ± 0.1	5.3 ± 0.9	62.3 ± 0.6	52.5 ± 0.1
24	20	22.6 ± 0.2	44.7 ± 0.1	10.6 ± 0.9	ND
	150	0.0 ± 0.0	6.2 ± 0.1	64.8 ± 0.9	48.1 ± 0.0
48	20	24.4 ± 1.1	48.5 ± 0.3	8.4 ± 1.2	ND
	150	0.0 ± 0.0	14.0 ± 1.1	55.1 ± 1.8	37.5 ± 4.2
72	20	20.7 ± 0.3	57.0 ± 0.6	5.0 ± 0.1	ND
	150	0.0 ± 0.0	23.0 ± 0.4	42.5 ± 1.2	15.9 ± 0.6

+DC: after deconjugation; ND: not determined

With increasing age of the latter cultures, no differences were observed in the initial conversion of ß-nortestosterone, whereas the subsequent formation of the polar metabolites clearly decreased (Fig. 4.1F, Table 4.1).

The initially formed metabolite (RT. 21 min) coeluted on HPLC with norandrostenedione. Its identity was further confirmed by GC/MS analysis, following derivatization of the two keto-groups with MOX (Fig. 4.2), resulting in an M^+ peak of 330 (272 + 2x29).

Using UV/VIS diode array equipment, the main deglucuronidated metabolite (RT. 15 min) was shown to have an absorption maximum at 244 nm. The ^{1}H-NMR spectrum of the purified metabolite is shown in Fig. 4.3A, as well as the effect of addition of CD_3OD on the spectrum (insert). Decoupling experiments showed that the NMR signal at 4.43 ppm was coupled to the signals at 1.40, 2.12 and 3.02 ppm. The latter two were coupled to each other, with a coupling constant J of about 19 Hz. The identity of the metabolite was further investigated by GC/MS, either directly or after

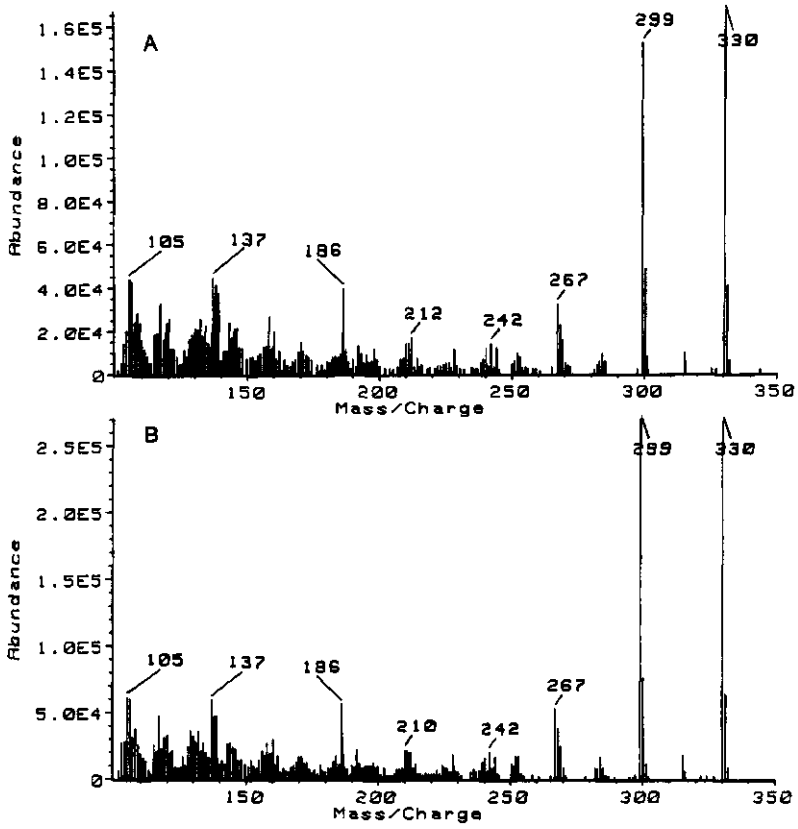

Fig. 4.2. GC/MS analysis of the initial major metabolite of ß-nortestosterone.
The metabolite eluting at 21 min was purified, derivatized with MOX (A) and compared to a reference standard of norandrostenedione (B). Spectra were recorded in full-spectrum GC/MS.

treatment with MOX and/or TMS to derivatize keto and/or hydroxy groups respectively. The mass spectrum of the underivatized compound showed an M^+-peak at 288 m/e (100%) and major fragments at 260 (27%), 147 (16%), 133 (17%) and 110 m/e (36%).

Treatment with MOX/TMS or only TMS resulted in M^+-peaks at 418 and 360 m/e. From these spectra the possible identity of the metabolite was thought to be 15α-hydroxy-norandrostenedione. A reference standard of this compound was shown to have identical retention times on HPLC, HPTLC and GLC as the *in vitro* metabolite. NMR and mass spectra of the *in vitro* metabolite (A) and the standard (B) are compared in Figs 4.3 and 4.4 respectively.

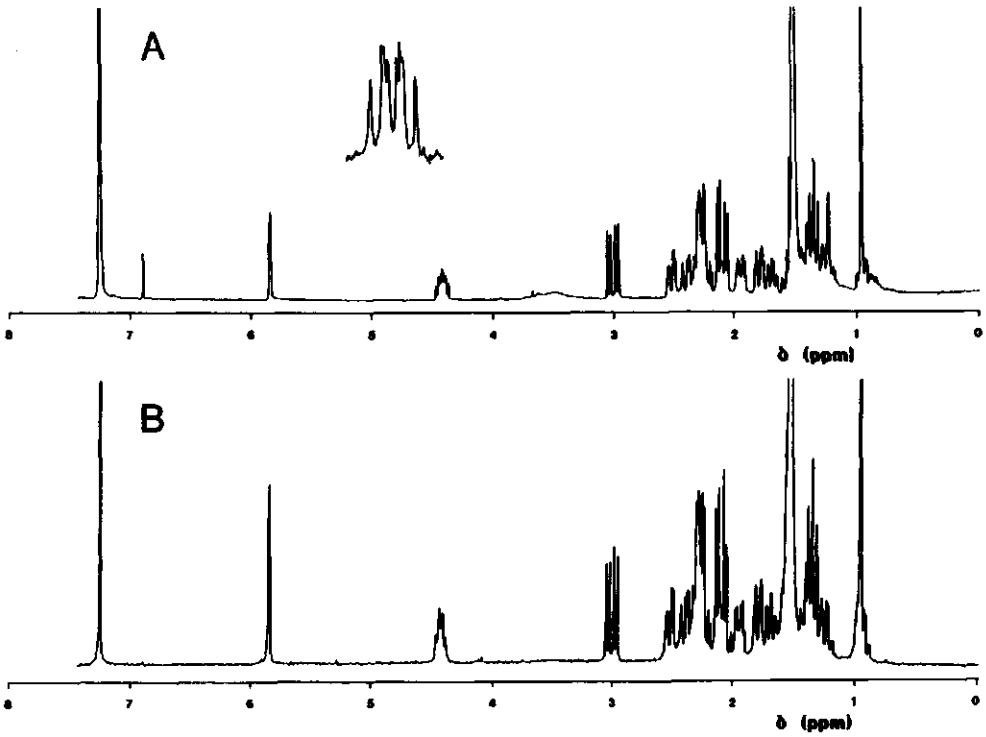

Fig. 4.3. Identification of 15α-hydroxy-norandrostenedione as an *in vitro* metabolite by ^{1}H-NMR. Spectra of the major *in vitro* metabolite after deconjugation (A), and a standard of 15α-hydroxy-norandrostenedione (B). The insert in (A) shows the effect of CD_3OD on the spectrum.

For validation of the *in vitro* results, three castrated male pigs were injected with ß-nortestosterone. For one pig, all urine samples were investigated for the presence of 15α-hydroxy-norandrostenedione, norandrostenedione and the parent compound. Based on the information from the *in vitro* experiments, glucuronides could be purified from the matrix by HPLC, prior to the ß-glucuronidase treatment. After extraction of the deconjugated metabolites with EtAc, HPTLC analysis revealed a blue spot with RF-values of 0.33 and 0.12 in respectively the first and second direction of development, identical with 15α-hydroxy-norandrostenedione. The intensity of this spot, which was not observed in urine samples taken before intramuscular injection of ß-nortestosterone, was most bright in the urine sample collected between 4-8 h after administration and gradually decreased in samples collected at later time intervals. A similar pattern and time course was obtained with GC/MS analysis (Fig. 4.5, Table 4.2).

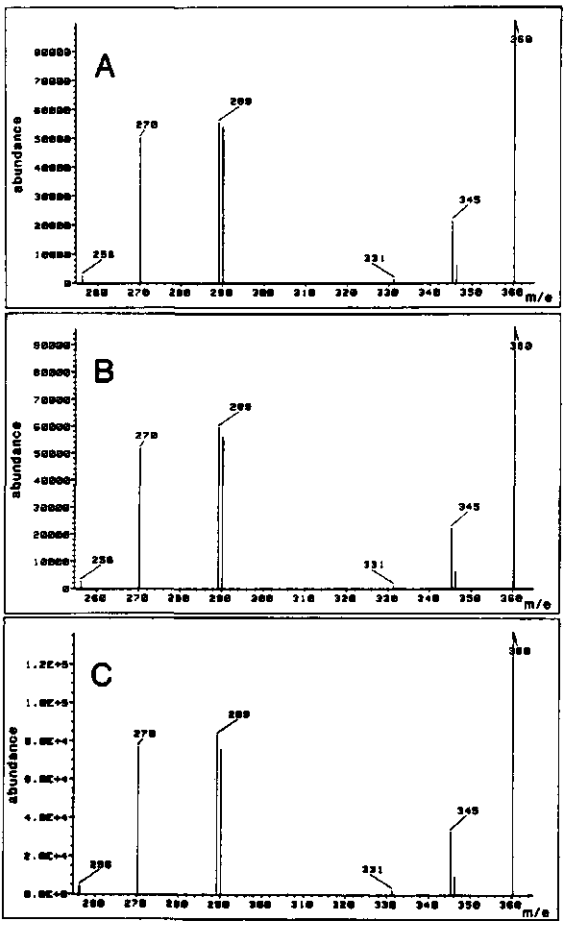

Fig. 4.4. Mass (MID) spectra of the purified major deconjugated metabolite of ß-nortestosterone generated by pig hepatocytes (A), a standard of 15α-hydroxy-norandrostenedione (B), and a deconjugated metabolite with the same retention time in the urine of pigs, injected with ß-nortestosterone (C).Compounds were derivatized with TMS, and the four major fragment-peaks were selected for comparison (MID).

The identity of the compound as 15α-hydroxy-norandrostenedione was further confirmed by GC/MS analysis (Fig. 4.4C). The metabolite in its glucuronidated, and to a smaller extent in its free form, could also be detected by HPTLC in urine samples of two other pigs, collected 4-8 h after injection with ß-nortestosterone, but not in samples collected before treatment.

The occurrence of norandrostenedione in the urine was investigated by GC/MS analysis. As shown in Fig. 4.5, a compound with an identical retention time as

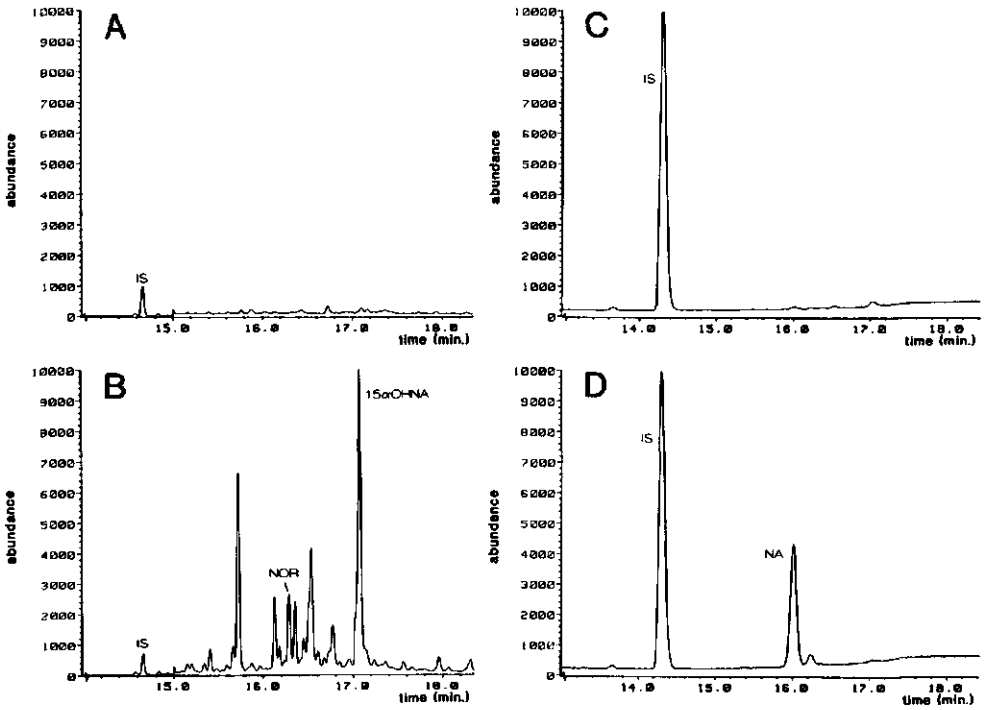

Fig. 4.5. Detection by gas-chromatography (MID) of 15α-hydroxy-norandrostenedione and norandrostenedione in urine samples of a pig treated with ß-nortestosterone.
Urine samples were collected before (A,C), or 4-8 h after injection with ß-nortestosterone (B,D). Samples were injected on HPLC and the glucuronide-fraction was incubated with ß-glucuronidase. Deconjugated compounds, extracted with EtAc, were derivatized with TMS and analyzed by GC/MS (A,B). The HPLC fraction containing norandrostenedione was collected and treated with MOX (C,D) before GC/MS analysis. The peaks of PCB-138 (IS) and those coeluting with ß-nortestosterone, norandrostenedione and 15α-hydroxy-norandrostenedione are indicated.

norandrostenedione (16.0 min) could be detected after (D), but not before (C) treatment with ß-nortestosterone. Its identity was confirmed by the similarity of its mass spectrum with that of a reference standard as shown in Figs 4.6B and 4.6A respectively.

The presence of a small amount of ß-nortestosterone in the urine was confirmed by GC/MS analysis, but only in the EtAc-extract obtained after deglucuronidation. In addition, in these samples a number of other spots and peaks were observed by HPTLC and GLC (Fig. 4.5) respectively, which were absent before treatment, and increased and subsequently decreased in samples collected at longer time intervals. These spots had not been observed after incubation of hepatocytes with ß-nortestosterone and were therefore not further investigated.

Table 4.2 Estimated concentrations of 15α-hydroxy-norandrostenedione, ß-nortestosterone and norandrostenedione in urine samples of a pig treated with ß-nortestosterone.
Concentrations of conjugated 15α-hydroxy-norandrostenedione and ß-nortestosterone, and norandrostenedione in urine samples were estimated by GC/MS analysis.

compound	interval (h after injection)					
	0-4	4-8	8-12	12-24	24-34	34-48
15αOHNA	3.3	8.5	4.8	1.7	0.2	0.1
NOR	0.5	0.6	0.4	0.2	0.1	---
NA	---	0.5	0.5	---	---	---

Concentrations are expressed as μg deconjugated compound/ml

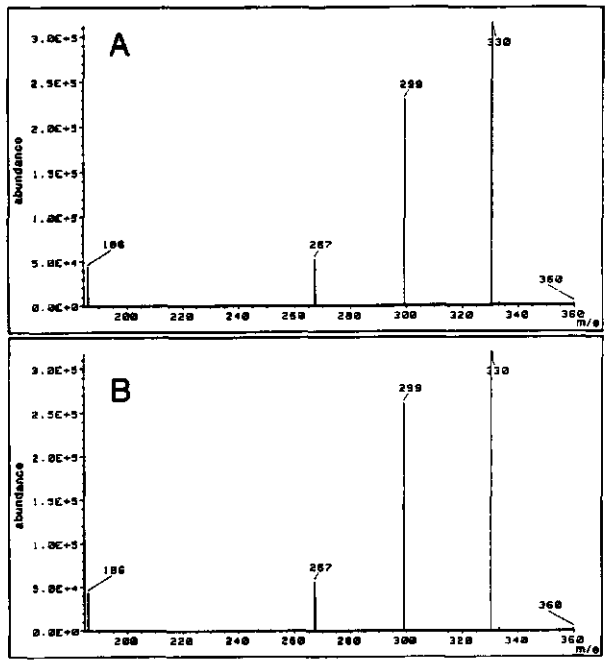

Fig. 4.6. Identification by GC/MS of norandrostenedione in the urine of pigs treated with ß-nortestosterone. Mass spectra of a standard of norandrostenedione (A), and a compound in the urine with similar retention time (B), after derivatization with MOX.

Discussion

The biotransformation of ß-nortestosterone by hepatocytes isolated from livers of a number of sows and castrated male pigs showed a reproducible pattern. This resulted initially in the formation of norandrostenedione, which was further metabolized into a number of polar compounds. The most important metabolite, which in its free form had a retention time of 15 min, was identified as 15α-hydroxy-norandrostenedione by its spectrometric properties (Fig. 4.7). The molecular mass of 288 of the deconjugated metabolite is consistent with a metabolite of norandrostenedione, containing an additional hydroxy group. The presence of this hydroxy and the two keto groups is confirmed by the GC/MS analysis after derivatization of the metabolite with TMS and MOX/TMS, showing M^+ peaks of 360 (288 + 72) and 418 m/e (288 + 72 + 2x 29). Furthermore, the fragments at 110 and 147 m/e in the mass spectrum of the underivatized compound, indicate that the A and B rings contain no other substituents than the keto group at position 3 (Zaretskii 1976). Together with the UV spectrum and the NMR signal at 5.85 ppm, belonging to the proton at C-4, this also confirmed the presence of the double bond.

Further information on the position of the hydroxy group came from the broad coupling of about 19 Hz, observed in the signals at 3.02 and 2.12 ppm in the NMR spectrum (Fig. 4.3A). Such a broad signal has been reported to be specific for the protons at C-16 in steroids with a keto group at C-17 (Leibfritz *et al.* 1982). The migration of the signal of the 16α-proton from about 2.5 to 3 ppm and the coupling of the signal at 4.43 ppm (H-15ß) with the signals at 3.02 ppm (H-16α), 2.12 ppm (H-16ß) and 1.40 ppm (H-14), strongly suggest that the hydroxy group should be at position 15α. More evidence for the α-orientation of the hydroxy group at C-15 comes from the only limited shift of the signal belonging to the protons at the methyl group (0.97 ppm) as compared to that in the ^{1}H-NMR spectrum of norandrostenedione (0.94 ppm), which in the case of a ß-orientation would have been much larger (Bridgeman *et al.* 1970). Finally, the coupling of the proton at 15ß with the hydroxy group at 3.5 ppm explains the extra splitting of the pattern at 4.43 ppm, which disappears upon addition of CD_3OD. The identity of the metabolite as 15α-hydroxy-norandrostenedione was subsequently confirmed by the similarity of the NMR and GC/MS spectra of this metabolite and a reference standard (Figs 4.3 and 4.4).

No substantial changes were observed in the metabolite pattern of ß-nortestosterone during the first 48 h of the cultures (Fig. 4.1, Table 4.1). In particular, no decrease was observed in the oxidation at C-17, which in the case of testosterone and rats was shown to be cytochrome P-450*b* related (Waxman 1988, Wood 1983). Regarding the previously reported decrease in cytochrome P-450 levels and ECOD-activity (Chapter 3), this may be an indication that other enzymes like 17ß-hydroxysteroid-dehydrogenase (Sonderfan *et al.* 1989) are involved in the oxidation of ß-nortestosterone to norandrostenedione. The age-dependant decrease in the oxidation of norandrostenedione at C-15 subscribes

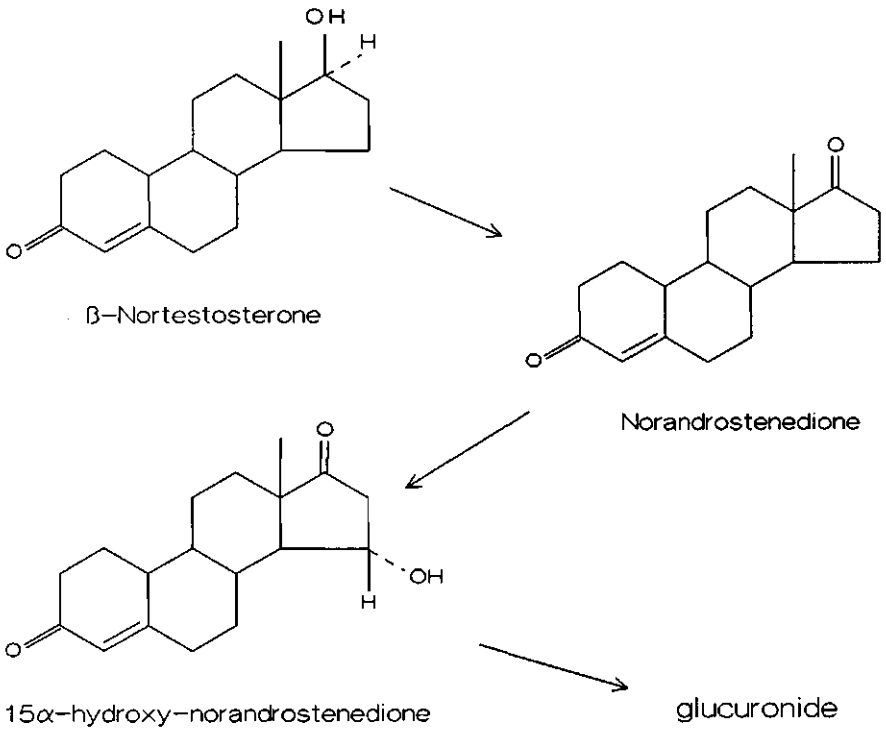

Fig.4.7. Major biotransformation pathway of ß-nortestosterone in primary cultures of pig hepatocytes.

the role of a cytochrome P-450 related activity in this conversion step.

The results from the *in vivo* experiment confirm the formation of norandrostenedione from ß-nortestosterone and its excretion by the cells, resulting in small quantities in the urine (Figs 4.5D and 4.6). The subsequent hydroxylation at C-15 and the almost complete glucuronidation are in agreement with the *in vitro* results. The less significant glucuronidation of the parent compound was not observed *in vitro*. The same was true for a number of other compounds observed in the HPTLC and GC/MS analysis, whose presence seemed to be related to the treatment with ß-nortestosterone. However, it is unclear whether these compounds are metabolites of ß-nortestosterone or compounds whose elimination is increased due to the treatment with this hormone.

The present study shows that the use of hepatocytes, preceding *in vivo* studies, facilitates the characterization and detection of important *in vivo* metabolites and their subsequent identification. In addition to these type of biotransformation studies, the use of hepatocytes can be helpful in the production of both standards and reference materials necessary for the validation of analytical methods.

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CHAPTER 5

THE USE OF PIG HEPATOCYTES FOR TOXICITY STUDIES OF VETERINARY DRUGS

A comparative study with furazolidone and other nitrofurans

This chapter is based on the following publication:

Hoogenboom L.A.P., Oorsprong M.B.M., Vliet T. van and Kuiper H.A. (1991). The use of pig hepatocytes for toxicity studies of veterinary drugs: a comparative study with furazolidone and other nitrofurans. *Toxicology in Vitro.*

THE USE OF PIG HEPATOCYTES FOR CYTOTOXICITY STUDIES OF VETERINARY DRUGS

A comparative study with furazolidone and other nitrofurans

Summary

The possible application of primary cultures of pig hepatocytes for cytotoxicity studies of veterinary drugs was investigated, using the nitrofurans furazolidone, furaltadone, nitrofurazone, nitrofurantoin and nitrovin. Leakage of lactate-dehydrogenase (LDH) from the cells into the medium could be measured at high concentrations of nitrofurantoin, nitrovin, nitrofurazone and acetaminophen, which was used as a positive control. After incubation of cells with high concentrations of all drugs intracellular LDH activities were lower than the control. The incorporation of ^{14}C-leucine into proteins, especially those excreted into the medium, was shown to be a more sensitive parameter than LDH-leakage and was decreased by all drugs. Intracellular GSSG-levels were increased upon exposure to all nitrofurans. However, only after incubation of cells with 500 μM nitrofurantoin intracellular GSH levels were decreased. In all other cases GSH-levels were unchanged or even elevated upon exposure. The most sensitive parameter measured, was the accumulation of pyruvate and lactate in the medium upon treatment with all nitrofurans except nitrovin. It was shown in the case of furazolidone, that this effect did not disappear immediately when the exposure was terminated and in addition accumulated upon repeated treatment of the cells, especially at low doses.

The results of the study clearly demonstrate that primary cultures of pig hepatocytes can be used for cytotoxicity studies of veterinary drugs.

Abbreviations: DMSO= dimethyl-sulfoxide; GSH= reduced glutathione; GSSG= oxidized glutathione; LDH= lactate-dehydrogenase (EC 1.1.1.27); PBS= phosphate buffered saline; PCA= perchloric acid; TCA= trichloro acetic acid

Introduction

In previous chapters we described the isolation of hepatocytes from pig livers and the biotransformation of sulphadimidine, furazolidone and ß-nortestosterone by these cells. In addition to biotransformation studies, intact hepatocytes offer the opportunity to study the toxic properties of xenobiotics and their underlying mechanisms, as has been shown with hepatocytes from rats and other laboratory animals (Fry & Bridges 1979; Jones *et al.* 1986; Ratanasavanh *et al.* 1988). However, hepatocytes from food-producing animals have not been used for such studies, despite the fact that procedures for their isolation from liver samples of calves (Shull et al. 1987), sheep (Demigne *et al.* 1986), goats (Aiello *et al.* 1989) and pigs (Pangburn *et al.* 1981; Bachorik *et al.* 1982) have become available. Since species-specific metabolites may be formed, the use of hepatocytes isolated from livers of farmanimals should be preferred when studying the toxicity of compounds used in veterinary practice. This is of particular interest regarding the possible presence of such metabolites as residues in animal products.

The aim of the present study was therefore to investigate the possible use of pig hepatocytes for studying the toxic properties of xenobiotics, in this case a number of nitrofurans, like nitrofurazone, nitrofurantoin, furazolidone, furaltadone, and nitrovin (Fig. 5.1). Nitrofurans are widely used as antibacterial and antiprotozoal agents in veterinary and human practice. However, following their application in man and animals, a number of very diverse toxic effects like peripheral neuropathy, degeneration of sexual glands (Paul & Paul 1964, Cohen 1978, Ali 1989), and hepatotoxic effects (Sharp *et al.* 1980, Black *et al.* 1980, Takabateke & Fukuhara 1978) have been observed. In addition, most of these compounds have been shown to posses mutagenic (McCalla 1983) and carcinogenic properties (Cohen 1978).

For studying the toxicity of nitrofurans with pig hepatocytes, LDH-release and protein-synthesis were selected as more general end-points of cellular damage, and concentrations of reduced (GSH) and oxidized (GSSG) glutathione in the cells, and of pyruvate and lactate in the medium as more specific endpoints. Intracellular GSH levels may decrease regarding the role of GSH as a protective agent either by trapping reactive intermediates of nitrofurans, as was shown for furazolidone (Vroomen *et al.* 1987; Vroomen *et al.* 1988), or as a substrate in the detoxification of hydrogen-peroxide by GSH-peroxidase, leading to the formation of GSSG (Holtzman 1981). In mammalian cells nitrofurans are primarily degraded by a reduction of the nitro-group (Vroomen *et al.* 1987; Boyd *et al.* 1979; Jonen 1980; Wang *et al.* 1974), initially leading to the formation of a radical nitro anion. Superoxide and eventually hydrogen peroxide may be formed due to reoxidation of this radical nitro anion by oxygen into the parent compound (redox-cycling). Reactive intermediates capable of binding covalently to proteins, DNA or GSH (Vroomen *et al.* 1987; Boyd *et al.* 1979; Vroomen *et al.* 1986) may be formed upon further reduction of the radical nitro anion.

A disturbance of the pyruvate metabolism has been reported after incubation of

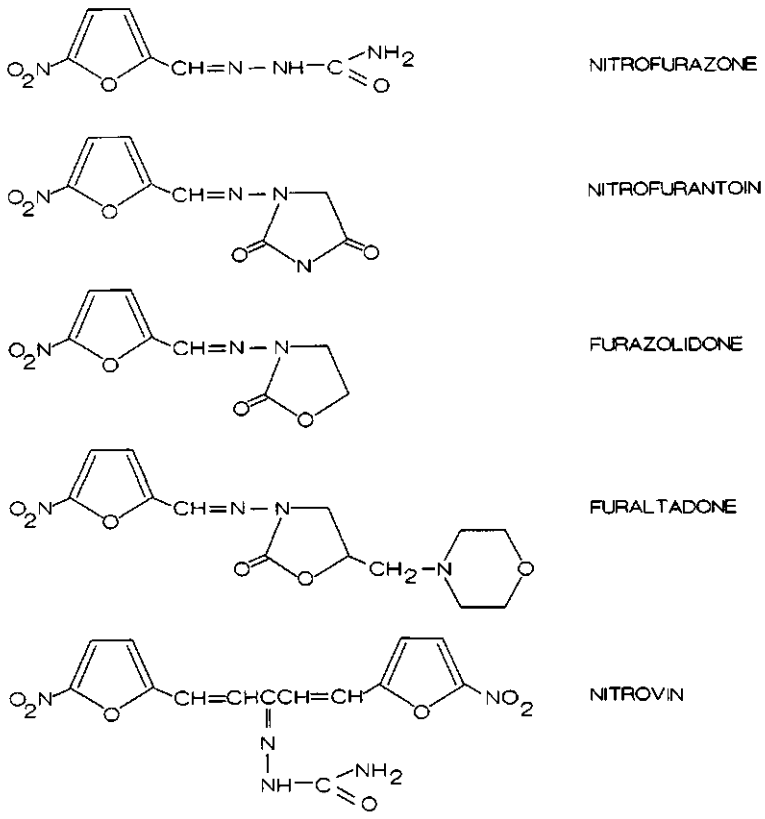

Fig. 5.1. Structures of the different nitrofurans used in the experiments described in this chapter.

bacteria (Asnis *et al.* 1957; Lu & McCalla 1978) and slices of various rat tissues, including liver (Paul *et al.* 1952; Paul *et al.* 1953) with nitrofurans. Paul *et al.* (1956), observing an inhibition of a crude preparation of the pyruvate dehydrogenase complex from pigeon breast muscle by nitrofurazone, postulated that the inhibition may be due to an effect of the drug on the electron-flow through the enzyme-complex. Lim *et al.* (1986) showed that exposure of isolated mouse liver mitochondria to nitrofurantoin and nitrofurazone resulted in an inhibition of state 3 respiration with complex I substrates, and presented evidence that reactive oxygen-species were responsible for the effect.

Regarding the importance of possible irreversible and accumulating effects with respect to the actual administration of nitrofurans *in vivo*, special attention was paid to the recovery of cells from the exposure to furazolidone as well as to the effect of a repeated treatment of cells with this compound. In addition such studies may give more information on the mechanisms underlying the toxic effects observed.

Materials & Methods

Materials

Furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidinone) and nitrovin were a gift from Orphahell BV (Mijdrecht, The Netherlands). Nitrofurazone (5-nitro-2-furaldehyde semicarbazone), nitrofurantoin (N-[5-nitro-2-furfurylidene]-1-amino-hydantoin), furaltadone (5-morpholinomethyl-3-[5-nitrofurfurylidene-amino]-2-oxazolidinone and acetaminophen were obtained from Sigma (St Louis, MO, USA). Williams' E, Hanks' balanced salts, trypan blue, penicillin/ streptomycin and foetal calf serum were purchased from Flow Labs (Rickmansworth, UK), collagenase type H, GSH, GSSG and analytical testkits for the determination of pyruvate and lactate from Boehringer (Mannheim, FRG) and L-[U-^{14}C]leucine (specific activity 12.65 GBq/ mmol) from Amersham (Amersham, UK).

Animals

Liver samples were taken from six to nine month old sows used for studies at the Department of Animal Husbandry at the Agricultural University in Wageningen. In most cases animals were pregnant for 10 or 40 days. Only animals known to be free from possible drug treatment during the past 4 weeks were used.

Isolation and culturing of hepatocytes

Hepatocytes were isolated from a liver sample of about 100 grams by a three-step perfusion technique as described in Chapter 2. About 3.5 million viable cells were seeded in uncoated 60 mm culture dishes and allowed to attach for 4 h before renewal of the incubation medium. Routinely cells were incubated and exposed in Williams' medium E supplemented with 5% foetal calf serum, 0.5 μg/ml insulin, 50 IU/ml penicillin and 50 μg/ml streptomycin, in a CO_2 incubator at 37 °C.

Exposure of cells

All test compounds, except furaltadone were dissolved in dimethyl-sulfoxide (DMSO) and added to the medium to obtain the desired drug concentration. The final DMSO concentration of 1% had no direct effect on the parameters measured. Furaltadone was dissolved in water, filtersterilized and added to the medium (10 μl/ml) which was supplemented with 1% DMSO. Nitrovin was dissolved in DMSO up to the highest possible concentration of 5 mM and therefore not tested at levels above 50 μM. Monolayer cultures of hepatocytes were exposed for 16 h, starting 26 h after plating. In the case of recovery experiments with furazolidone, cells were exposed to the drug, washed twice with medium and incubated for another 8 h without the drug. At that time, incubation was either terminated or continued for another 16 h after replacement of the medium. During the same experiment, the effect of a repeated exposure was investigated. Therefore cells were incubated in the presence of different concentrations

of furazolidone for 16 h, washed twice, incubated for 8 h without the drug and subsequently exposed to the same drug concentrations as during the first interval. Single treated cells were handled in the same way, *i.e.* medium renewed and washed at the same time-points, but only exposed during the second interval.

At the end of the incubation period, samples of the medium were taken for determination of the LDH-release and the protein synthesis. Cells were washed twice with phosphate buffered saline (PBS), scraped from the dishes in 1 ml of PBS with a rubber policeman, and resuspended using a Teflon pestle. For determination of the intracellular LDH-activity, a 100 μl aliquot was diluted with 400 μl PBS containing 1% Triton X-100 and homogenised by ultrasonic treatment. Except for the samples used for the LDH-release, all samples were stored in liquid nitrogen until analysis.

Protein synthesis

For measurement of the protein synthesis, cells were incubated with medium containing ^{14}C-labelled leucine (1850 Bq/ml) and the test compounds. At the end of the incubation, medium and cells were collected as described. Protein was precipitated by addition of PCA (final concentration 3%) and the supernatant was used for determination of the GSH-content. The pellet was washed three times with 7% TCA and solubilized in 1 ml of 5% sodium-dodecyl-sulphate/0.5 N NaOH. Samples of 0.75 ml were mixed with 3 ml scintillation cocktail (Minisolve; Zinsser Analytical, Maidenhead, UK) and counted in a Philips liquid scintillation counter (type PW 4700). In the case of cellular protein, part of the samples were used for a protein determination. Figures for ^{14}C-leucine incorporation into proteins present in the medium and in the cells were corrected for cellular protein and subsequently compared with the adequate controls which were set to 100%.

Assays

Lactate-dehydrogenase activity (EC 1.1.1.27) in medium and cells was measured as described by Jauregui *et al.* (1981). Pyruvate and lactate in the medium were measured with Boehringer test-kits. Absolute amounts were calculated from calibration curves with known amounts of lactate and pyruvate in medium. Intracellular GSH was measured by the method of Reed *et al.* (1980), using a modified HPLC-method as described previously (Chapter 3). Protein in cells and medium was measured by the method of Lowry, as modified by Peterson (1977).

Reproducibility

All results presented were shown to be reproducible in at least one additional experiment with cells obtained from a liver sample of another pig. The significance of the observed differences between drug treated cells and the controls was statistically tested using the Student's t-test. For statistical analysis of the effect of a repeated exposure on the pyruvate metabolism an ANOVA-analysis was used.

Table 5.1. Effect of furazolidone, nitrofurantoin, nitrofurazone, furaltadone, nitrovin and acetaminophen on LDH, protein synthesis and intracellular GSH and GSSG in pig hepatocytes (mean ± SEM; n=3). Results marked with * are significantly different from the control ($p<0.05$).

compound	concentration	LDH (absolute)		LDH (relative)	GSH	GSSG	protein synthesis	
		medium	cells	medium	cells		medium	cells
	(μM)	(U/mg cellular protein) (x 0.001)		(% of total)	(nmol/mg protein)		(% of total)	
blanc (1% DMSO)		29 ± 3	0.62 ± 0.02	4.45 ± 0.24	49.1 ± 3.2	0.39 ± 0.04	100 ± 3	100 ± 4
Furazolidone	15	28 ± 1	0.67 ± 0.02	4.04 ± 0.04	53.8 ± 0.9	0.43 ± 0.01	111 ± 4	114 ± 1
	50	29 ± 1	0.72 ± 0.01	3.91 ± 0.11	69.1 ± 1.4 *	0.58 ± 0.01 *	85 ± 4 *	114 ± 2
	150	28 ± 0	0.70 ± 0.01	3.68 ± 0.14	68.0 ± 0.1 *	0.72 ± 0.08 *	58 ± 4 *	100 ± 4
	500	31 ± 1	0.48 ± 0.02 *	6.03 ± 0.11 *	56.3 ± 1.1	0.81 ± 0.06 *	42 ± 5 *	59 ± 1 *
Furaltadone	50	30 ± 3	0.67 ± 0.04	4.23 ± 0.34	59.7 ± 3.2	0.53 ± 0.04	81 ± 3 *	96 ± 1
	500	30 ± 3	0.53 ± 0.02 *	5.45 ± 0.34 *	58.8 ± 1.4	1.41 ± 0.12 *	34 ± 2 *	47 ± 2 *
Nitrofurantoin	50	26 ± 2	0.57 ± 0.02	4.32 ± 0.24	47.8 ± 3.2	0.42 ± 0.04	86 ± 4	93 ± 3
	500	100 ± 12 *	0.38 ± 0.03 *	21.19 ± 3.19 *	29.1 ± 2.4 *	1.46 ± 0.14 *	8 ± 1 *	12 ± 1 *
Nitrofurazone	50	31 ± 2	0.69 ± 0.01	4.10 ± 0.21	56.3 ± 1.8	0.42 ± 0.03	94 ± 4	104 ± 2
	500	42 ± 2 *	0.46 ± 0.01 *	8.37 ± 0.21 *	54.8 ± 1.6	1.27 ± 0.01 *	28 ± 2 *	42 ± 1 *
Nitrovin	5	31 ± 1	0.65 ± 0.02	4.59 ± 0.21	70.0 ± 4.2	0.61 ± 0.02 *	83 ± 3 *	104 ± 1
	15	33 ± 1	0.58 ± 0.01	5.41 ± 0.15 *	105.8 ± 4.9 *	1.18 ± 0.11 *	50 ± 4 *	87 ± 3
	50	46 ± 5 *	0.44 ± 0.01 *	9.48 ± 0.78 *	47.8 ± 2.0	1.10 ± 0.05 *	7 ± 1 *	27 ± 2 *
Acetaminophen	1000	27 ± 2	0.65 ± 0.01	4.04 ± 0.31	50.1 ± 1.1	0.37 ± 0.05	85 ± 7	102 ± 3
	3000	25 ± 1	0.53 ± 0.03	4.47 ± 0.15	6.3 ± 2.6 *	0.17 ± 0.04	45 ± 5 *	62 ± 4 *
	10000	66 ± 13 *	0.27 ± 0.02 *	19.37 ± 2.44 *	2.1 ± 0.3 *	0.10 ± 0.05	64 ± 9 *	30 ± 1 *

Results

As described in Chapter 2, hepatocytes isolated from a piece of pig liver, were shown to posses a high viability and were able to form monolayer cultures required for the present studies. The decision to start the exposition of cells to the drugs after a 24 h recovery period was based on two observations. First of all, this period allowed the restoration of intracellular GSH levels, which decreased markedly during the isolation of the cells (Chapter 3). Secondly, contrary to the cytochrome P-450 related biotransformation reactions, the degradation of furazolidone was shown not to decrease during the first 96 h of the cultures (Chapters 3 and 9).

Table 5.1 shows the effect of acetaminophen, used as a positive control, and the nitrofurans on LDH-leakage, protein synthesis and intracellular glutathione-status. A clear visible effect could only be observed after exposure to 10 mM acetaminophen, where cells were no longer stretched and partly detached. Exposure to this drug resulted in a dose related decrease of the GSH concentration, a reduction of the intracellular LDH-activity and an inhibition of the protein synthesis. The relatively high levels of radiolabelled proteins in the medium at 10 mM as compared to 3 mM were probably due to cell detachment. At this dose there was an increase in the LDH-activity in the medium.

Slightly increased LDH-activity in the medium was also observed at the highest dose-levels of nitrofurazone, nitrovin, nitrofurantoin and, after correction for the decreased total enzyme activity on the dish, also for furazolidone. Intracellular LDH-activities were more clearly lowered at these concentrations of the drugs. This correlated very well with the reduced incorporation of ^{14}C-leucine into proteins, as measured in the cellular fraction (corr. coef. 0.96). At lower doses the latter effect could only be measured on the proteins excreted into the medium, which contributed for about 15% to the total amount of ^{14}C-leucine incorporation in the case of cells incubated with 1% DMSO. Exposure to nitrofurans resulted in an increase in intracellular GSSG at the highest dose levels. However, only after exposure to 500 μM nitrofurantoin, intracellular GSH-levels were lower than the control. In all other cases GSH-levels were similar or even elevated, as demonstrated by the more than twofold increase after incubation of cells with 15 μM nitrovin.

Fig. 5.2 shows the effect of nitrofurazone, nitrofurantoin, furaltadone and furazolidone on pyruvate and lactate concentrations in the medium. When combined (Fig. 5.2C), there was a clear dose-related accumulation of these compounds up to the highest drug levels tested, except for nitrofurantoin, where a maximum effect was seen at 150 μM. Pyruvate levels alone followed a similar pattern (Fig. 5.2A), whereas lactate levels seemed to reach a plateau at the higher drug concentrations (Fig. 5.2B). As a result there was a dose related increase in pyruvate/lactate ratio's (Fig. 5.2D), although large variations for the different drugs were observed. Nitrovin, tested at 15 and 50 μM, had no effect on pyruvate and lactate levels (data not shown).

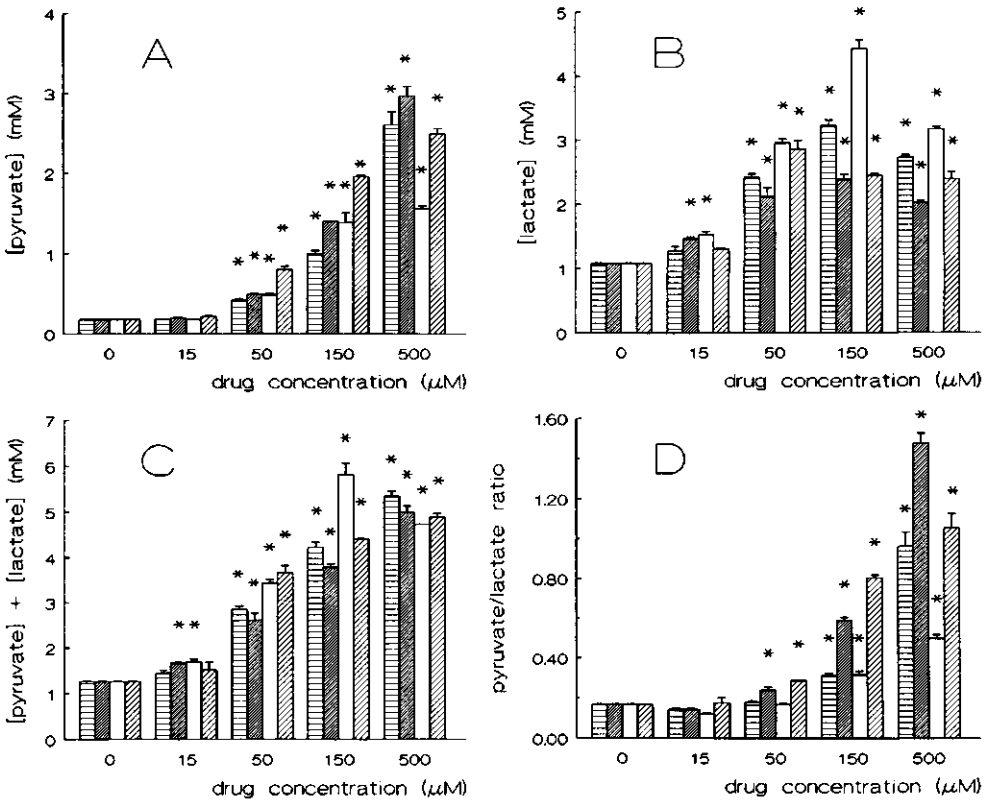

Fig. 5.2. Pyruvate (A) and lactate (B) concentrations in the medium as well as their sum (C) and ratio (D) after incubation of pig hepatocytes for 16 h with various concentrations of furazolidone (eeeee), furaltadone (eeeee), nitrofurantoin (eeeee) and nitrofurazone (eeeee). Results are expressed as mean ± SEM (n=3). Concentrations marked with * are statistically higher from the control ($p<0.05$).

The reversibility of some of the effects was investigated for furazolidone. As shown in Table 5.2, intracellular LDH-activity was fully restored within 24 h after ending the exposure. The amount of radiolabelled leucine present in proteins excreted into the medium was still low at the highest dose-level after 24 h. However, when the figures of cells and medium were combined, overall protein-synthesis at the dose of 500 μM was 75 ± 3 and 95 ± 1% of the control after 8 and 8 + 16 h respectively. Intracellular GSH-levels tended to be elevated in cells after exposure to the drug, even after 24 h.

Table 5.2 Reversibility of cell damage due to furazolidone exposure, after a recovery period of eight (+8) or eight plus sixteen h (+8+16). Results shown in Tables 5.1 and 5.2 were from experiments performed with cells isolated from the same liver (mean ± SEM; n=3). Results marked with * are significantly different from the control ($p<0.05$).

conc.	recovery interval	cellular LDH	protein synthesis		cellular GSH
			medium	cells	
(μM)	(h)	(U/mg protein)	(% of control)		(nmol/mg protein)
0	+8	0.78 ± 0.02	100 ± 5	100 ± 2	54.8 ± 3.0
50	+8	0.85 ± 0.04	68 ± 0 *	106 ± 3	70.5 ± 1.2 *
500	+8	0.63 ± 0.01 *	28 ± 2 *	85 ± 2 *	76.6 ± 4.4 *
0	+8+16	1.10 ± 0.00	100 ± 0	100 ± 1	65.5 ± 1.6
50	+8+16	1.44 ± 0.08	85 ± 8	111 ± 2 *	79.3 ± 1.3 *
500	+8+16	1.10 ± 0.03	33 ± 4 *	117 ± 1 *	75.0 ± 2.1

In order to investigate the reversibility of the effect of furazolidone on the pyruvate metabolism, cells were exposed to various concentrations of the drug for 16 h, washed and subsequently incubated in medium without the drug for 8 h, followed by a renewal of the medium and an additional incubation for 16 h without the drug (Fig. 5.3). During the first 8 h of incubation in renewed medium, the rate of accumulation of pyruvate and lactate seems to be unchanged as compared to the 16 h incubation in the presence of the drug. After the second renewal of the medium, a substantial decrease in pyruvate accumulation was observed in the medium at all concentrations whereas the effect on lactate was still clearly present, especially at the highest dose rate.

When cells were exposed to furazolidone for a second interval of 16 h after a recovery interval of 8 h, there was a clear accumulation of the effect at lower dose-levels (Fig. 5.4). However, only a small effect of the repeated exposure was observed at higher concentrations.

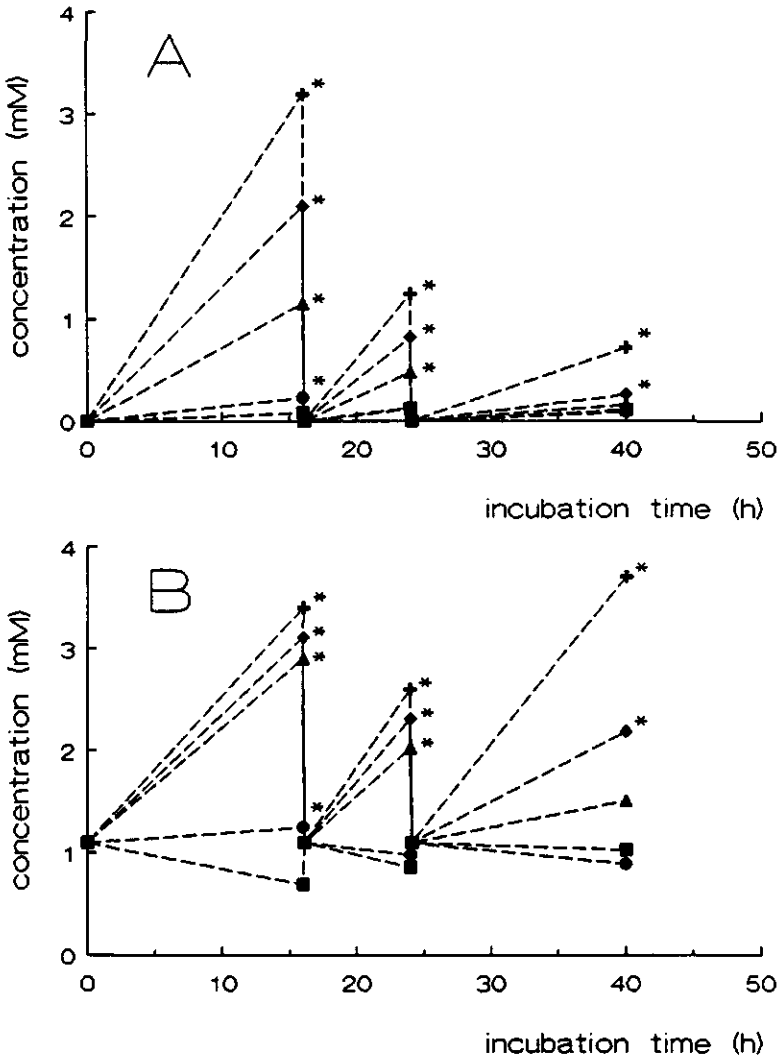

Fig. 5.3. Recovery of cells from the effect of furazolidone on the pyruvate metabolism. Cells were exposed to 0 ■ , 15 ● , 50 ▲ , 150 ♦ , or 500 + μM of the drug for 16 h. At 16 h and again at 24 h the medium was replaced by medium without drug. Levels of pyruvate (A) and lactate (B) were measured in media samples taken at 16, 24 and 40 h. Results are expressed as the mean for triplicate dishes (n=3). For clarity the SEM has been omitted from the figure. Concentrations marked with * are significantly higher than the control ($p<0.05$).

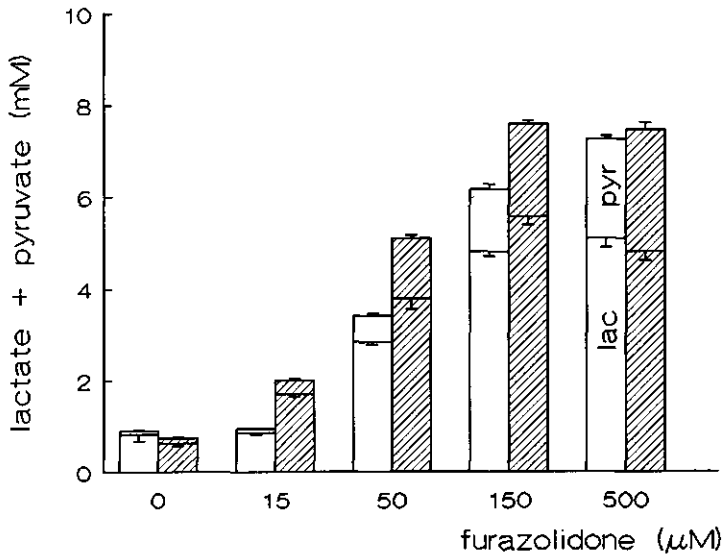

Fig. 5.4. Accumulation of pyruvate and lactate in the medium after a single (▭) or repeated (▨) exposure to furazolidone. Cells were incubated in the presence of different concentrations of the drug for 16 h, washed twice, incubated for 8 h without the drug and subsequently exposed to the same drug concentrations as during the first interval for another 16 h. In the case of a single exposure (▭) cells were similarly treated but only exposed during the second interval. Bars represent the combined average concentrations of lactate (lac) and pyruvate (pyr), both with their own SEM (n=3). The dose dependant differences in pyruvate and lactate concentrations, both separate and combined, between single and repeatedly treated cells were statistically significant ($p<0.05$; ANOVA).

Discussion

The present study shows that the end-points selected could easily and reproducibly be measured in monolayer cultures of pig hepatocytes. Therefore the model could be used to study the cytotoxicity of a number of widely used nitrofurans.

Incubation of pig hepatocytes with the known hepatotoxic agent acetaminophen resulted in a decrease in intracellular GSH and the subsequent increase of LDH-activity in the medium as was previously reported for hepatocytes from rats (Hayes *et al.* 1986; Mitchell *et al.* 1985) and hamsters (Boobis *et al.* 1986).

Among the different end-points selected, changes in LDH-activity in the medium and

the cells, which are considered to be an indication for membrane-damage, could only be observed at high doses of the drugs. The decrease in the intracellular LDH-activity cannot be explained by leakage of the enzyme into the medium, and may rather be due to an effect of the test compounds on either the activity or the *de novo* synthesis of the enzyme. The latter theory is supported by the good correlation between the decrease in intracellular LDH-activity and the inhibition of the protein synthesis. Furthermore, synthesis of LDH by the cells during the exposure intervals was clearly demonstrated from the increase in intracellular LDH during the ageing of the cultures (0.62 ± 0.04 and 1.10 ± 0.00 U/mg protein at 44 and 68 h after isolation respectively; Tables 5.1 and 5.2).

For testing the toxicity of the nitrofurans, the inhibition of the incorporation of ^{14}C-leucine into cellular proteins and especially into those excreted into the medium, appears to be a more sensitive end-point (Table 5.1). The more pronounced effect of the drugs on the proteins excreted in the medium may be explained by a selective reduction in the synthesis of these proteins or an effect of the test compounds on their excretion by the cells. The results of the recovery experiment with furazolidone (Table 5.2) seem to support the latter hypothesis, since 24 h after withdrawal of the drug, the overall protein synthesis seemed to be almost restored while the amount of radiolabelled leucine in proteins excreted into the medium was still reduced. At this point it is unclear whether there is a relation between this effect and the decreased plasma protein levels that have been measured in the case of chickens (Ali 1984), turkeys (Pang *et al.* 1982; Simpson *et al.* 1979), and goats (Mustafa *et al.* 1987) treated with furazolidone.

The increased levels of intracellular GSSG after exposure of pig hepatocytes to nitrofurans (Table 5.1) are indirect evidence for the detoxification of reactive oxygen species, generated by redox-cycling of the radical nitro-anion with oxygen. The formation of the radical nitro-anion was recently demonstrated in rat hepatocytes incubated with nitrofurazone and nitrofurantoin (Rao *et al.* 1988). The decreased intracellular GSH levels observed in the case of nitrofurantoin are in agreement with results reported for rat hepatocytes (Rossi *et al.* 1988) and perfused rat liver (Akerboom *et al.* 1982, Hoener *et al.* 1989). The absence of GSH-depletion after exposure of cells to other nitrofurans was unexpected and contradictory to the decrease observed after treatment of a perfused rat liver with nitrofurazone (Hoener 1988). It is possible that a loss of GSH caused by an increased formation of GSSG and possibly GSH-conjugates is compensated by an adequate reduction of GSSG to GSH by GSSG-reductase and furthermore an elevated synthesis of GSH by the cells. The active synthesis of GSH by these cells has already been demonstrated in chapter 3 and may also explain the elevated GSH-levels observed in some cases at the end of the exposure interval (Table 5.1) or after a recovery period (Table 5.2). The use of an incubation medium like Williams' E, which contains high concentrations of the amino-acids required for GSH-synthesis, might therefore be one of the possible explanations for the

discrepancies between the results of our experiments and those reported by other authors.

This is the first report on a disturbance of the metabolism of pyruvate in cultured mammalian cells incubated with nitrofurantoin, nitrofurazone, furazolidone and furaltadone (Fig. 5.2). This parameter turned out to be the most sensitive end-point measured for detecting the cytotoxicity of nitrofurans. The slow recovery of the cells from the damage as well as the accumulation of the effect upon repeated exposure to furazolidone point in the direction of an irreversible binding of furazolidone or a metabolite to the pyruvate-dehydrogenase complex as suggested by Paul *et al.* (1956). A similar slow recovery of the decreased glucose consumption and citrate formation was observed with testis tissues isolated from rats killed either during the treatment with nitrofurazone, nitrofurantoin or furazolidone, or after a recovery period (Paul *et al.* 1953). It is of interest to investigate whether this inhibition underlies the elevated plasma levels of pyruvate and lactate that have been reported for man (Paul & Paul 1964) and turkey (Ali & Bartlet 1982), although in the latter case the authors themselves ascribed the effect to the reduced feed intake after drug treatment.

In comparing the different nitrofurans tested, it appears that exposure of cells to nitrovin results in somewhat different biological effects than the other compounds. Effects on LDH-activity in medium and cells, and on protein synthesis were observed at relatively low doses, whereas the drug appeared to have no effect on the metabolism of pyruvate. Furthermore, nitrovin had a striking effect on GSH-levels, as discussed before. Of the other four compounds, nitrofurantoin seems to have the largest impact on the cells, as demonstrated by the LDH-leakage, the GSH-depletion and the peak in the effect on the pyruvate metabolism at 150 μM. The higher sensitivity for this drug may be explained by the fact that nitrofurantoin is a more potent inhibitor of GSSG-reductase than the other compounds (Buzard & Kopko 1963, Ondarzo & Abney 1970; Grinblat *et al.* 1989).

From the results presented above, it is clear that most of the effects observed with other *in vitro* models could be reproduced with cultures of pig hepatocytes. In general however, results from *in vitro* studies will have to be validated by *in vivo* studies. As indicated above, some of the observed effects might be related to effects that have been noticed after treatment of animals. However, further *in vivo* studies will have to reveal the biological significance of the various effects. In this regard, the observation of an accumulating inhibitory effect after repeated exposure is particularly interesting, since it may have consequences for the administration regime of these compounds in the veterinary practice. The results of the present study clearly demonstrate that hepatocytes isolated from a sample of pig liver, can be used for detecting and comparing the (hepato)toxic properties of veterinary drugs, therefore avoiding possible extrapolation problems due to species differences. Furthermore, these cells may be very useful for studying in more detail the mechanisms underlying the observed effects.

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CHAPTER 6

THE USE OF PIG HEPATOCYTES TO STUDY THE INHIBITION OF MONOAMINE OXIDASE BY FURAZOLIDONE

This chapter is based on the following publication:

Hoogenboom L.A.P., Tomassini O., Oorsprong M.B.M. and Kuiper H.A. The use of pig hepatocytes to study the inhibition of monoamine oxidase by furazolidone. *Fd Chem. Toxicology* (in press).

THE USE OF PIG HEPATOCYTES TO STUDY THE INHIBITION OF MONOAMINE OXIDASE BY FURAZOLIDONE

Summary

Primary cultures of pig hepatocytes were used to examine the irreversible inhibition of monoamine oxidase (MAO), observed in tissues of a number of different animal species after oral treatment with furazolidone. The rapid biotransformation of the MAO-substrate p-tyramine by intact cells could effectively and irreversibly be inhibited with the known MAO-inhibitors iproniazid and clorgyline. Incubation of cells with ß-hydroxyethylhydrazine but also 3-amino-2-oxazolidinone, which were previously proposed as the metabolites of furazolidone responsible for the *in vivo* effect, resulted in an irreversible inhibition of the MAO-activity. Incubation of cells with furazolidone also resulted in a dose related inhibition, but this effect was completely reversible upon withdrawal of the drug. A similar MAO-inhibition was observed after treatment of cells with nitrofurazone and furaltadone but not nitrofurantoin.

The results obtained with intact cells were confirmed by studies with 13,000 g pellets of homogenates made from cells preincubated for 24 h with the compounds, showing an irreversible type of inhibition in the case of iproniazid and 3-amino-2-oxazolidinone, but not in the case of furazolidone.

The present study shows that hepatocytes are capable of transforming 3-amino-2-oxazolidinone, but not furazolidone itself, into a potent irreversible type of MAO-inhibitor.

Abbreviations used: MAO= monoamine oxidase; AOZ= 3-amino-2-oxazolidinone; HEH= ß-hydroxyethylhydrazine; LDH= lactate-dehydrogenase

Introduction

Oral treatment of human patients (Pettinger *et al.* 1968), and animals like poultry (Ali *et al.* 1980; Ali *et al.* 1982), ducklings (Ali *et al.* 1982), and rats (Palm *et al.* 1967; Stern *et al.* 1967; Yeung & Goldman 1981), with furazolidone, nowadays widely used as a veterinary drug, has been shown to result in the inhibition of monoamine oxidase activity [EC 1.4.3.4] (MAO) in tissues from these animals. Since the inhibitory effect was shown to accumulate upon repeated exposure and disappeared only gradually after termination of the treatment (Ali *et al.* 1980; Palm *et al.* 1967; Stern *et al.* 1967; Pettinger *et al.* 1968), it is thought to be due to the irreversible binding of a metabolite of furazolidone to the MAO enzyme(s). Stern *et al.* (1967) hypothesized that this metabolite might be ß-hydroxyethylhydrazine (HEH), formed by ring cleavage of 3-amino-2-oxazolidinone (AOZ), which is derived from the side-chain of furazolidone by hydrolysis of the azomethine bond (Fig. 6.1). This hypothesis is supported by the fact that MAO-inhibition was observed in tissues of rats after oral treatment with AOZ and HEH, but not after treatment with nitrofurans containing different side-chains, like nitrofurantoin, nitrofurazone and furaltadone (Palm *et al.* 1967; Stern *et al.* 1967). Furthermore, the proposed active metabolite, HEH, was shown to be a potent MAO-inhibitor in isolated rat liver mitochondria, whereas AOZ and furazolidone gave negative results (Stern *et al.* 1967). The fact that the MAO-inhibition by furazolidone may be related to the possible transformation of its side-chain into a hydrazine, is of particular interest, since AOZ may not only be released from the parent compound, but also from protein-bound metabolites of the drug, which can be present as residues in edible tissues of farmanimals (Vroomen *et al.* 1986, 1988; Chapter 8). Since in chickens the MAO-inhibition was observed only after oral administration of furazolidone, but not after intramuscular

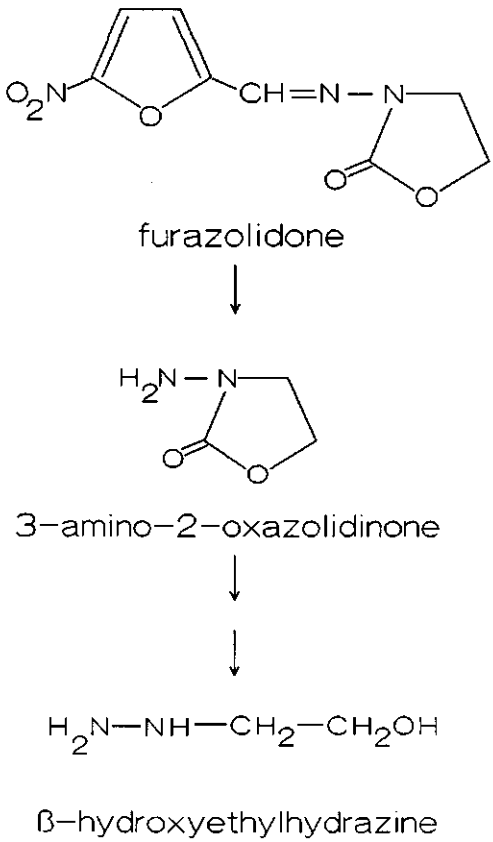

Fig. 6.1. Proposed pathway for the degradation of furazolidone, leading to the formation of ß-hydroxyethylhydrazine (Stern *et al.* 1967).

injection (Ali *et al.* 1982), it has been proposed that the ultimate active metabolite is formed in the G.I. tract, most likely by bacteria. Results from experiments with chickens and ducklings (Ali *et al.* 1980; Ali *et al.* 1982), where the bacterial activity was reduced by simultaneous treatment with neomycin, seemed to support this hypothesis, but were contradicted by the results of an experiment with germfree rats (Yeung & Goldman 1981). Furthermore, both Stern *et al.* (1967) and Cohen (1979) showed that such a difference between oral and intramuscular, or intraperitoneal treatment was less obvious in the case of rats.

Aim of the present study was to further investigate the MAO-inhibiting properties of furazolidone using intact pig hepatocytes instead of cell fractions. This approach offers the advantage of a model with an intact biotransformation potential, and also might give a better insight on the actual effect of the MAO-inhibition on the activity of the cell towards specific MAO substrates. Furthermore, as shown before (Chapter 5), monolayer cultures can easily be washed and are therefore very suitable for studying the recovery of the cells from the observed effects, thereby supplying important information to explain their underlying mechanisms.

Previously, Cohen (1979) showed that the decreased MAO-activity in brain homogenates of rats treated with furazolidone or HEH, was non-specific with regard to the A and B forms of the enzyme. Therefore, the MAO-activity was determined by incubating the cells with ^{14}C-labelled p-tyramine, a well known substrate for both A and B types of the MAO-enzymes (Houslay & Tipton 1976), followed by the selective extraction of the deaminated metabolites at pH 5, as described by Otsuka and Kobayashi (1964). Using this method, the effects of the known MAO-inhibitors clorgyline and iproniazid were compared with those of HEH, AOZ and furazolidone, as well as a number of other nitrofurans, paying special attention to the reversibility of the observed inhibition.

Materials & Methods

Chemicals

Collagenase type H was obtained from Boehringer (Mannheim, FRG), multiwell tissue culture dishes from Costar (Badhoevedorp, The Netherlands), Williams' medium E, Hanks' balanced salts, trypan blue, penicillin/streptomycin and fetal calf serum from Flow Labs (Rickmansworth, UK), insulin, iproniazid, clorgyline, ß-hydroxyethylhydrazine, nitrofurazone, furaltadone, nitrofurantoin and p-tyramine from Sigma (St. Louis, USA). Furazolidone and 3-amino-2-oxazolidinone were gifts from Orphahell BV (Mijdrecht, The Netherlands). ^{14}C-labelled p-tyramine (spec. act. 1.63 GBq/mmol) was purchased from NEN (Boston, USA).

Animals

Liver samples were taken from six to nine month old sows (Dutch Landrace x Yorkshire) used for studies at the Department of Animal Husbandry at the Agricultural University in Wageningen, The Netherlands.

Isolation of hepatocytes

Hepatocytes were isolated from a piece of pig liver by a three-step perfusion method, as described in Chapter 2. Cells were diluted to a density of about 0.5 million per ml of incubation medium, Williams' medium E supplemented with 5% fetal calf serum, 0.5 μg/ml insulin, 50 IU/ml penicillin and 50 μg/ml streptomycin. Of this suspension 0.5 ml was seeded per well of a 24-multiwell dish. After 4 h the medium was replaced by 0.25 ml new medium and the cells were incubated for another 16-24 h before experiments were started.

MAO-inhibition assay

Inhibition of MAO-activity was measured as described by Otsuka and Kobayashi (1964). In short, cells were washed once with Williams' E and incubated with 250 μl of Williams' E supplemented with the drugs dissolved in dimethyl-sulfoxide (DMSO; final concentration in the medium 1%). After preincubation (usually one hour), 50 μl of Williams' E containing 600 μM ^{14}C-labelled p-tyramine (diluted to a spec. act. of 10 MBq/mmol) was added. At the end of the incubation interval (normally 20 or 30 min) a sample of 200 μl was taken, diluted with 800 μl 0.5 M citric acid and mixed with 2 ml toluene/ethylacetate (1:1 v/v) containing 0.6% 2,5-diphenyl-oxazole (PPO). After centrifugation and freezing of the water layer, the supernatant was aspirated, mixed with 2 ml of scintillation cocktail (Minisolve, Zinser, FRG) and counted in a Philips liquid scintillation counter (PW 4700). Using this method about 70% of the metabolites were recovered with the first extraction.

In the case of recovery experiments cells were incubated with the drug, washed twice with Williams' E and incubated in 250 μl plain medium for another 1 or 24 h, before the addition of the solution containing p-tyramine as described above.

MAO-inhibition in cell homogenates was determined by incubating cells in 60 mm culture dishes with drugs for 24 h, washing them twice with Williams' E followed by another incubation without drugs for 2 h. Cells were washed twice with phosphate-buffered saline and scraped from the dishes in 1 ml of a phosphate buffer (KPi, 0.1 M, pH 7.4) using a rubber policeman. Cells were disrupted by ultrasonic treatment and the resulting homogenates centrifuged at 13,000 g for 10 min (4 °C). The supernatant was aspirated and the pellet was dissolved in 1 ml KPi. MAO-activity was measured by adding 100 μl of this suspension to 400 μl of KPi (0.1 M, pH 7.4), containing 125 μM ^{14}C-labelled p-tyramine (37 °C). After 30 min a sample of 200 μl was taken and treated as described above. Protein in the 13,000 g fractions was determined by the method of Lowry as modified by Peterson (1977).

Leakage of the cytosolic enzyme lactate-dehydrogenase into the medium was determined by the method of Jauregui *et al.* (1981) as described in Chapter 5.

Calculations and statistics

All measurements were corrected for a blanc, *i.e.* a sample taken at the start of the incubation. In most cases the fractions of p-tyramine metabolized by treated cells are expressed in relation to the fractions metabolized by the appropriate controls. In the other cases, where the results are presented as the fraction of p-tyramine metabolized (Fig. 6.4), a correction was made for the 70% of metabolites recovered by the method used. All incubations were performed in triplo and controls were included in each multiwell-dish. The results from all experiments were checked for their reproducibility in at least one other experiment with cells isolated from the liver of another pig. Differences observed between treated and untreated cells were tested for significance using the two-tailed Student's t-test.

Results

Using the method of selective extraction of radiolabelled metabolites, the biotransformation of p-tyramine by pig hepatocytes could easily be measured. After incubation of 4 h old monolayer cultures with 100 μM p-tyramine for 20 min, 55 ± 2% of the substrate was metabolized into extractable metabolites. This fraction decreased to 33 ± 3% and 20 ± 2% in 26 and 50 h old cultures. Therefore, the standard incubation time of 20 min, used when working with 24-28 h old cultures, was prolonged to 30 min when cells were incubated for periods of 24 h. Within a 20 min incubation interval, very similar amounts of p-tyramine were deaminated by 24 h old cell cultures, isolated from the three other pigs used for the experiments described in this study, being respectively 36 ± 0, 29 ± 2, and 36 ± 0%.

Fig. 6.2 shows the biotransformation of p-tyramine both in the presence of the MAO-inhibitors clorgyline and iproniazid and after an additional recovery interval of 24 h. The MAO-activity could be completely reduced by these drugs and this activity was only slowly restored upon withdrawal of the drugs from the medium.

Exposure of cells for 1 h to HEH resulted in a marked inhibition of the MAO-activity, which similar to iproniazid and clorgyline, recovered only slowly upon withdrawal of the agent from the medium (Fig. 6.3A). A further increase of the MAO-inhibition was observed upon prolonged incubation in the presence of this compound, especially at low concentrations (Fig. 6.3B). Incubation of cells with AOZ for 1 h resulted only in a slight inhibition of the biotransformation of p-tyramine (Fig. 6.3C).

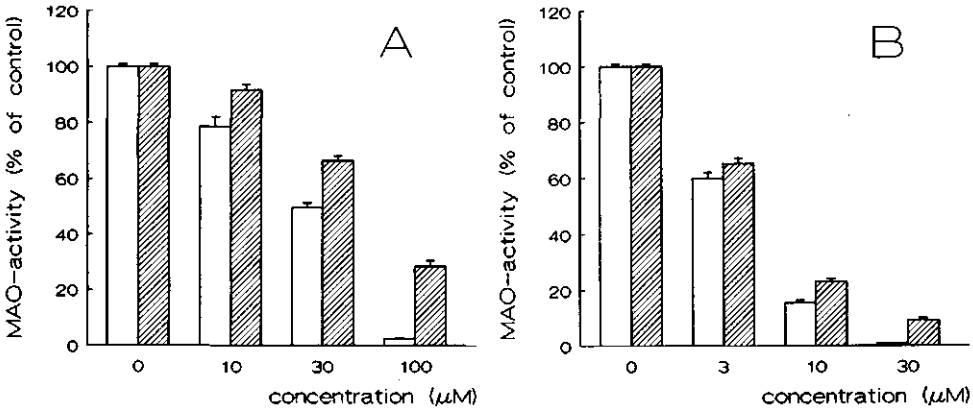

Fig. 6.2. Biotransformation of p-tyramine both in the presence of clorgyline (A) or iproniazid (B), or after an additional recovery interval of 24 h.
Cells were preincubated for 1 h with the drugs (and 1% DMSO). At that time p-tyramine was added (□) or cells were washed twice and incubated without drugs for 24 h prior to the addition of p-tyramine (▨). Results are presented as the fraction of the control (mean ± SEM; n=3). The fractions of p-tyramine metabolized by the control cells were 36.0 ± 0.3 and 25.2 ± 0.2% without or after a recovery period. For both drugs differences with the control were significant at all concentrations tested (two-tailed Student's t-test; $p<0.05$).

However, as shown in Fig. 6.3D, there was a clear and irreversible inhibition after an incubation interval of 24 h.

Incubation of cells with different concentrations of furazolidone resulted in a dose-related decrease in the amount of extractable metabolites (Fig. 6.4). The MAO-activity was however completely restored after exposure of cells to the drug for 1 h followed by a recovery interval of 1 h (Table 6.1). Furthermore, when cells were exposed to furazolidone for 24 h, the inhibition that was observed after 1 h in the presence of 500 μM, could no longer be measured (Table 6.1). After an additional recovery period of 1 h there was even an increase in the amount of extractable metabolites formed by the cells.

Similar to furazolidone, the biotransformation of p-tyramine by the cells was decreased in the presence of nitrofurazone and furaltadone but only to a minor extent in the presence of nitrofurantoin (Table 6.2). The good solubility of furaltadone in water made it possible to check the use of DMSO as a solvent for the other compounds. As indicated in Table 6.2, DMSO slightly decreased the MAO-activity, but the relative fractions metabolized, as compared to the appropriate controls, were not influenced by this effect of the solvent.

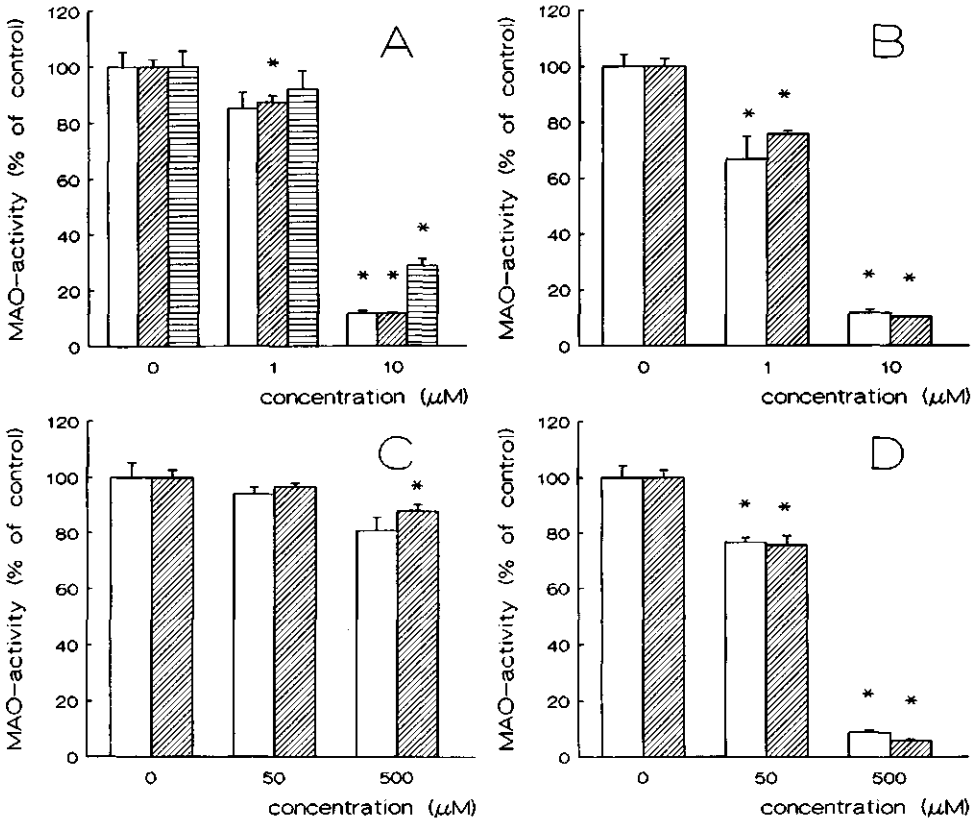

Fig. 6.3. The effect of ß-hydroxy-ethylhydrazine (A,B) and 3-amino-2-oxazolidinone (C,D) on the deamination of p-tyramine.
Cells were incubated with the compounds (and 1% DMSO) for 1 (A,C) or 24 (B,D) h. At that time p-tyramine was added (□) or cells were washed and incubated without the drug for 1 (▨) or 24 (▤) h prior to the incubation with p-tyramine. Results are expressed as the fraction of the control (mean ± SEM; n=3). Values marked with * were significantly different from the control (two-tailed Student's t-test; $p<0.05$). The fractions of p-tyramine metabolized by control cells were 29.2 ± 1.6, 34.4 ± 0.9, and 29.2 ± 1.6% after incubation for 1 h followed by recovery intervals of respectively 0, 1 and 20 h, and 31.9 ± 1.4, and 32.2 ± 0.9% after incubation for 24 h followed by recovery intervals of respectively 0 and 1 h.

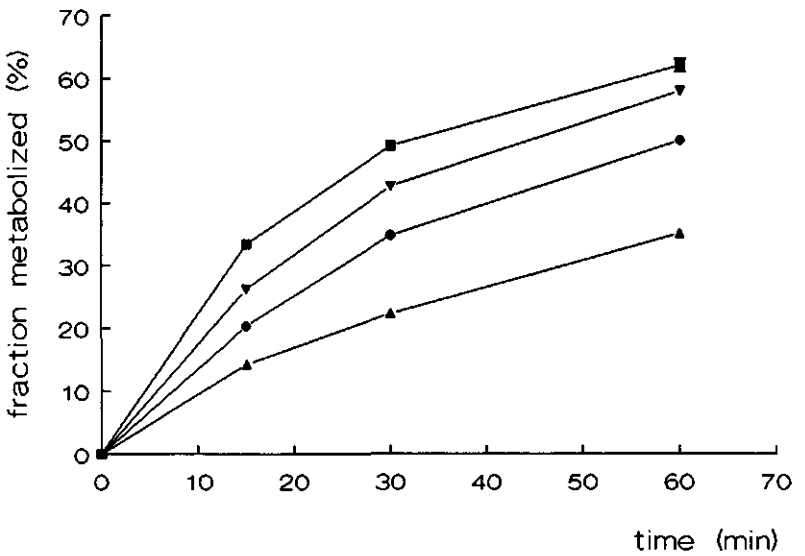

Fig. 6.4. Biotransformation of p-tyramine by monolayer cultures of pig hepatocytes in the absence or presence of different concentrations of furazolidone.
Cells were preincubated in medium containing 1% DMSO and 0 (■), 50 (▼), 150 (●) or 500 (▲) μM furazolidone for 1 h. At that time p-tyramine was added and samples were taken after another 15, 30 or 60 min. Results are presented as the mean ± SEM (n=3), unless the size of the symbol was larger than the SEM. At all concentrations of furazolidone and at each time interval, the differences with the control were statistically significant (two-tailed Student's t-test; $p<0.05$).

In none of the experiments described in this Chapter, exposure of cells resulted in clear visible effects, like rounding up or detachment of cells. Furthermore, exposure to AOZ had no effect on levels of either intracellular or extracellular LDH-activity (data not shown). However, since a decreased biotransformation of p-tyramine by intact cells may be caused by not observable cytotoxic effects, other than a direct inhibition of the MAO-enzyme, some additional experiments were performed with 13,000 g pellet fractions of cells, homogenized after their incubation for 24 h in the presence of furazolidone, AOZ or iproniazid, followed by a recovery interval of 2 h. As shown in Table 6.3, exposure to both iproniazid and AOZ resulted in a clear decrease in the MAO-activity, whereas in the case of furazolidone no effect could be measured.

Table 6.1. The effect of a short or long preincubation with furazolidone on the biotransformation of p-tyramine by pig hepatocytes, and the effect of a recovery interval.
Cells were preincubated in the presence of furazolidone for 1 or 24 h. At that time p-tyramine was added or cells were washed twice and incubated without drug for 1 h prior to the addition of p-tyramine (+rec). Results are presented as the fraction of the control (mean ± SEM; n=3). Values marked with * are significantly different from the control (two-tailed Student's t-test; $p<0.05$). The fractions of p-tyramine metabolized by the controls are presented in the legends of Fig. 6.3.

Concentration (μM)	MAO-activity (% of control)			
	1 h	24 h	1 h + 1 h rec.	24 h + 1 h rec.
0	100.0 ± 5.3	100.0 ± 4.3	100.0 ± 2.6	100.0 ± 2.6
50	91.9 ± 6.2	98.8 ± 6.2	94.9 ± 5.7	99.3 ± 3.5
500	55.3 ± 3.2 *	115.0 ± 3.2	102.9 ± 0.2	146.5 ± 1.8 *

Table 6.2. The metabolism of p-tyramine in the presence of furazolidone, nitrofurazone, nitrofurantoin and furaltadone, and the effect of the solvent DMSO.
Cells were preincubated with different concentrations of the drugs (and 1% DMSO) for 1 h, prior to the incubation with p-tyramine for 20 min. The effect of the water-soluble furaltadone was also studied in the absence of 1% DMSO. Results are presented as the fraction of the control (mean % SEM; n=3). Values marked with * are significantly different from the control (two-tailed Student's; $p<0.05$). The amount of tyramine metabolized by the controls was 36.2 ± 0.3 and 40.5 ± 0.3% of the initially added dose, in the presence or absence of DMSO respectively.

Compound	Concentration (μM)	MAO-activity (% of control) with DMSO		without DMSO	
Control		100.0 ± 0.9		100.0 ± 0.7	
Furazolidone	50	85.2 ± 1.4	*		
	500	42.1 ± 0.5	*		
Nitrofurazone	50	86.4 ± 2.7	*		
	500	38.3 ± 2.7	*		
Nitrofurantoin	50	97.7 ± 1.7			
	500	94.3 ± 1.4	*		
Furaltadone	50	77.6 ± 1.2	*	73.9 ± 0.9	*
	500	36.1 ± 0.2	*	35.3 ± 0.2	*

Table 6.3. MAO-activity in 13,000 g pellets of cells, homogenized after incubation for 24 h in the presence of furazolidone, 3-amino-2-oxazolidinone (AOZ) or iproniazid, followed by a recovery interval of 2 h.
Results were corrected for the amount of protein and are expressed as the fraction of the control (mean ± SEM; n=3). In the case of the control 45.4 ± 0.3% of the p-tyramine was deaminated. Values marked with * were significantly different from the control (two-tailed Student's t-test $p<0.05$)

Compound	Concentration (μM)	MAO-activity (% of control)	
1% DMSO		100.0 ± 1.1	
Furazolidone	50	92.8 ± 3.2	
	500	107.3 ± 4.2	
AOZ	50	74.9 ± 2.9	*
	500	9.2 ± 0.4	*
Iproniazid	10	4.9 ± 0.5	*

Discussion

The present study demonstrates that pig hepatocytes are a useful model to study the potency of drugs to inhibit MAO-activity. First of all it was shown that the method generally used for tissue homogenates (Otsuka and Kobayashi 1964), can easily be applied with this model with high reproducibility both within and between cell cultures of different animals. The specificity of the system was shown by the fact that the MAO-related formation of extractable metabolites of p-tyramine could be completely blocked with the MAO-inhibitors clorgyline and iproniazid (Fig. 6.2). The slow recovery of the effect is in agreement with the irreversible binding of these drugs to the MAO-enzyme, the recovery being dependent on the *de novo* synthesis of the enzyme. Further evidence that this effect is actually due to the inhibition of the MAO-activity and not to another cytotoxic effect, is presented by the inhibited activity in 13,000 g pellets of cells, homogenized after their incubation with iproniazid (Table 6.3). The almost complete inhibition observed with clorgyline appears to be contradictory to the supposed selectivity of this compound towards type A MAO-activity (Johnston 1968), since p-tyramine can be metabolized by both the A and B type MAO-enzymes (Houslay & Tipton 1976), and since both forms have been shown to be present in pig liver (Ekstedt 1979). However, as shown by Yang & Neff (1974), the specificity of clorgyline for

MAO-A is concentration dependant, since at higher dose levels it also inhibited MAO-B activity. An additional explanation may be a higher affinity of MAO-A towards p-tyramine, as shown in the case of rat liver (Fowler & Tipton 1981); as a result p-tyramine could be selectively deaminated by the A-form at the concentrations used in our experiments, especially regarding a possible concentration gradient over the cell membrane. In our studies the inhibition by clorgyline could, however, not be circumvented by increasing the concentration of p-tyramine from 100 μM up to 3 mM (data not shown). However, since the *in vivo* inhibition by furazolidone has been shown to be non-specific with regard to the two forms of MAO-activity (Cohen 1979), the possible specificity of p-tyramine should not be crucial for the outcome of the present study.

The rapid and irreversible inhibition of the MAO-activity after exposure of cells to HEH (Figs 6.3A and 6.3B), is in agreement with the results from experiments with rats and rat liver mitochondria (Stern *et al.* 1967, Cohen 1979). The MAO-inhibition caused by AOZ (Figs 6.3C and 6.3D) has been demonstrated *in vivo* but could up till now not be reproduced *in vitro* (Stern *et al.* 1967). The fact that in our study the inhibition becomes more clear after prolonged incubation with AOZ (Figs 6.3C and 6.3D), supports the hypothesis that AOZ has to be transformed into an active metabolite. This would explain the negative results obtained with isolated mitochondria, either because the incubation time was to short, or because these organelles were unable to activate AOZ. The reduced metabolism of p-tyramine observed with 13,000 g' pellets of cells pretreated with AOZ (Table 6.3), further demonstrates that the effect of AOZ is specific.

The dose-related inhibition of the metabolism of p-tyramine in the presence of furazolidone, following preincubation with the drug for 1 h (Fig. 6.4), differed in two ways from the MAO-inhibition observed *in vivo*. First of all, the inhibition disappeared completely upon withdrawal of the drug from the medium (Table 6.1), whereas *in vivo* only a slow recovery was observed (Ali *et al.* 1980; Palm *et al.* 1967; Stern *et al.* 1967; Pettinger *et al.* 1968). Secondly, in contrast to the *in vivo* situation (Palm *et al.* 1967; Stern *et al.* 1967), the inhibition was also observed with the nitrofurans nitrofurazone and furaltadone (Table 6.2). A similar conclusion could be drawn from the results obtained with intact cells (Table 6.1) and cellular fractions (Table 6.3) of cells, that had been incubated with furazolidone for 24 h. Although apparently the inhibition in the presence of the drug was no longer observed, the MAO-activity in intact cells was still suppressed in a reversible way as shown upon withdrawal of the drug from the medium. The reason for the increased formation of extractable metabolites of p-tyramine at the highest dose-level is unclear, but may be due to the inhibition of other enzymes involved in the metabolism. It can however be concluded that, contrary to AOZ, prolonged incubation of cells with furazolidone did not result in the formation of the metabolites, or at least adequate amounts of them, required for a detectable irreversible inhibition of the MAO-activity. This cannot be ascribed to a lack of

biotransformation of the drug, since similar studies revealed that during a 24 h incubation interval, about 50 and 10% of the furazolidone was transformed at 50 and 500 μM respectively (see Chapter 7).

The reversibility of the observed inhibition by furazolidone and the fact that the more cytotoxic agent nitrofurantoin (Chapter 5) had little effect, indicate that the inhibition is rather specific and not due to a general disruption of the cell functioning. Furthermore it may be ascribed to the nitrofuran-part of the drug and therefore be related to the reported competitive inhibition of rabbit liver monoamine oxidase by a number of nitro aromatic compounds, including nitrofurazone (Kimes & Carr 1982). This type of MAO inhibition by furazolidone has not been shown before, but its importance *in vivo* is questionable, since first of all relatively high concentrations are required, and secondly there is no accumulation of the effect.

The use of pig hepatocytes in this study allowed the elucidation of the discrepancy between the results of previous *in vivo* and *in vitro* studies, revealing that AOZ but not furazolidone can be transformed by these cells into active MAO-inhibiting metabolites. Therefore the results are in favour of the hypothesis of Stern *et al.* (1967) and support the role of the G.I. tract in the transformation of furazolidone into active metabolites. However, regarding the sensitivity of the azomethine bond to acid hydrolysis (Paul *et al.* 1960; Buzard *et al.* 1956), this role may be limited to the release of AOZ from furazolidone, and its metabolites, in the stomach. Evidence for this hydrolysis has actually been obtained after oral treatment of pigs (Tennent & Ray 1971) and mice (Loserth *et al.* 1988) with furazolidone, and after incubation of the drug in ruminal fluid (Hunder *et al.* 1987). Since mammalian cells are apparently able to transform AOZ into a MAO-inhibiting metabolite, the need for a bacterial activation in the G.I. tract (Ali *et al.* 1980; Ali *et al.* 1982) has become questionable.

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CHAPTER 7

THE USE OF PIG HEPATOCYTES TO STUDY THE ROLE OF GLUTATHIONE ON THE BIOTRANSFORMATION AND TOXICITY OF FURAZOLIDONE

This chapter is based on the following publication:

Hoogenboom L.A.P., Kammen M. van, Huveneers-Oorsprong M.B.M. and Kuiper H.A. The use of pig hepatocytes to study the role of glutathione on the biotransformation and toxicity of furazolidone. Manuscript presented to *Toxicology In Vitro* for publication.

THE USE OF PIG HEPATOCYTES TO STUDY THE ROLE OF GLUTATHIONE ON THE BIOTRANSFORMATION AND TOXICITY OF FURAZOLIDONE

Summary

Incubation of monolayer cultures of pig hepatocytes with furazolidone resulted in increased intracellular GSSG-levels but no decreased GSH-levels. A clear decrease in GSH-levels was observed at higher drug concentrations when either the synthesis of GSH was blocked with BSO or the reduction of GSSG by BCNU. Furthermore, when cells containing ^{35}S-labelled GSH were exposed to furazolidone, an increased dose-related loss of radiolabelled GSH was observed. The loss was balanced by increased levels in the medium of GSSG and a second compound, probably CySSG. The biotransformation of ^{14}C-furazolidone by cells resulted partly in the formation of the open-chain cyano-metabolite as well as a large number of more hydrophilic unknown metabolites. No evidence was obtained for the excretion by the cells of the glutathione-conjugate that was previously detected in microsomal incubations. This could however be due to the unstable nature of the conjugate, as demonstrated by its rapid disappearance when added to the medium. In the presence but not the absence of cells, this partly resulted in the formation of the cyano-metabolite, which upon prolonged incubation was further metabolized into more polar metabolites.

An increased LDH-leakage at high drug concentrations could only be observed when GSH-levels were kept at a low level with BSO. This effect was not paralleled by an increased formation of protein-bound metabolites of furazolidone and of the cyano-metabolite. GSH-depletion had no effect on the inhibition of the pyruvate metabolism by furazolidone, the most sensitive end-point up till now.

No evidence was obtained for the presence of reactive thiol-conjugates of furazolidone in the protein fraction of cells incubated with the drug. It is concluded that GSH has an active role in the protection of cells against the cytotoxic effects of furazolidone related to oxidative stress, but that no evidence could be obtained for the formation and excretion of reactive thiol-conjugates.

Abbreviations used: GSH/GSSG= reduced/oxidized glutathione; PBS= phosphate buffered saline; BSO= buthionine-D,L-sulfoximine; BCNU= 1,3-bis(2-chloroethyl)-1-nitrosourea; DMSO= dimethyl-sulfoxide; GSF (MSF)= glutathione (mercaptoethanol) conjugate of furazolidone: MSH= mercaptoethanol; LDH= Lactate-dehydrogenase (EC 1.1.1.27)

Introduction

Furazolidone [N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidinone] is a widely used veterinary drug, belonging to the class of the nitrofurans. After oral administration to pigs, the drug is extensively metabolized, partly resulting in the formation of the so-called open-chain cyano metabolite, as well as drug-derived metabolites that cannot be extracted from tissue macromolecules (Vroomen *et al.* 1986, 1987a). Both these type of metabolites and the cyano-metabolite could be detected after incubation of rat and pig liver microsomes with the drug (Vroomen *et al.* 1987b, 1987c). However, their formation was almost completely reduced in the presence of physiological concentrations of reduced glutathione (2-10 mM), due to the formation of a glutathione-conjugate (Vroomen *et al.* 1988). Subsequent studies revealed that the furazolidone derived part, the acrylonitrile-derivative, of this and other thiol-conjugates can be released and bind again to other thiol-group containing compounds, including protein, and possibly DNA (Fig. 7.1). Subsequently, it has been suggested that such reactive thiol-conjugates might be responsible for the formation of unextractable furazolidone-derived metabolites in tissues of rats, fed with muscle tissue from a piglet that had been treated with the drug (Vroomen *et al.* 1990). Regarding the potential health risk for the consumer of edible products containing these type of metabolites, it is important to obtain more information on their possible formation and presence in tissues of treated animals, especially considering the relative long half-life of bound metabolites (Vroomen *et al.* 1986).

In previous chapters we described the isolation of hepatocytes from a sample of pig liver, and their use for studying the biotransformation and toxicity of veterinary drugs. Since hepatocytes are in general considered to be a more complete *in vitro* model than liver microsomes, this model was used to investigate the possible formation of thiol conjugates of furazolidone. Incubation of rat hepatocytes with the nitrofurans nitrofurantoin, nitrofurazone and nifurtimox has been shown to result in the formation of the radical nitro anion (Rao *et al.* 1988), as observed in studies with microsomes (Peterson *et al.* 1979, 1982, Docampo *et al.* 1981). This radical nitro anion may be further reduced, resulting in the formation of other reactive intermediates, or be oxidized by oxygen, resulting in the formation of reactive oxygen species (Fig. 7.1). In intact cells, GSH may be involved in the scavenging of reactive intermediates of drugs, but also in the detoxification of H_2O_2 (Orrenius *et al.* 1983, Meister & Anderson 1983). The GSSG produced in the latter process is normally reduced by GSSG-reductase (EC 1.6.4.2), but can also be excreted by the cell. Such an increased excretion of GSSG and decrease in tissue GSH-levels has been reported for isolated perfused rat livers and rat hepatocytes exposed to nitrofurantoin (Akerboom *et al.* 1982, Hoener *et al.* 1989, Rossi *et al.* 1988), and nitrofurazone (Hoener 1988), and after treatment of rats with nifurtimox (Dubin *et al.* 1983). Incubation of pig hepatocytes with nitrofurazone,

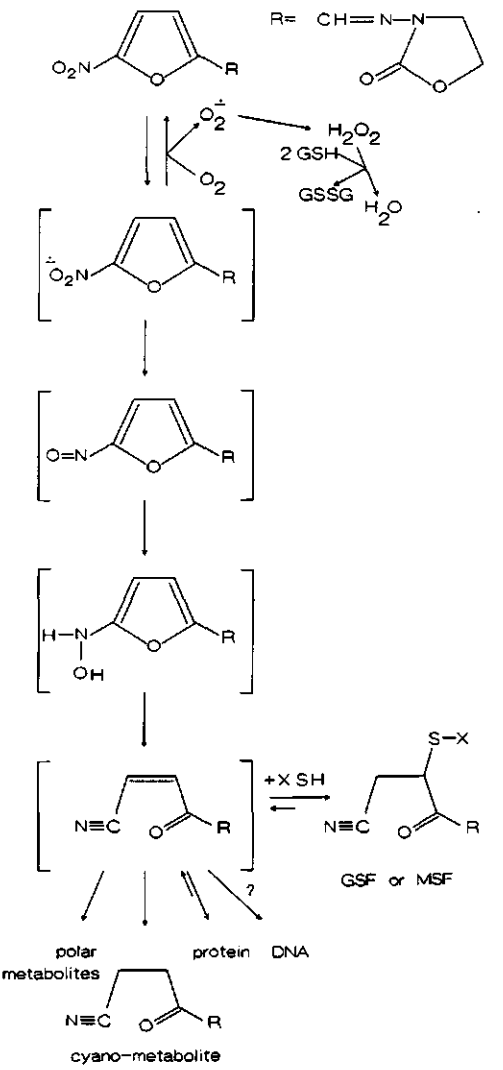

Fig. 7.1. Proposed metabolic pathway of furazolidone by pig liver microsomes and the role of glutathione (GSH) in the detoxification of both reactive drug intermediates and oxygen radicals.

furaltadone, furazolidone and nitrofurantoin also resulted in increased intracellular GSSG, but only in the latter case this was accompanied by decreased GSH-levels (Hoogenboom *et al.* 1991). It was hypothesized that the absence of a clear effect of furazolidone on intracellular GSH-levels, might be due to an increased synthesis of GSH by the cells. In addition to more direct studies on the formation of thiol-conjugates of furazolidone, this possibility was investigated, as well as the possible protective role of GSH against the toxic effects of the drug, since indirect evidence for the formation and role of thiol-conjugates might be obtained from such studies.

Materials and Methods

Chemicals

Collagenase type H, GSH, GSSG and NADPH were purchased from Boehringer (Mannheim, FRG), fetal calf serum, penicillin/streptomycin and Williams' medium E from Flow Labs (Rickmansworth, UK), (1,3-bis(2- chloroethyl))-1-nitrosurea) (BCNU) from Bristol-Meyers (Toronto, Canada), insulin and buthionine-D,L-sulfoximine (BSO) from Sigma (St. Louis, USA), and ^{35}S-methionine (spec act 8 GBq/mmol) from Amersham (Amersham, UK). ^{14}C-Furazolidone, labelled in the nitrofuran-ring (spec act 1.75 GBq/mmol) (Amersham, Amersham, U.K.) and furazolidone were gifts from Orphahell (Mijdrecht, The Netherlands).

Hepatocyte isolation and culturing

Pig hepatocytes were isolated from livers of 6 to 12 months old sows (Dutch Landrace x Yorkshire) as described in Chapter 2. Cell suspensions were diluted to a density of 1.25 to 1.5 million viable cells/ml of medium and seeded into 60 mm tissue culture dishes (2.5 ml/dish). Unless indicated, Williams' medium E supplemented with 5% fetal calf serum, 0.5 μg/ml insulin, 50 IU/ml penicillin and 50 μg/ml streptomycin was used for culturing the cells.

Exposure of cells

In most experiments, treatment with furazolidone was started with 28 h old monolayer cultures. In some studies, cells were pretreated for 24 h with BSO (4 mM) or ^{35}S-methionine (160 kBq/ml), starting 4 h after the plating of the cells. Following preincubation, cells were rinsed twice with Williams' medium E and incubated in this medium for 1 h prior to drug treatment. Incubation of cells with furazolidone was started by replacement of the old medium with plain Williams' E containing the indicated drug concentrations. Unless indicated, this was done by the addition of DMSO-stock solutions of the drug (final DMSO-concentration in the medium 1%). In some experiments 40 μM BCNU (from a DMSO-stock solution) or 4 mM BSO (from a stock solution in Williams' E) were also dissolved in the medium.

Following exposure, media samples were collected and the cells were rinsed twice with PBS and scraped from the dishes in 1 ml of PBS using a rubber policeman. Cells were either used directly or stored in liquid nitrogen until analysis.

Assays

Protein-levels were measured by the method of Lowry as modified by Peterson (1977), and LDH-activity in medium and cells as described by Jauregui *et al.* (1981). GSH-levels were determined based on the method by Reed *et al.* (1980), with slight modifications in the HPLC-procedure as described in Chapter 3. In experiments with

^{35}S-labelled GSH, fractions of 0.25 ml were collected, mixed with scintillation cocktail (Minisolve, Zinsser Analytical, Maidenhead, UK), and counted in a Philips liquid scintillation counter (type PW 4700). Pyruvate and lactate concentrations in the medium were determined enzymatically as described in Chapter 5.

Biotransformation studies

The ^{14}C labelled open-chain cyano metabolite of furazolidone was made by incubation of the Ames' tester strain *Salmonella typhimurium* TA 100 with 50 μM ^{14}C-furazolidone for 1 h, and purified by extraction and HPLC as described by Vroomen *et al.* (1987a). The mercaptoethanol (MSF) and glutathione (GSF) conjugates of furazolidone were purified from a microsomal incubation (Vroomen *et al.* 1987b, 1988), and stored in a formic-acid/NaOH buffer (5 mM; pH 3.6) at -40 °C. In this way the conjugates were shown to be stable for at least 6 months.

For studying the degradation of furazolidone, the cyano-metabolite and thiol conjugates (MSF, GSF), compounds were dissolved in Williams' E without the use of DMSO. However, in control experiments no effect of this solvent on the metabolite pattern of furazolidone could be observed. At the indicated time intervals, media samples of 190 μl were taken, instantaneously mixed with 10 μl 1% formic acid and injected on an HPLC system equiped with a PRP-1 column (250 x 4.6 mm I.D. (Hamilton, Reno, USA) and coupled to an on line radioactivity detector (LB506C; Berthold, Wildbad, FRG). Compounds were eluted using a linear 22 min gradient of formic acid/NaOH (0.1 M; pH 3.6): water: acetonitrile 50/925/25 (v/v) and formic acid/NaOH (0.1 M; pH 3.6): water: acetonitrile 50/650/300 at a flow rate of 0.8 ml/min. For exchange studies, samples of the medium were mixed with mercaptoethanol to a final concentration of 10 mM, incubated at room temperature for 45 min, mixed with 1% formic acid and analyzed. For confirmation of the identity of the cyano-metabolite, a sample of the medium was extracted with ethyl-acetate. After evaporation of the organic solvent under nitrogen, the residue was dissolved in water/acetonitrile 7/3 (v/v) and injected on an HPLC system, equiped with an Hypersil 5 ODS column (250 x 4.6 mm I.D.; Chrompack, Middelburg, The Netherlands) and coupled to UV/VIS diode array equipment (Hewlett-Packard 1040 A) (eluens water/acetonitril 8/2). The identity of the metabolite was also confirmed by ^{1}H-NMR as described previously (Vroomen *et al.* 1987a).

For determination of the binding of ^{14}C-labelled metabolites of furazolidone to TCA-precipitable material, cells were washed with PBS (2x), scraped from the dishes in 1 ml PBS, and mixed with TCA to a final concentration of 0.7%. The precipitate was collected by centrifugation, washed with ice-cold methanol (3x) and ethanol (2x), dissolved in 5% sodium-dodecyl-sulphate and 0.4 N NaOH, mixed with scintillation cocktail and counted.

For examination of the presence of exchangeable protein-bound metabolites, cells were incubated with 50 μM ^{14}C-furazolidone (1,75 GBq/mmol) for 6 or 24 h, and the

protein collected and washed as described above. However, after the third clean-up with methanol, the protein was resuspended by ultrasonic treatment in 1.5 ml phosphate buffer (0.1 mM, pH 7.4) containing 10 mM mercaptoethanol. After an incubation interval of 1 h at room temperature, the mixture was extracted with EtAc (3x 2 ml). The organic phase was taken to dryness, and the residue dissolved in eluent and investigated by HPLC for the presence of the mercaptoethanol-conjugate (Vroomen *et al.* 1987b). The protein was precipitated with TCA and washed twice with methanol. The washstep and the protein fraction were counted as described above. The method was checked with microsomal protein, that had been incubated with MSF as described previously (Vroomen *et al.* 1988) and gave consistent results.

Salmonella/microsome test

The glutathione and mercaptoethanol-conjugate, made by microsomal incubations as described above, were tested for their genotoxic properties in the *salmonella* mutagenicity test (Maron & Ames 1983), as described previously (Vroomen *et al.* 1988). MSF and GSF were synthesized, purified and tested on the same day. Compounds were preincubated for 1 h at 37 °C with tester strain TA100 in the absence of S9-mix, in concentrations up to 5 μg per plate. Furazolidone was used as a positive control.

Results

The hypothesis that the previously observed lack of effect of furazolidone on intracellular GSH-levels (Chapter 5) could be due to an increased GSH-synthesis and/or an effective reduction of GSSG to GSH by the cells, was investigated by incubating hepatocytes with furazolidone either in the presence of BSO, an inhibitor of the GSH-synthesis (Griffith & Meister 1979), or BCNU, an inhibitor of GSSG-reductase (Babson & Reed 1978). Table 7.1 shows the results of two experiments with cells isolated from livers of different pigs. As observed before (Chapters 3 and 5), intracellular GSH-levels increased during the exposure interval of 20 h, from about 30 nmol/mg protein till about 60 nmol/mg protein. This increase was apparently not influenced by furazolidone, despite the presence of elevated intracellular GSSG-levels at the highest dose-level. The increase in GSH-concentrations during ageing could effectively be inhibited by incubating the cells with 4 mM BSO. Incubation of cells with furazolidone under these conditions, resulted in a dose-related decrease in intracellular GSH-levels. Exposure of cells to BCNU (40 μM) did not affect absolute GSH-levels. Simultaneous treatment of cells with BCNU and furazolidone resulted in elevated GSH-levels at the concentration of 50 μM, whereas a decrease was observed at 500 μM (Table 7.1).

Table 7.1 Effect of furazolidone on intracellular GSH-levels, either in the absence or presence of BSO or BCNU.
Monolayer cultures of pig hepatocytes, isolated from livers of two different pigs, were incubated with furazolidone for 20 h, starting 28 h after their isolation, either in the absence or presence of BSO (4 mM), or BCNU (40 μM). Results are expressed as the mean ± SEM (n=3).

Inhibitor	[Fur] (μM)	experiment 1 GSH	experiment 2 GSH	experiment 2 GSSG
		(nmol/mg protein)		
t=28h		29 ± 3	35 ± 1	0.4 ± 0.1
none	0	57 ± 5	53 ± 2	1.0 ± 0.1
	50	62 ± 6	62 ± 1	1.3 ± 0.0
	500	61 ± 0	54 ± 2	3.7 ± 0.5
BSO	0	25 ± 1	29 ± 1	0.6 ± 0.1
	50	19 ± 1	23 ± 1	0.5 ± 0.0
	500	6 ± 1	6 ± 1	0.1 ± 0.0
BCNU	0	71 ± 5	55 ± 2	1.1 ± 0.1
	50	111 ± 1	94 ± 2	2.0 ± 0.0
	500	30 ± 3	41 ± 3	7.0 ± 1.3

In order to exclude possible artefacts related to the use of inhibitors, like BSO and BCNU, the effect of furazolidone on GSH-levels was studied in hepatocytes that had been preincubated in the presence of ^{35}S-methionine for 24 h, resulting in the labelling of the intracellular GSH. As shown in Fig. 7.2A, exposure to furazolidone resulted in a clear and dose-related loss of labelled GSH from the cells, despite the absence of an effect on absolute GSH-concentrations. Investigation of media samples collected at the end of the incubation interval, revealed that the label was excreted into the medium and could be recovered in four peaks, one of them coeluting with GSSG (see insert Fig. 7.2B). A second peak eluted just before the GSH-peak and might be CySSG, the disulfide of cysteine and GSH (Reed *et al.* 1980, Fariss *et al.* 1983). As shown in Fig. 2B there was a dose-related increase in the levels of these two labelled compounds in the medium, which almost matched the loss of ^{35}S-GSH by the cells. The increased levels of these compounds in the medium could also be observed in the HPLC-chromatograms obtained by UV-detection (data not shown).

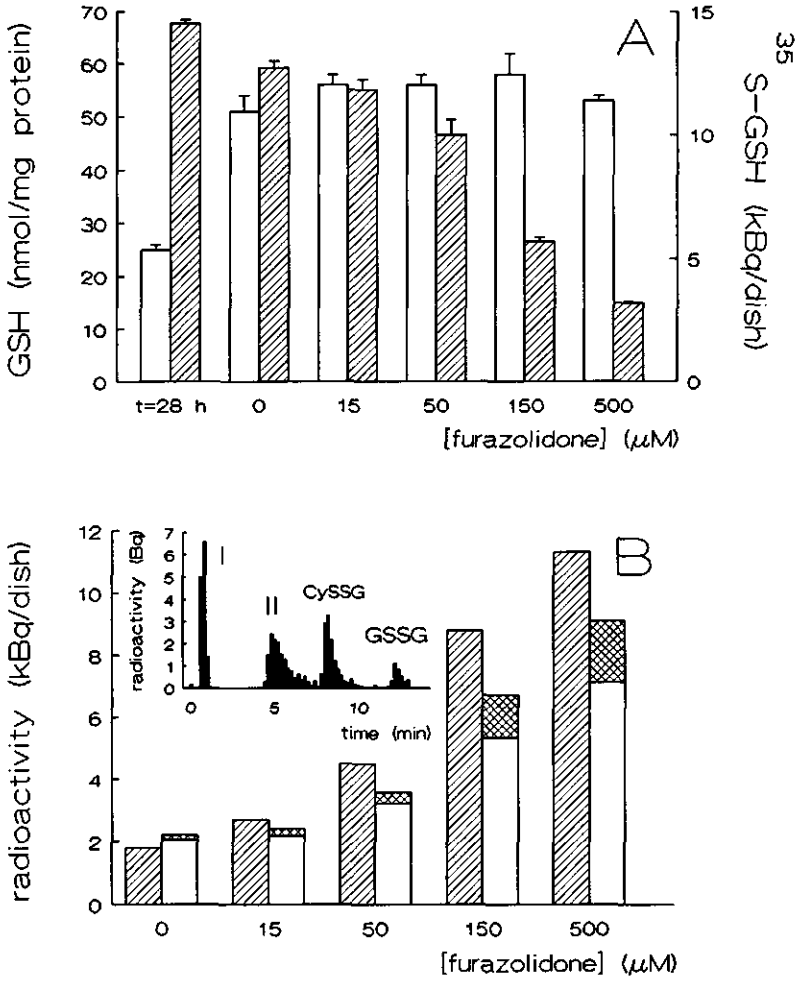

Fig. 7.2. The effect of furazolidone on levels and turn-over of intra-cellular GSH (A), and its excretion into the medium (B).
Cells, containing ^{35}S-GSH, were exposed to the drug for 20 h. Before (t=28 h) and after exposure, levels of intracellular GSH (□) and ^{35}S-GSH (▨) were measured (A) (mean ± SEM; n=3). Medium samples were pooled and investigated by HPLC (as shown for 500 μM in insert; RT GSH 10.1 min). The amount of label recovered in the GSSG (▩) and "CySSG" (□) peaks were compared with the amount of label lost by the cells as GSH (▨).

The possible formation of thiol-conjugates of furazolidone was investigated by incubating monolayer cultures with 50 μM ^{14}C-labelled drug (Fig. 7.3). HPLC-analysis of media samples taken after 7 (A), 26 (B) and 50 (C) h revealed that respectively 73, 97 and 100% of the drug was transformed, resulting in the formation of a large number of more hydrophilic compounds. The open-chain cyano metabolite (retention time 20 min) could be identified by its UV and NMR spectrum (data not shown), but was only present in low amounts. Incubation of cells with purified cyano-metabolite (5 μM), revealed however that about 72 and 97% of this compound was transformed into more polar metabolites after respectively 12 and 36 h (Fig. 7.3D). The detection of GSF in these media samples was hampered by the presence of a number of compounds with similar retention time (14.7 min; see Fig. 7.4A). Therefore, samples taken after 10 and 28 h (Fig. 7.3E) were incubated with 10 mM MSH for 45 min, allowing the possible exchange from GSF into MSF, which could be detected more easily. This indirect approach did not result in the formation of detectable amounts of MSF. The effectiveness of this approach was shown by the fact that MSF could easily be detected when a similar medium sample was enriched with GSF to a final concentration of 0.5 μM, prior to the incubation with 10 mM MSH (Fig. 3F).

Subsequent experiments revealed that GSF, purified from microsomal incubations, was highly unstable when added to monolayer cultures, initially leading to the formation of two compounds eluting after about 25 min (Fig. 7.4A) and eventually more polar metabolites as well as the cyano-metabolite (Fig. 7.4B), as confirmed by its chromatographic behaviour and UV-spectrum (data not shown). Similar results were obtained with the mercaptoethanol conjugate of furazolidone (MSF) (Fig. 7.4C). In the absence of cells, GSF (Fig. 7.4D) and MSF (data not shown) also disappeared rapidly, but in this case no cyano-metabolite could be detected. The cyano-metabolite was formed when GSF was preincubated for 15 min in medium, which was subsequently added to the cells, indicating that this metabolite is formed from a degradation product of GSF. Polar metabolites but not the cyano-metabolite were formed when cells were incubated with GSF in medium enriched with 1 mM GSH (data not shown).

In order to obtain further information on the possible interaction between GSH and furazolidone, the biotransformation and toxicity of the drug in hepatocyte cultures containing different levels of intracellular GSH was investigated. Cells were incubated with 0, 50 or 500 μM ^{14}C-labelled furazolidone for 3 (Table 7.2) or 16 h (Table 7.3), after or during treatment with BSO. Pretreatment of cells with BSO, resulting in decreased GSH-levels, led to a marked increase in the LDH-leakage at the highest drug level, as compared to cultures that were not pretreated (Tables 7.2 and 7.3). Simultaneous treatment with BSO and furazolidone for 3 h had no effect on LDH-leakage, despite a slight decrease in GSH-levels (Table 7.2). The incubation of cells with furazolidone resulted in the formation of protein-bound metabolites (Table 7.2), but the extent of binding decreased, rather than increased, in cultures with low GSH-levels. Similarly, BSO-pretreatment did not result in an increased accumulation

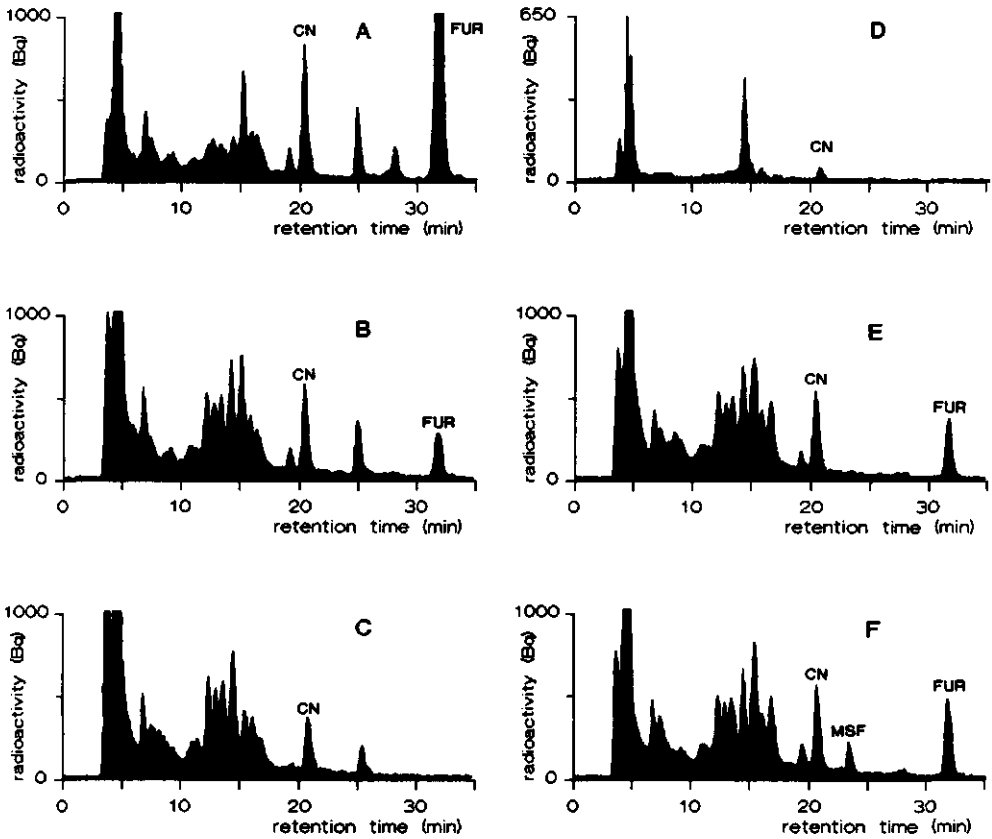

Fig. 7.3. Biotransformation of furazolidone (FUR) and its cyano-metabolite (CN) by monolayer cultures of pig hepatocytes.
Cells were incubated in the presence of 50 μM ^{14}C-labelled furazolidone for 7 (A), 26 (B) or 50 (C) h, or 5μM of the cyano-metabolite for 36 h (D). The excretion of GSF by cells incubated with furazolidone was examined by incubating a medium sample taken at t=26 h with 10 mM MSH for 1 h prior to HPLC-analysis (E). As a control a similar sample was spiked with 0.5 μM GSF prior to the incubation with 10 mM MSH (F).

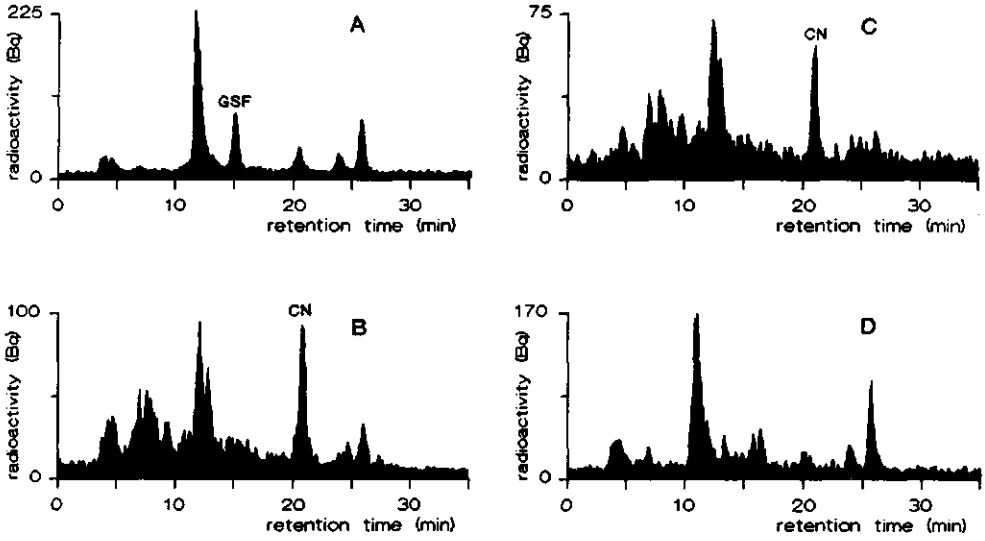

Fig 7.4 Fate of GSF (A,B,D) and MSF (C) incubated in the presence or absence of cells. GSF or MSF was added to the medium to a final concentration of 5 μM in the presence (A,B,C) or absence (D) of cells. Samples were taken after 5 (A) or 60 (B,C,D) min and analyzed by HPLC.

of pyruvate and lactate after exposure of cells to 50 μM furazolidone for 16 h, and actually resulted in a decrease at the highest dose level (Table 7.3). BSO-pretreatment had no effect on the amount of drug transformed, nor did it result in the increased formation of the cyano-metabolite. In addition, no effect on metabolite patterns of furazolidone, nor on the biotransformation of the cyano-metabolite could be observed (HPLC-chromatograms not shown).

Table 7.2 Influence of intracellular GSH-levels on LDH-release caused by furazolidone and on the level of binding of reactive intermediates of the drug to cellular protein.
Cells were incubated with various doses of furazolidone for 3 h. BSO (4 mM) was added to the medium either before (24 h), or during the treatment with furazolidone. Results presented as mean ± SEM for n=3.

Treatment with BSO	[Fur] (μM)	GSH (nmol/mg protein)	LDH-leakage (% of total)	Covalent Binding (nmol/mg protein)
none	0	34 ± 2	1.8 ± 0.1	
	50	34 ± 2	1.9 ± 0.1	0.12 ± 0.01
	500	27 ± 1	2.3 ± 0.1	0.35 ± 0.02
before	0	4 ± 1	5.3 ± 0.3	
	50	3 ± 0	6.0 ± 0.6	0.12 ± 0.01
	500	1 ± 0	63.7 ± 1.6	0.25 ± 0.01
during	0	25 ± 2	1.7 ± 0.2	
	50	27 ± 1	1.6 ± 0.1	0.13 ± 0.01
	500	16 ± 1	2.5 ± 0.2	0.34 ± 0.02

[1] GSH-levels at t=0 were 28 ± 1 and 5 ± 1 nmol/mg protein for cells preincubated without and with BSO respectively.

The possible presence of reactive protein-bound metabolites in cells exposed to furazolidone was investigated by incubating the washed protein in the presence of 10 mM mercaptoethanol for 1 h. Although part of the label could be released and extracted with EtAc, no MSF could be detected in these extracts (detection limit 0.25%) (Table 7.4). With the same method, the acrylonitrile derivative could be released from microsomal protein, that had been incubated with MSF, as described previously by Vroomen *et al.* (1988).

Both GSF and MSF, purified from microsomal incubations were tested twice in the *salmonella* mutagenicity test in doses up till 5 μg/plate, but their mutagenicity could not be confirmed. In all four tests, furazolidone gave a clear positive result at a dose of 10 μg/plate, similar to those reported previously (Vroomen *et al.* 1988). In all cases the reversibility of the thiol-bond was confirmed by incubation of MSF or GSF with GSH or MSH respectively.

Table 7.3. Influence of intracellular GSH-levels on the LDH-release and the accumulation of pyruvate and lactate in the medium following incubation of cells with furazolidone, as well as on the biotransformation of the drug and the formation of the cyano-metabolite.
Cells were incubated with various doses of furazolidone for 16 h. Pretreatment of cells with BSO (4 mM) for 24 h was either stopped 1 h before or continued during the treatment with furazolidone. Results presented the mean ± SEM for n=3.

Treatment with BSO	[Fur] (μM)	GSH (nmol/ mg protein)	LDH-leakage (% of total)	Pyruvate+ Lactate (mM)	Furazolidone metabolized (%)	[Cyano] (μM)
none	0	66 ± 6	1.6 ± 0.1	0.8 ± 0.1		
	50	68 ± 8	1.5 ± 0.1	2.8 ± 0.1	51 ± 2	1.8 ± 0.1
	500	72 ± 4	1.7 ± 0.1	3.9 ± 0.1	7 ± 1	2.0 ± 0.1
before	0	15 ± 2	1.0 ± 0.1	0.7 ± 0.1		
	50	7 ± 1	1.2 ± 0.1	3.0 ± 0.1	52 ± 2	1.3 ± 0.1
	500	4 ± 0	43.8 ± 6.8	2.4 ± 0.2	6 ± 1	1.8 ± 0.1
before	0	10 ± 2	1.9 ± 0.2	0.4 ± 0.0		
and during	50	5 ± 1	2.0 ± 0.2	3.0 ± 0.1	52 ± 4	1.8 ± 0.2
	500	ND[2]	86.5 ± 3.2	2.4 ± 0.1	3 ± 2	3.1 ± 0.7

[1] GSH-levels at t=0 were 53 ± 2 and 17 ± 2 nmol/mg protein for cells preincubated without or with BSO respectively.
[2] not detected

Table 7.4. Exchange of protein-bound metabolites of furazolidone with mercaptoethanol. Cells were incubated with 50 μM furazolidone for 6 or 24 h.
Cellular protein was washed with methanol and incubated with MSH (10 mM) at pH 7.4 for 1 h. Following extraction with EtAc, the protein was precipitated and washed twice with methanol. The EtAc-extract was analyzed by HPLC and the fraction coeluting with MSF was collected ("MSF").

Incubation time (h)	Radioactivity in fractions (% of total) protein	methanol	EtAc	"MSF"
6	91.6	6.3	2.2	ND
	87.2	9.8	3.0	ND
24	93.3	5.6	1.0	ND
	93.0	6.0	1.0	ND

[1] ND: not detected. Detection limit 0.25%
[2] 100% matches 3.9 and 3.8, and 3.5 and 3.8 nmol at 6 and 24 h respectively.

Discussion

This study shows that pig hepatocytes are capable of biotransforming furazolidone into the open chain cyano-metabolite, a large number of unknown more hydrophilic metabolites (Fig. 7.3), and protein-bound metabolites (Table 7.2). The transformation of the cyano-metabolite into more hydrophilic metabolites, shows that it is not an end-metabolite, as concluded from studies with cellular fractions (Abraham *et al.* 1984, Tatsumi *et al.* 1981, Vroomen *et al.* 1987b, 1987c). The relatively low levels of this intermediate in the plasma of pigs treated with furazolidone (Vroomen *et al.* 1987a) may therefore as well be due to its further degradation rather than to its low formation.

As hypothesized before (Chapter 5), the exposure of pig hepatocytes to furazolidone results in a loss of intracellular GSH, which is however compensated by an increased synthesis of GSH. This is demonstrated both by the dose-related decrease in GSH-levels in the presence of BSO (Table 7.1), as well as the dose-related loss of ^{35}S-labelled GSH in the absence of an effect on absolute GSH-levels (Fig. 7.2A). The increased synthesis of GSH may underly the almost doubled GSH-levels observed in cells after combined treatment with 50 μM furazolidone and 40 μM BCNU (Table 7.1), and after treatment with nitrovin (Chapter 5). The active synthesis of GSH by hepatocytes is well documented (Meredith & Reed 1982, Orrenius *et al.* 1983), and was previously shown in ageing monolayer cultures of pig hepatocytes (Chapter 3). However, an increased synthesis of GSH by cells in response to the effect of xenobiotica has only been described after exposure of human fibroblasts to diethyl-maleate (Bannai 1984). Regarding the sometimes doubled GSH-levels, it appears that the effect cannot be explained by feed-back regulation of the synthesis of GSH, but may also be due to an additional increase in the uptake of one of the precursors of GSH-synthesis, as shown in the case of fibroblasts (Bannai 1984).

At least part of the increased loss of GSH can be ascribed to the formation and excretion of GSSG, formed in the detoxification of reactive oxygen species (Fig. 7.1). First of all, increased GSSG-levels were measured in cells exposed to high concentrations of the drug (Table 7.1). Secondly, about 40% of the ^{35}S-labelled GSH lost by the cells, was recovered in the medium as GSSG (Fig. 7.2B), considering the fact that only 50% of the GSSG is derivatized with the method of Reed *et al.* (1980). Thirdly, when the adequate reduction of GSSG was further impaired by simultaneous incubation of the cells with BCNU, GSH-levels could no longer be completely restored at the highest furazolidone level. Finally, the fact that GSH primarily protected the cells against membrane damage at high concentrations of furazolidone (Tables 7.2 and 7.3) supports the role of GSH in the detoxification of reactive oxygen species (Fig. 7.1).

From the results presented in Fig. 7.2B, it appears that the remaining part of the GSH-loss is balanced by increased levels of CySSG in the medium, which, according to Farris and Reed (1983), can be ascribed to the excretion of GSH by the cells and its subsequent exchange with the cystine in the medium. A similar increased loss of GSH

has been reported after treatment of rats with nifurtimox (Dubin *et al.* 1983), and to a lesser extent after exposure of perfused rat livers to nitrofurantoin (Hoener *et al.* 1989) and nitrofurazone (Hoener 1988). The mechanism behind this effect remains unclear, since it is apparently not due to an increased membrane-leakage, as shown by the results presented by Dubin *et al.* (1983), and those in Tables 7.2 and 7.3.

The failure to detect a glutathione conjugate of furazolidone in samples of the incubation medium (Fig. 7.3), might be related to its unstability under these conditions (Fig. 7.4). Nevertheless, the excretion of such a conjugate by the cells seems unlikely since an almost tenfold reduction in intracellular GSH did not result in increased levels of protein bound metabolites (Table 7.2), as in the case of microsomal incubations (Vroomen *et al.* 1987b, 1987c). The formation of GSF cannot entirely be excluded by this observation, since the conjugate may act as a carrier of reactive metabolites within the cell, exchanging its furazolidone-derived part with cellular proteins (Vroomen *et al.* 1988). However, this would have to result in the formation of stable protein-adducts, since the furazolidone-derived part of protein bound metabolites was no longer exchangeable with mercaptoethanol (Table 7.4). The fact that, contrary to studies with microsomes (Vroomen *et al.* 1987b, 1987c), decreased GSH-levels did not result in the increased formation of the cyano-metabolite, might be explained by the formation of this metabolite from a compound, formed by the spontaneous degradation of the thiol-conjugates in the medium (Fig. 7.4). However, since GSF is apparently not excreted by the cells (see above), the indirect formation of the cyano-metabolite from GSF should occur within the cell, which seems unlikely considering the high intracellular GSH-levels (5-10 mM), and the fact that low levels of GSH (1 mM) in the medium prevented the formation of the cyano-metabolite from GSF or MSF completely. In summary, the excretion but also the formation of a glutathione-conjugate of furazolidone by the cells appears to be highly questionable.

The fact that GSH protects the cells primarily against effects related to oxidative stress, like membrane-damage, supports the conclusions from previous studies with nitrofurantoin (Boyd 1980, Rossi *et al.* 1988). However, from the present study it is also evident that even in GSH-depleted cells (Table 7.2), high concentrations of furazolidone are required for these type of effects. Therefore, disturbance of cellular functions, possibly due to the binding of reactive intermediates of the drug to cellular proteins, appear to be of more relevance under these conditions, especially since they may accumulate upon repeated exposure. An example of such an effect is the inhibition of the pyruvate metabolism by relatively low levels of furazolidone (Chapter 5), an effect that appears to be due to the binding of a metabolite of furazolidone to the pyruvate dehydrogenase complex (Paul *et al.* 1953, Paul & Paul 1964).

In summary, the present study clearly demonstrates that exposure of cells to furazolidone results in an increased loss and subsequent synthesis of cellular GSH. At least part of this loss could be ascribed to the increased formation of GSSG and possibly GSH, but no evidence could be obtained for the formation of a glutathione

conjugate of furazolidone. The presence of exchangeable thiol conjugates in the protein fraction could not be demonstrated, nor could the genotoxic potential of these type of compounds (Vroomen *et al.* 1988) be confirmed. However, given the relatively high bioavailability of bound residues of furazolidone (Vroomen *et al.* 1990) and the mutagenic and carcinogenic potential of the parent compound (McCalla 1983, Food and Drug Administration 1976), further investigations on the toxic potency of bound residues are required.

In addition this study demonstrates that pig hepatocytes are a very suitable model for this type of mechanistic studies, especially regarding the great opportunities to integrate biotransformation and toxicity studies.

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CHAPTER 8

THE USE OF PIG HEPATOCYTES TO STUDY THE NATURE OF PROTEIN-BOUND METABOLITES OF FURAZOLIDONE

A new analytical method for their detection

This chapter is based on the following publication:

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THE USE OF PIG HEPATOCYTES TO STUDY THE NATURE OF PROTEIN-BOUND METABOLITES OF FURAZOLIDONE

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Summary

The biotransformation of furazolidone, both *in vivo* and *in vitro*, results in the formation of so-called bound metabolites. Following the incubation of pig hepatocytes with ^{14}C-furazolidone, a dose and time related increase in the formation of bound metabolites was observed. After withdrawal of the drug from the medium, levels decreased gradually to about 50% within 36 h. Using a newly developed method, it was shown that in the case of cells and liver microsomes at least 70% of the bound residues still contains the 3-amino-2-oxazolidinone (AOZ) side chain of furazolidone. With liver samples of piglets that had been treated orally with the drug for 10 days, followed by withdrawal periods of 2 h or 10 days, fractions of releasable AOZ were respectively 23 and 15%, equivalent to levels of respectively 3.83 and 0.26 μg furazolidone per gram of tissue.

The release of the AOZ side-chain of furazolidone from at least part of the bound metabolites, finally proves their drug-related nature and subscribes the requirement for both adequate withdrawal periods and useful control methods. The relative simplicity of the newly developed method makes it an ideal tool for this purpose.

Abbreviations: AOZ= 3-amino-2-oxazolidinone; NPAOZ= 3[[(2-nitrophenyl)methylene]-amino]-2-oxazolidinone; EtAc= ethyl-acetate; NBA= 2-nitrobenzaldehyde

Introduction

The biotransformation of veterinary drugs by the animal may lead to the formation of so-called bound metabolites, next to more readily excreted metabolites (Baer *et al.* 1977; Rico and Burgat-Sacaze 1984; Lu *et al.* 1988). This has been demonstrated in studies with radioactive labelled compounds, where part of the label could not be extracted from the tissues, and was therefore assumed to be associated with tissue macromolecules such as DNA or proteins. As a result the rate of clearance of these type of metabolites from the body may depend on the turnover of tissue proteins and be relatively low. Long withdrawal periods will be required in the absence of information on the identity and properties of bound residues, especially in the case of parent compounds with genotoxic and carcinogenic properties. However, in most cases the control on their maintenance will be difficult, due to the absence of adequate analytical methods.

Aim of the present study was to further investigate the nature of protein-bound metabolites of the antibacterial drug furazolidone. Vroomen *et al.* (1986) showed that, following the oral treatment of piglets with ^{14}C-labelled furazolidone, the parent compound itself had a short plasma half-life (1 h) and could not be detected in tissues, even when an animal was killed within two hours after the last treatment. However, radiolabelled compounds with unknown identity were present at high concentrations in all tissues and could only partly be extracted with water and organic solvents. During a withdrawal period of 14 days, levels of both total and bound radiolabelled compounds decreased, but could still be detected in all tissues at μg per gram levels.

Using pig liver microsomes, strong evidence was obtained that the binding of metabolites to protein was due to the formation of a reactive site in the nitro-furan-part as a result of the reduction of the nitro-group (Vroomen *et al.* 1987), leaving the 3-amino-2-oxazolidinone

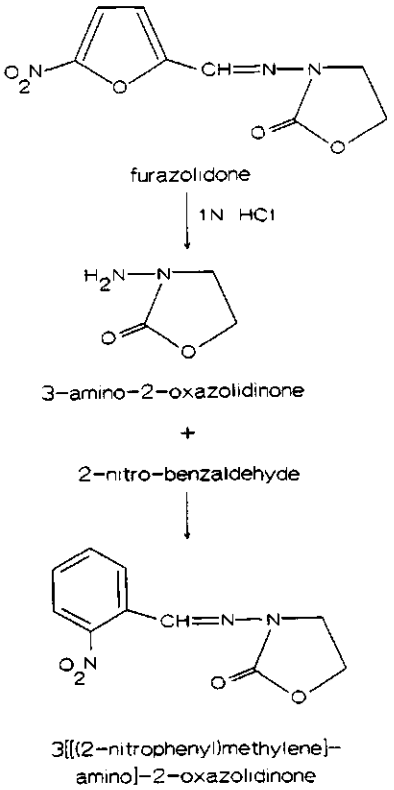

Fig. 8.1. Mechanism behind the proposed method for the release and detection of 3-amino-2-oxazolidinone (AOZ) released from furazolidone and metabolites.

(AOZ) side-chain unaltered (see Fig. 7.1). Therefore, we developed a new method for the release of the AOZ side-chain under mild acid conditions and its subsequent detection after its derivatization with 2-nitrobenzaldehyde to 3[[(2-nitrophenyl)-methylene]-amino]-2-oxazolidinone (NPAOZ) (Fig. 8.1). For studying the formation and identity of bound metabolites, the use of pig hepatocytes was preferred above microsomes, since this *in vitro* model has been shown to be more suitable for studying the biotransformation and toxicity of furazolidone (Chapter 7).

Materials and methods

Materials

2-Nitrobenzaldehyde was purchased from Aldrich Chemicals (Milwaukee, USA), Collagenase type I and insulin from Sigma (St. Louis, USA), NADPH and porcine pepsin (2500 U/mg) from Boehringer (Mannheim, FRG), Williams' medium E, fetal calf serum and penicillin/streptomycin from Flow Labs. (Rickmansworth, UK). 3-Amino-2-oxazolidinone, furazolidone and [^{14}C]furazolidone labelled in the nitrofuran ring (1.75 GBq/mmol) were gifts from Orphahell BV (Mijdrecht, The Netherlands). [^{14}C]Furazolidone labelled in the oxazolidinone part (27 MBq/mmol) was a gift from Norwich Eaton Pharmaceuticals Inc. (Norwich, New York, USA). All other chemicals used were of reagent grade.

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

For the synthesis of NPAOZ, 10.2 mg of 3-amino-2-oxazolidinone (AOZ) was incubated with 15.2 mg of 2-nitro-benzaldehyde (NBA) in 40 ml 0.1 N HCl for 1 h at room-temperature. The reaction product was extracted with 40 ml of EtAc, taken to dryness under nitrogen and redissolved in 4 ml acetonitrile/water 1/1. The product was further purified on a semi-preparative Lichrosorb RP-18 column (250 x 10 mm I.D.; Hibar), eluted with acetonitrile/water 4/6 (v/v) at a flow-rate of 2.5 ml/min. The collected fractions were taken to dryness, redissolved in 10 ml water and extracted with 10 ml EtAc, which was subsequently evaporated. The identity of the product was confirmed by ^{1}H-NMR-analysis, dissolved in $CDCl_3$, using a Bruker CXP-300. Signals and interpretation: 8.26 (dd), 8.05 (dd), 7.66 (dt) and 7.53 (dt) ppm: protons at the phenyl-ring; 8.21 (s) ppm: proton at the azomethine-bond; 4.59 (dd) and 3.98 (dd) ppm: protons in the oxazolidinone-ring.

Hepatocyte isolation and culturing

Hepatocytes were isolated from liver samples of 5-9 month old sows (Dutch Landrace x Yorkshire) as described in Chapter 2. Cells were plated in 60 mm culture

dishes at a density of 1.25 million per ml (2.5 ml/dish) using Williams' medium E supplemented with 5% fetal calf serum, 0.5 μg/ml insulin, 50 IU/ml penicillin and 50 μg/ml streptomycin. After 4 h the medium was renewed and routinely cells were incubated for another 16-24 h before exposure to furazolidone.

Exposure of cells

Exposure of cells was started by replacement of the medium with Williams' E containing 25 or 50 μM furazolidone. An adequate amount of a stock solution of the ^{14}C-labelled drug in acetone was taken to dryness under nitrogen and the drug was redissolved in the medium, that was subsequently sterilized through a 0.2 μm filter. In some cases furazolidone was added from a stock solution in dimethyl sulfoxide (DMSO) to a final solvent concentration in the medium of 1%. Furazolidone labelled in the nitrofuran-ring was diluted with unlabelled drug to a specific activity of 33 MBq/mmol as checked by HPLC.

Following the incubation of cells with ^{14}C-furazolidone, the medium was aspirated and 1 ml was extracted with 2 ml of EtAc (3x). The extract was analyzed by HPLC as described in Chapter 3, and the HPLC-fraction containing the parent compound was collected and assayed for the amount of radiolabel, similar to aliquots of the EtAc-fraction and the remaining water phase. Cells were either washed twice with Williams' E and incubated for another 12 to 70 h, or washed three times with phosphate buffered saline (PBS) and scraped from the dishes in 1 ml PBS using a rubber policeman. Protein was precipitated with 2 ml of icy-cold methanol, collected by centrifugation, than washed with 2 ml of respectively methanol (2x), ethanol (2x) and diethyl-ether (2x), and redissolved in sodium-dodecyl-sulphate/NaOH (5%/0.4N). Part of this solution was counted for the amount of radioactivity and part was used for a protein determination, using the method of Lowry as modified by Peterson (1977).

The amounts of radiolabel present in the EtAc-extract and remaining water-layer of the medium, and the label present in the redissolved cellular protein and various solvents, used to wash the protein, were combined. The fraction of drug metabolized was calculated from the amount of radiolabel present in the HPLC-fraction containing the furazolidone, as compared to this overall amount of radiolabel. Both the total recovery of radiolabel and the recovery of the parent compound varied between 90 and 95%.

Microsomal Incubation

Microsomes, prepared from the liver of a sow (Chapter 3), were incubated with 50 μM [^{14}C]furazolidone, labelled in the AOZ side chain, under anaerobic conditions as described by Vroomen *et al.* (1987). Standard conditions were: 1 mM NADPH, 3 mM $MgCl_2$, 0.1 M potassium phosphate buffer (pH 7.4), and 1.5 mg protein per ml. After bubbling of the mixture with argon on ice, and preincubation at 37 °C for 2 min, the reaction was started by adding 10 μg/ml of a DMSO stock solution of furazolidone

(5 mM). After an incubation period of 10 min, the protein was precipitated with methanol and washed with methanol (2x), ethanol (2x) and diethyl-ether (2x).

Liver samples

Liver samples were obtained from two male piglets (initial age and weight: 5 weeks, 6 kg), that had been treated orally with furazolidone, labelled in the AOZ side-chain, for 10 days (75 mg/day), followed by a withdrawal period of either 2 h or 10 days, as described by Vroomen *et al.* (1986). The tissues had been stored at -40 °C for six years with occasional thawing. For the experiments, samples of about 1 g were frozen in liquid nitrogen, pulverized and washed with 6 ml of respectively methanol (3x), ethanol (2x) and diethyl-ether.

Release and detection of 3-amino-2-oxazolidinone (AOZ)

Following extraction with organic solvents, pellets containing the protein of cells, microsomes or livers were resuspended in water using ultrasonic treatment. Aliquots of these suspensions were mixed with 2-nitrobenzaldehyde (from a stock solution of 50 mM in DMSO) and HCl, and subsequently incubated in a waterbath at the indicated temperature and incubation time. Typical conditions were 2 ml of incubation mixture containing 5-10 mg protein in 0.1N HCl and 0.5 mM NBA, incubated for 20 h at 37 °C. In one experiment pepsin was added to a final concentration of 1 mg/ml. Following incubation, samples were extracted twice with 2 ml EtAc. The combined organic phases were evaporated under nitrogen at 40 °C in about 45 min. The excess NBA was eliminated by redissolving the residue in 2 ml EtAc, and evaporation of the organic phase. The residue was redissolved in 0.25 ml acetonitrile/water 1/1 (v/v) and analyzed by HPLC, using a C18-spher column (250x4.6 mm I.D., Chrompack, The Netherlands) and acetonitrile/water 3/7 (v/v) as eluent (1 ml/min). Compounds were detected at 275 nm (Spectroflow 783, Applied Biosystems), and the fraction containing NPAOZ was collected, mixed with scintillation cocktail (Minisolve, Zinsser, FRG) and counted in a Philips liquid scintillation counter (type PW 4700). For some experiments the HPLC-system was coupled to an on-line radioactivity detector (LB 506C; Berthold, FRG) or diode-array equipment (HP1041A; Hewlett Packard). The identity of the reaction product was confirmed by comparison with the standard of NPAOZ.

Following the extraction with EtAc, the protein in the remaining incubation mixture was precipitated and washed with methanol and redissolved in sodium-dodecyl-sulphate/NaOH (5%/0.4N). Both the protein fraction and washfractions as well as 50 ml of the EtAc-fraction redissolved in acetonitrile/water were mixed with scintillation cocktail and counted. Levels of released AOZ were calculated as the fraction of the radioactivity present in the NPAOZ-peak as compared to the combined radioactivity present in the EtAc, protein and wash fractions.

All solutions containing NPAOZ were protected from the light as much as possible, regarding suspected light sensitivity of this compound.

Results

As shown in Table 8.1, the biotransformation of furazolidone by monolayer cultures of pig hepatocytes at different ages, resulted in the formation of protein-bound metabolites. At the end of the incubation period, 3-4% of the metabolites could not be extracted from the protein fraction by subsequent treatment with methanol, ethanol or diethylether, and were therefore regarded as protein-bound. The capacity of the cultures to metabolize the drug was relatively low during the first 24 h after the isolation of the cells, as compared to the next 72 h. This was partly reflected in the levels of bound metabolites, reaching an optimum in cells incubated during the second interval of 24 h. During this interval there was a dose-related increase in the amount of bound metabolites up to the concentration of 150 μM, accompanied by a decrease in the fraction of drug that was transformed. Insulin, penicillin, streptomycin and fetal calf serum, which were omitted from the medium in further experiments, did apparently not affect the biotransformation of the drug (Table 8.1).

Table 8.1. Biotransformation of furazolidone by monolayer cultures of different ages, and the formation of bound metabolites. Following an incubation period of 24 h in Williams' E containing 5% fetal calf serum, penicillin/streptomycin, insulin and the indicated levels of furazolidone, concentrations of the parent drug in the medium and of protein bound metabolites in the cells were determined. Results are expressed as the mean ± SEM (n=3).

Concentration (μM)	Culture Age (h)	Drug metabolized (% of initial)	bound metabolites (nmol/mg protein
50	4-28	57.7 ± 2.9	0.36 ± 0.02
15	28-52	87.1 ± 1.0	0.30 ± 0.01
50	28-52	80.5 ± 1.1	0.70 ± 0.05
50 [1]	28-52	81.0 ± 3.5	0.69 ± 0.08
150	28-52	55.4 ± 2.2	1.48 ± 0.01
500	28-52	16.9 ± 0.0	1.36 ± 0.02
50	52-76	81.0 ± 1.3	0.49 ± 0.04
50	76-100	73.5 ± 3.4	0.53 ± 0.04

[1] Cells were incubated in Williams' E without any other additions.

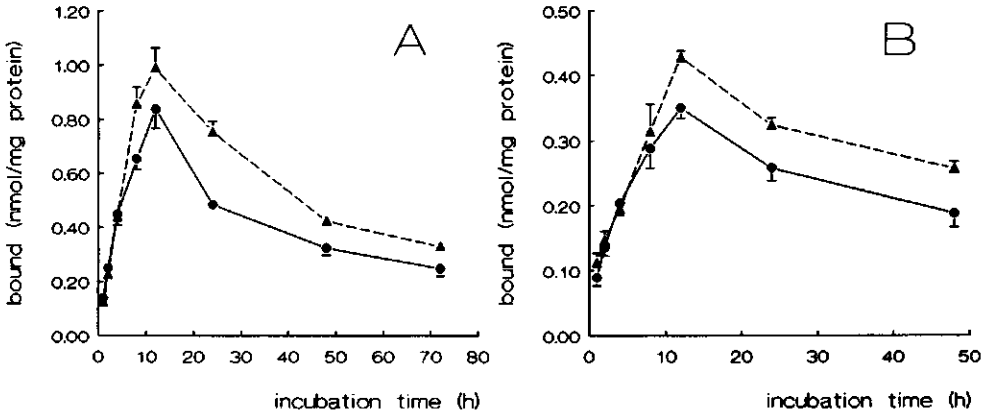

Fig. 8.2. Time related formation and loss of bound metabolites in monolayer cultures of pig hepatocytes incubated with furazolidone.
Cells isolated from livers of two different animals were incubated in medium containing either 50 (A) or 25 (B) μM [^{14}C]furazolidone, labelled in the nitrofuran part (▲) or the amino-oxazolidinone part (●) of the molecule. After 12 h, cells in the remaining dishes were washed and incubated in normal medium. The medium in these dishes was renewed at 24 and 48 h. Results are expressed as the mean ± SEM (n=3).

When hepatocytes were incubated for different time intervals (1-12 h) in the presence of 50 μM furazolidone, labelled in either the nitrofuran or amino-oxazolidinone part of the molecule, there was a clear time-related increase in the levels of protein-bound metabolites (Fig. 8.2A). Similar results were obtained in a second experiment (Fig. 8.2B) where cells, isolated from the liver of another pig, were incubated in the presence of 25 μM of the drug. When the exposure interval was ended after 12 h by replacement of the medium, levels of bound metabolites decreased gradually to respectively 40% (50 μM) and 57% (25 μM) after 36 h. Although levels of bound residues tended to be somewhat higher for the drug labelled in the nitro-furan-ring, the time-related increase and subsequent decrease was very similar for both types of labelled drug.

A new method was developed for the release and detection of the AOZ side-chain of furazolidone, as presented in Fig. 8.1. Incubation of AOZ in 0.1N HCl in the presence of 2-nitrobenzaldehyde resulted in the quick formation of a compound identified as NPAOZ, which could be extracted with EtAc, and analyzed by HPLC. When the same method was applied on washed protein of hepatocytes that had been incubated with furazolidone labelled in the AOZ side-chain, a radioactive labelled compound coeluting with NPAOZ (Fig. 8.3A and B), and with identical UV-spectrum

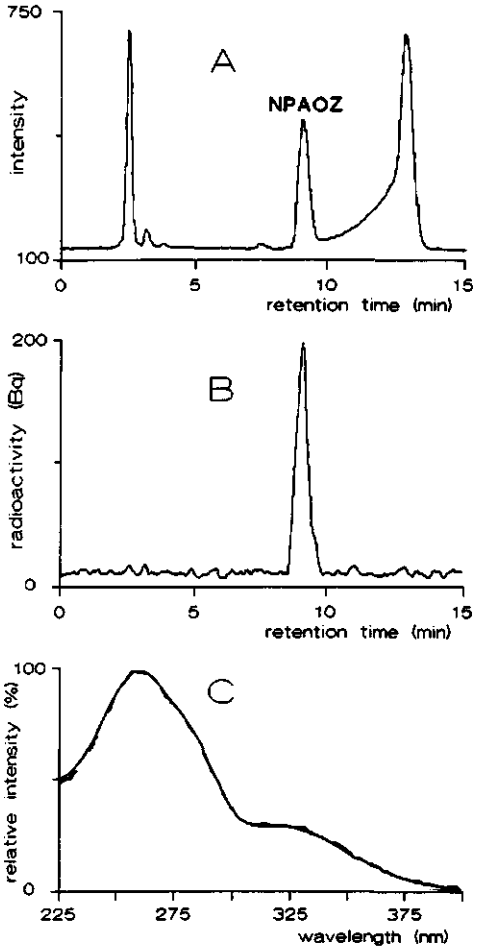

Fig. 8.3. Detection of 3-amino-2-oxazolidinone released from protein of hepatocytes incubated with furazolidone. Cells were incubated with 50 μM furazolidone labelled with ^{14}C in the oxazolidinone side-chain. Washed protein was incubated in 0.1N HCl at 37 °C in the presence of NBA for 24 h, and extracted with EtAc. The extract was analyzed by HPLC using detection by UV at 275 nm (A), radioactivity (B), or diode-array (C). In the latter case the UV-spectrum (——) was compared with that of a standard of NPAOZ (---).

(Fig. 8.3C) could be detected in the EtAc-extract. Unlabelled NPAOZ was formed when using protein of cells that had been incubated with furazolidone labelled in the nitrofuran-ring (data not shown). NPAOZ was also formed when the method was applied on furazolidone, its cyano-metabolite or protein of microsomes, that had been incubated with furazolidone (data not shown).

When the same method was used for liver samples of piglets that had been treated with furazolidone, labelled in the AOZ side-chain (Vroomen *et al.* 1986), radiolabelled NPAOZ could easily be identified, not only with a sample from the animal killed 2 h after the last treatment but also with a sample of the animal killed after a withdrawal period of 10 days (Fig. 8.4).

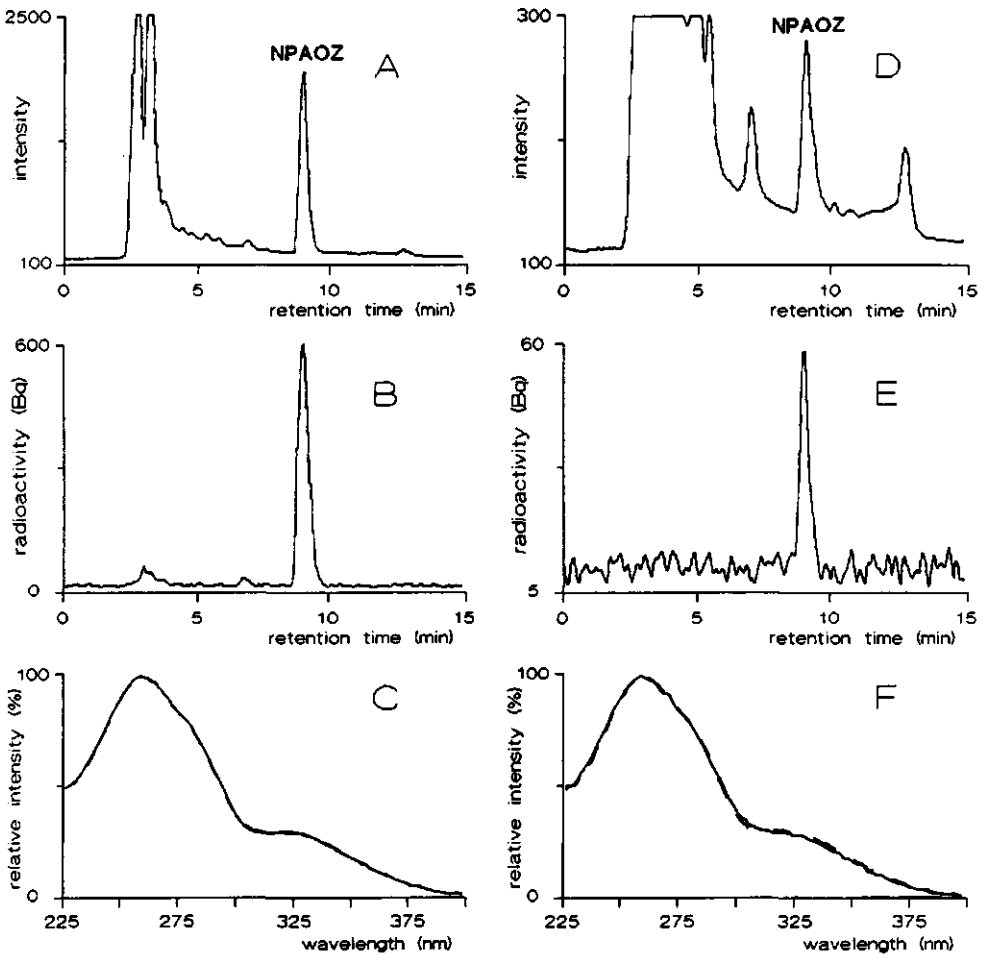

Fig. 8.4. Detection of 3-amino-2-oxazolidinone released from protein of livers from animals treated with furazolidone. Piglets were treated orally with furazolidone labelled with ^{14}C in the AOZ side-chain for 10 days and killed either 2 h (A-C) or 14 days (D-E) after the last treatment. Liver samples of 1 gram were washed and incubated with NBA in the presence of 0.1N HCl. NPAOZ was extracted and analyzed by HPLC as described under Fig. 8.3.

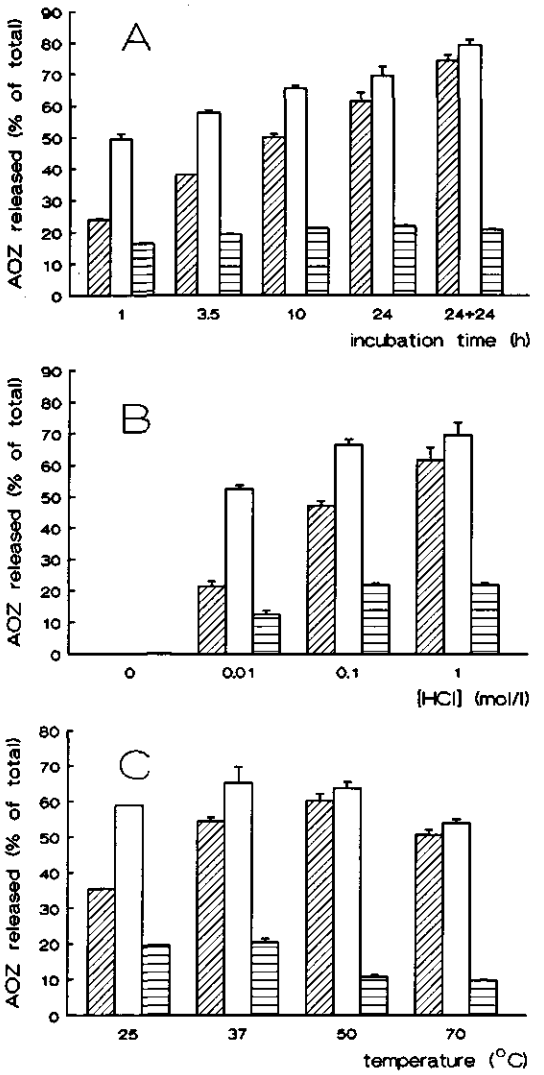

Fig. 8.5. Effect of incubation time, HCl concentration and temperature on the release of 3-amino-2-oxazolidinone from protein of microsomes (▨), cells (□) or liver samples (▤). Washed protein was incubated in the presence of 0.5 mM NBA and 1N HCl at 37 °C for 24 h, unless indicated. Incubation time (A), HCl-concentration (B) and temperature (C) were subsequently varied. Incubation mixtures contained 2.3, 1.7 and 14.6 mg of protein from microsomes, cells and liver respectively, containing respectively 1.1, 1.7 and 0.7 nmol of bound metabolites per mg protein. The liver used was obtained from the animal killed 2 h after the last treatment with furazolidone. Results are expressed as the mean ± SEM for n=2.

Further experiments were conducted to search for optimum conditions for the assay, with respect to the amount of label that could be released as NPAOZ (Fig. 8.5). As shown in Fig. 8.5A, there was a time related increase in the fraction of bound residues that could be hydrolysed to AOZ in the case of protein from microsomes and cells, up to a maximum of about 60% at 24 h (0.1N HCl; 37 °C; 24 h). With liver samples, only 20% of the bound radioactivity could be released as NPAOZ. Following an additional

incubation of the extracted protein for another 24 h, some additional NPAOZ was formed, resulting in maximum total levels of about 75, 80 and 25% for respectively microsomal, cellular and liver protein. Reduction of the concentration of HCl (Fig. 8.5B) led to a decrease in the levels of NPAOZ, whereas almost no NPAOZ could be detected in the absence of HCl. Increasing the incubation temperature was especially beneficial in the case of microsomes (Fig. 8.5C), but with cellular and liver samples the effect was less clear. Addition of pepsin to an incubation mixture with a liver sample (37 °C, 0.1N HCl, 24 h) resulted in a release of 26% of NPAOZ as compared to 24% in the absence of the enzyme, whereas as a result of the treatment with pepsin, the fraction of label detected in the methanol-precipitable fraction after the incubation period decreased markedly from 41% to 9%. Incubating a liver sample with 0.05, 0.5 or 5 mM NBA (0.1N HCl, 37 °C), resulted in a dose-related increased release of NPAOZ from 9 to 22%, and 26% respectively. However, in the latter case the large amount of NBA could no longer be effectively removed and therefore disturbed the UV-detection. In the absence of NBA about 62% of the label could be recovered in the precipitable protein fraction, as compared to 56% after incubation with 0.5 mM NBA, indicating that the release of AOZ from the protein is not caused by NBA.

Optimum conditions were used to determine the amount of total and bound residues, as well as the amount of releasable side-chain in liver samples of piglets killed at day 0 and day 14 of the withdrawal period (Table 8.2). For a comparison, a blanc liver sample was spiked with protein from hepatocytes incubated with [^{14}C]furazolidone, equivalent with an overall binding of 67 pmol/mg protein. From these results it appears that the almost 50x dilution of cellular protein with liver protein does not affect the release of AOZ. As shown before, the fraction of bound metabolites in the liver samples that can be released does not exceed 25% and is even lower in the liver of the animal killed at day 14. However, absolute amounts of releasable AOZ reached levels of almost 4 μg furazolidone equivalents per gram of tissue just after the last treatment, and although markedly decreased, amounts equivalent with 0.25 μg of furazolidone could still be released as AOZ from one gram of tissue following the withdrawal period.

Discussion

The results of the present study show that pig hepatocytes are a useful model to study the formation and identity of protein-bound metabolites of veterinary drugs, especially considering the often required use of radiolabelled compounds for these type of studies. The time and dose related formation of bound metabolites, following the biotransformation of furazolidone by hepatocytes (Table 8.1, Fig. 8.2), is in agreement

Table 8.2. Levels of total, bound and AOZ-related residues in livers of piglets treated with furazolidone for 10 days, and killed after withdrawal periods of 0 and 14 days.
Liver samples (0.5 g) were homogenized, washed with organic solvents, and incubated in 0.1N HCl for 24 h in the presence of NBA. As a control, cells, that had been incubated with furazolidone were treated similarly either in the absence or presence of 0.5 g blanc liver. Results are expressed as absolute amounts for duplicate determinations (single for total residues). Levels of bound residues were 490 and 68 pmol per mg of protein for liver samples of animals killed at day 0 (2 h) and day 14 respectively.

Sample	Total (nmol)[1]	Bound (nmol)[1]	NPAOZ released (nmol)[1]	(% of bound)
Liver day 0	62.49	36.9 ± 0.0	8.5 ± 0.0	23.1 ± 0.1
	(28.12)	(16.59)	(3.83)	
Liver day 14	5.53	4.0 ± 0.0	0.6 ± 0.0	14.6 ± 0.5
	(2.49)	(1.79)	(0.26)	
Liver+cells	8.51	4.5 ± 0.1	2.4 ± 0.0	53.3 ± 0.7
cells	8.73	4.0 ± 0.0	2.4 ± 0.1	59.7 ± 3.2

[1] numbers between brackets are levels expressed as μg furazolidone equivalents per gram of tissue.

with previous result from both *in vivo* and *in vitro* studies with microsomes (Vroomen *et al.* 1986, Vroomen *et al.* 1987). Furthermore, the decrease in the levels of bound metabolites during the recovery period (Fig. 8.2), most likely due to the turn-over of cellular protein, indicates that this *in vitro* model can also be used to study the identity of metabolites that are released from the protein and may be similar to those present as extractable residues in tissues during the withdrawal period.

The fact that, similar to previous studies (Chapter 3), the biotransformation of furazolidone did not decrease during the first 100 h of the cultures (Table 8.1), as observed with cytochrome P-450 related enzyme activities (Chapters 3 and 4), is in agreement with the results from studies with pig liver microsomes, showing that cytochrome P-450 enzymes are not involved in the degradation of this drug (Vroomen *et al.* 1987). The similar time course in both the formation and degradation of bound metabolites, as observed with the two differently labelled furazolidone preparations (Fig. 8.2), indicates that the two parts of the molecule are not separated from each other and supports the hypothesis that the bound metabolites still closely resemble the general structure of the parent compound. The latter conclusion is supported by the release and detection of the AOZ side-chain from metabolites bound to cellular protein

(Fig. 8.3). The effectiveness of the newly developed method for the release and detection of AOZ is demonstrated by the similarity in the HPLC retention time and UV-spectrum of the reaction product formed during incubation of protein-bound metabolites with HCl and NBA, and NPAOZ. Final proof comes from the fact that this product was only radiolabelled when furazolidone, used for the incubation of the cells, was labelled in the AOZ side-chain and not when labelled in the nitrofuran-part.

Under currently optimal conditions, evidence was obtained that at least 75-80% of the bound residues in the case of microsomes and cells, and 15-25% in the case of livers of treated animals, still posses the intact AOZ side-chain. It seems unlikely that the differences observed between hepatocytes and microsomes on one hand, and livers from treated animals on the other hand, may simply be due to the efficiency of the method. A more likely explanation for this discrepancy may be a difference in the nature of part of the bound metabolites. First of all, part of the drug and/or its metabolites might be completely metabolized *in vivo*, possibly at other sites than the liver, followed by the use of the label for the synthesis of endogenous compounds, including amino acids and proteins. On the other hand, regarding the acid-lability of the azomethine bond in the furazolidone molecule (Buzard *et al.* 1956, Paul *et al.* 1960), part of the drug may have been hydrolysed in the stomach and the released AOZ subsequently been transformed into a compound, capable of binding to tissue macromolecules. Evidence for the cleavage of the azomethine-bond *in vivo* has been presented by Tennent and Ray (1971), and Loserth *et al.* (1988). Furthermore, the subsequent formation of a reactive metabolite from AOZ appears to underly the irreversible type of inhibition of mono-amine oxidase (MAO) activity by furazolidone, as observed in tissues of a number of different animal-species, including man (Palm *et al.* 1967, Stern *et al.* 1967, Pettinger *et al.* 1968, Ali and Bartlet 1982). Strong support for this hypothesis was supported by the MAO-inhibition observed in both rats (Stern *et al.* 1967), as well as pig hepatocytes (Chapter 6) exposed to AOZ.

There is now substantial evidence that at least part of the bound metabolites of furazolidone still contains the AOZ side-chain, and that the conditions for the release of AOZ closely resemble those in the stomach. Therefore, the toxic properties of AOZ should be further investigated, especially considering the fact that ß-hydroxyethyl-hydrazine has been postulated to be an intermediate in the activation of AOZ into a MAO-inhibiting compound (Stern *et al.* 1967, see Fig. 6.1), and considering the mutagenic (Bos *et al.* 1983) and possible carcinogenic (Innes *et al.* 1969) properties of the latter compound, and the carcinogenic potency of many other hydrazines (Moloney and Prough 1983). Furthermore, the release of AOZ from protein-bound metabolites in the stomach, and its subsequent transformation into a reactive metabolite capable of binding covalently to protein, might underly the formation of bound metabolites as observed in rats, that had been fed with meat from a piglet treated with furazolidone (Vroomen *et al.* 1988a). This explanation offers a good alternative for the theory that the bound residues in the rat are due to the the release and absorption of reactive

thiol-conjugates from the protein of the piglet, which can subsequently bind to the protein of the rat (Vroomen *et al.* 1988b), a mechanism for which no support could be obtained in previous studies with pig hepatocytes (Chapter 7).

In the absence of better information on the properties of AOZ, that can be used for determining maximum tolerance levels, the presence of bound residues of furazolidone in edible products should be reduced as much as possible. Previously, a withdrawal period of 30 days was set in the Netherlands for pigs treated with furazolidone (Anonymous 1987), based on the genotoxic and carcinogenic properties of the parent compound (Klemencic & Wang, 1978; McCalla, 1983; Food and Drug Administration, 1976), and the lack of information on the identity and biological properties of protein bound metabolites. The results presented in this study support the use of such long withdrawal periods (Table 8.2.). The relatively simple method presented in this paper may now be helpful in further establishing and controlling adequate withdrawal periods.

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CHAPTER 9

SUMMARY AND CONCLUDING REMARKS

SUMMARY AND CONCLUDING REMARKS

The three main objectives of this study were, (1) to investigate the possibility to isolate viable hepatocytes from liver samples of pigs, (2) to study their use for biotransformation and toxicity studies, and (3) to demonstrate the value of this model, in particular in the field of residue toxicology.

The main result from this study was the fact that hepatocytes can be successfully isolated from samples of pig livers obtained at the slaughterhouse, and that the cells retained a high viability as demonstrated by parameters like the exclusion of trypan blue. Furthermore, the cells were able to attach themselves to untreated culture dishes, and to form monolayer cultures, which could be kept alive for at least seven days.

Even more important was the fact that the cells retained the capability of biotransforming xenobiotics into metabolites that were also observed *in vivo*.

Initial studies with suspension cultures (Chapter 3) demonstrated that these cells were capable of biotransforming a number of different substrates by very diverse pathways, like the deethylation of 7-ethoxy-coumarin and subsequent glucuronidation of the product 7-hydroxy-coumarin, the acetylation of sulfadimidine and deacetylation of N^4-acetyl-sulfadimidine, and the transformation of furazolidone, partly resulting in the formation of the open chain cyano-metabolite. *In vivo* the N^4-acetyl metabolite of sulfadimidine has been shown to be the only significant metabolite. In the case of furazolidone, the only *in vivo* metabolite identified sofar, is the cyano-metabolite, although similar to *in vitro* it appears to be formed in relatively small amounts.

In comparison with suspension cultures, monolayer cultures have a number of additional advantages, such as the extra viability criterium of the ability of cells to form a monolayer, the longer survival of the cells, and the physical separation between the cells and the culture medium. However, by the results from studies with hepatocytes from other animal-species, it was shown that the prolonged use of monolayer cultures is often limited by a decrease in the activity of certain biotransformation enzymes (Chapter 1). Therefore, special attention was paid to the possible existence and impact of this phenomenon in monolayer cultures of pig hepatocytes, by incubating cells with the substrates described above. There was a decrease in absolute levels of cytochrome P-450, and in the cytochrome P-450 related deethylation of 7-ethoxy-coumarin. In the

case of the N^4-acetylation of sulfadimidine, an increase in the activity was observed, which was paralleled and possibly caused by a decrease in the reverse activity, *i.e.* the deacetylation of the N^4-acetyl metabolite. Furazolidone was converted relatively slowly during the first 24 h of the monolayer cultures, as compared to the next 72 h, where little variation was observed. Therefore, the effect of ageing apparently varies from one compound to another, which may be a strong argument for the use of freshly isolated cells. However, a recovery period may allow the cells to recover from the isolation procedure. It was *e.g.* shown that intracellular levels of reduced glutathione (GSH) were sometimes very low in freshly isolated cells, but increased rapidly during the first 24-48 h of the cultures (Chapters 3, 5 and 7). Since GSH has an important role in protecting the cells against the adverse effects of many xenobiotics (see also Chapter 7), freshly isolated cells may be oversensitive to the toxic properties of certain drugs. Further experiments were therefore primarily performed with monolayer cultures, after a 24 h recovery period.

In the case of the anabolic steroid ß-nortestosterone, a substantial change in the metabolite pattern, due to the ageing of the cells, was only observed after 72 h (Chapter 4). This compound was rapidly oxidized by the cells, initially resulting in the formation of norandrostenedione, which was than hydroxylated and glucuronidated at its C-15 position. No differences were observed between cells from female and castrated male pigs, which is consistent with the "feminizing" effect of castration. In the absence of essential information on the *in vivo* biotransformation of this compound, three pigs were injected with the hormone. The fact that the two main metabolites could subsequently be identified in the urine of these pigs, clearly demonstrates the value of the model for the identification of important metabolites.

The high viability of the cells made the model very useful for toxicity studies. In Chapter 5, a number of different cellular functions were chosen as end-points to study and compare the effects of five different nitrofuran drugs. These parameters, *i.e.* the leakage from the cells of the cytosolic enzyme lactate dehydrogenase (LDH), due to membrane damage, the incorporation of ^{14}C-leucine into newly synthesized proteins, the intracellular levels of reduced and oxidized glutathione, and the metabolism of pyruvate, could all be measured easily and reproducibly. Using these parameters, clear similarities but also differences were observed between the drugs. At the highest dose levels there was a small but significant increased leakage of LDH, especially in the case of nitrofurantoin. With all drugs, this was accompanied by a decrease in intracellular LDH-levels, that could only partly be accounted for by the loss into the medium. This might be explained by the decreased incorporation of ^{14}C-labelled leucine into proteins after exposure to all nitrofurans. This inhibiting effect was especially clear on the proteins excreted into the medium. Furthermore, incubation with all nitrofurans resulted in an increase in intracellular levels of oxidized glutathione (GSSG), which is in agreement with the fact that reduction of the nitro-group of these drugs may result in

redox-cycling and the subsequent formation of H_2O_2 (Fig. 7.1). Somewhat unexpected was the fact that, with the exception of nitrofurantoin, there was no decrease in intracellular GSH levels. The most sensitive, and possibly most specific parameter, was the inhibition of the pyruvate metabolism by all nitrofurans, except nitrovin, as concluded from the accumulation of pyruvate and lactate in the medium. In a more detailed study with furazolidone, it was shown that the cells recovered only very slowly from the latter effect, which is consistent with previous results from *in vivo* studies. These and some of the other effects, might underlie some of the many adverse effects reported in both human and animal patients treated with these type of drugs, like *e.g.* the increased plasma levels of pyruvate and lactate. However, in general such *in vivo* information was too limited to completely validate the model at this point, and further *in vivo* studies should be carried out for this purpose. Nevertheless, the study demonstrates the value of the model for studying the toxicity and underlying mechanisms of xenobiotics.

In Chapter 6, another specific toxic effect of furazolidone observed *in vivo* but not *in vitro*, *i.e.* the inhibition of the enzyme monoamine oxidase (MAO), was studied in more detail. In this study, intact cells were used instead of homogenates for measuring the MAO-inhibition, thereby offering the advantage of the presence of an intact biotransformation potential. Using p-tyramine as a substrate for the MAO-enzymes, the effect of the known inhibitors clorgyline and iproniazid could easily be measured. Incubation of hepatocytes with furazolidone resulted in a thusfar unknown reversible MAO-inhibition. However, the irreversible inhibition reported in *in vivo* studies, was only observed after incubation of cells with 3-amino-2-oxazolidinone (AOZ), the side chain of furazolidone, and the proposed metabolite ß-hydroxyethylhydrazine (Fig. 6.1). The time-related increase in the MAO-inhibition by 3-amino-2-oxazolidinone, indicated that the compound must be transformed into a metabolite capable of inactivating MAO-enzymes irreversibly, possibly by covalent binding. The results from this study are in support of the hypothesis of Stern *et al.*, with regard to the role of the side-chain in the MAO-inhibition observed *in vivo*.

The great perspectives of the model in the field of residue toxicology are further demonstrated in Chapters 7 and 8, where the different features of the model were used to obtain more information on the identity and properties of the protein-bound metabolites of furazolidone. As a follow-up of the microsomal studies performed by Vroomen *et al.*, the role of glutathione in the biotransformation and toxicity of this drug was further investigated (Chapter 7). Using ^{14}C-labelled furazolidone, the drug was shown to be extensively transformed by monolayer cultures, resulting in the formation of the cyano metabolite, a large number of more hydrophilic compounds, and protein-bound metabolites. These results are in agreement with previous *in vivo* studies, and confirm the value of the model.

Similar to the studies described in Chapter 5, furazolidone had apparently no effect

on intracellular GSH-levels. However, both by inhibition of the GSH-synthesis and by labelling of the intracellular GSH with ^{35}S, it could be demonstrated that exposure of cells to furazolidone actually resulted in an increased loss of intracellular GSH, but that this effect was compensated for by an increased GSH synthesis. The increased GSH loss was accompanied by increased medium concentrations of GSSG and a second compound, most likely formed after excretion of GSH by the cells. However, no evidence could be obtained for the formation and excretion of the glutathione-conjugate of furazolidone that was previously detected in microsomal incubations. Similarly, the presence of unstable protein-bound thiol conjugates could not be demonstrated. Therefore, it seems unlikely that these type of metabolites are responsible for the previously observed high bioavailability of protein-bound metabolites in rats, that had been fed with muscle tissue of a piglet treated with furazolidone, as well as the subsequent formation of protein-bound metabolites in tissues of those rats.

An alternative explanation for this observation was given in Chapter 8, where the time- and dose-related formation of protein-bound metabolites of furazolidone in pig hepatocytes was studied in more detail. Furthermore, by using ^{14}C-furazolidone, either labelled in the nitrofuran or in the oxazolidinone part of the molecule, strong indications were obtained for the presence of both parts of the parent drug in protein-bound metabolites. Therefore, a new method for the detection of these type of compounds was developed, based on the release and detection of the 3-amino-2-oxazolidinone side-chain of furazolidone. Using this relatively simple method it was demonstrated that at least 75% of the metabolites bound to protein of hepatocytes still contained this side-chain, showing for the first time that at least part of these metabolites resemble the original structure of the parent compound. With the same method 3-amino-2-oxazolidinone could be released from 15-25% of the protein-bound metabolites present in liver samples from piglets treated with ^{14}C-furazolidone, even after a storage period of seven years at -40 °C.

The difference between *in vivo* and *in vitro* studies, with respect to the fraction of bound metabolites from which AOZ can be released, might be ascribed to the role of the stomach in the *in vivo* biotransformation of furazolidone. The acid hydrolysis of the azomethine bond of furazolidone may result in the release of 3-amino-2-oxazolidinone (Fig. 9.1), which is subsequently absorbed and metabolized into a compound, possibly via ß-hydroxyethylhydrazine (Fig. 6.1), capable of binding covalently to proteins (type I). As pointed out in Chapter 6, there are strong indications that this mechanism underlies the irreversible type of MAO-inhibition. The bound metabolites observed *in vivo* would therefore partly be related to the formation of a reactive intermediate from the side-chain (type I), and partly to intermediates containing an intact AOZ side-chain, because they are formed by the reduction of the nitro-group (type II), as shown in Fig. 7.1. In the case of incubation of furazolidone with microsomes and hepatocytes at pH 7, it seems likely that the formation of bound metabolites can be ascribed to the latter type of reactive intermediates (type II). Since AOZ can only be released from this type of

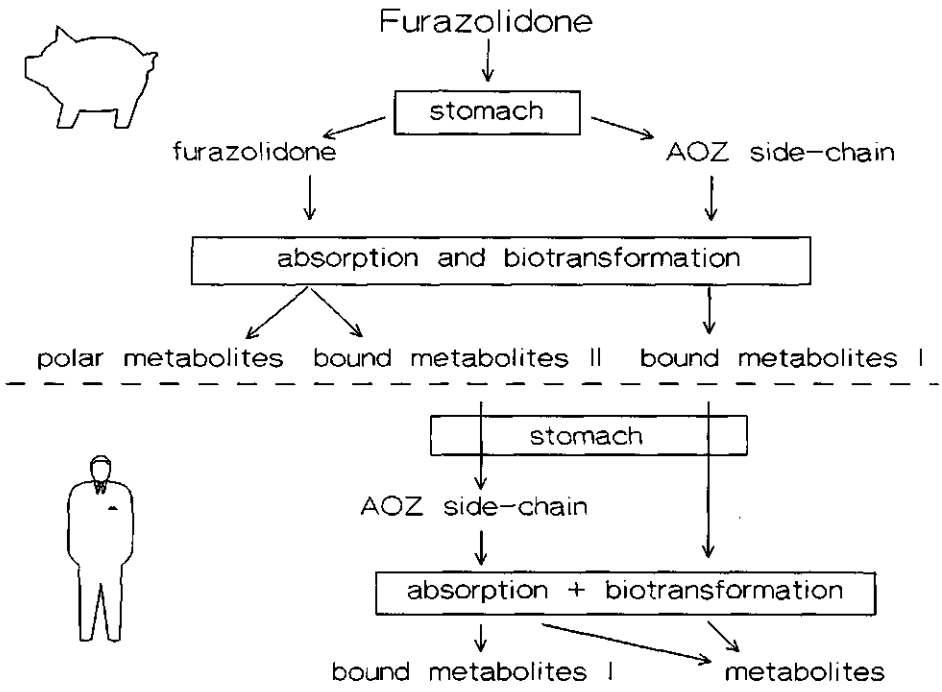

Fig. 9.1 Schematic presentation of the possible mechanisms underlying the formation of bound residues of furazolidone in the target animal and possible fate of bound metabolites in the consumer of products containing such residues.

metabolites, this difference could explain the discrepancy with *in vivo*. At this point it cannot be excluded that a similar mechanism, *i.e.* the acid hydrolysis of the azomethine bond and subsequent biotransformation of AOZ, is responsible for the high bioavailability of bound residues and the formation of new bound metabolites as observed in rats after consumption of meat from pigs treated with furazolidone.

This is the first time that, based on experimental data, strong indications are obtained for the fact that bound residues of a compound may still exert a toxic action, once orally absorbed by the consumer. Therefore, the presence of such protein-bound residues should be reduced as much as possible, and adequate analytical methods, such as presented in this study, should be developed in order to control this. It should be mentioned, that the possible toxic potential of bound-metabolites of other compounds, depends very much on the structure and properties of those compounds, and should

therefore be considered from one case to another. In general these results subscribe the importance of including metabolites in the risk-assessment of compounds used in veterinary practice.

The results of the present study clearly demonstrate the large potential of *in vitro* models with hepatocytes from large domestic animals. Regarding the high costs and special requirements that go along with *in vivo* studies with larger animals, the use of such *in vitro* models may eventually not only result in a reduction in the number of animals used for experiments, but even more, offer an improved chance to obtain the more detailed information on the species-related biotransformation and toxicity of xenobiotics, as requested more and more by regulatory authorities. In general, the use of such models may therefore contribute to the development of new drugs with a minimal health risk for the consumer and less adverse effects for the animal.

Furthermore, this study shows that knowledge on the identity and nature of metabolites, may result in the development of new analytical methods, that can be used to control the sound use of drugs and growth-promoting agents in veterinary practice.

CHAPTER 10

SAMENVATTING EN SLOTBESCHOUWINGEN

SAMENVATTING EN SLOTBESCHOUWINGEN

De drie hoofddoelstellingen van deze studie waren, (1) het onderzoeken van de mogelijkheid om levensvatbare hepatocyten te isoleren uit een stuk varkenslever, (2) het bestuderen van de mogelijkheden van dit model voor biotransformatie en toxiciteitsonderzoek en (3) het aantonen van de waarde van dit soort modellen, met name voor residu-toxicologisch onderzoek.

Een essentieel resultaat van dit onderzoek is het feit dat het mogelijk is om hepatocyten te isoleren uit een stuk varkenslever verkregen via het slachthuis en dat deze cellen een hoge levensvatbaarheid bezitten, zoals onder meer blijkt uit de trypaan blauw exclusie test. Verder bleken de cellen in staat om zich te hechten aan onbehandelde weefselkweekplaten en zodoende monolayers te vormen die tenminste gedurende een periode van zeven dagen in leven konden worden gehouden.

Nog belangrijker was het feit dat de cellen hun vermogen behielden om xenobiotica om te zetten in metabolieten die ook bij *in vivo* studies zijn aangetroffen.

Inleidende studies met suspensie cultures (Hoofdstuk 3), lieten zien dat de cellen in staat waren om een aantal verschillende stoffen om te zetten langs zeer verschillende biotransformatie routes, zoals de deëthylering van 7-ethoxy-coumarine en glucuronidering van het hierbij gevormde 7-hydroxy-coumarine, de acetylering van sulfadimidine en deacetylering van N^4-acetyl-sulfadimidine en de biotransformatie van furazolidon, gedeeltelijk resulterend in de vorming van de cyano-metaboliet. Uit *in vivo* studies met sulfadimidine is gebleken dat de N^4-acetyl metaboliet de enige metaboliet van betekenis is. In het geval van furazolidon is de enige geïdentificeerde *in vivo* metaboliet de cyano-metaboliet, die echter net als *in vitro* in relatief kleine hoeveelheden gevormd lijkt te worden.

In vergelijking met suspensie cultures hebben hepatocyten in monolayer cultures een aantal aanvullende voordelen. Zo vormt het vermogen van de cellen om een monolayer te vormen een extra criterium voor de kwaliteit van de cellen, kunnen de cellen gedurende een aantal dagen in leven worden gehouden en is er sprake van een duidelijke scheiding tussen de cellen en het kweekmedium. Echter, uit studies met hepatocyten van andere dieren is gebleken dat de mogelijkheden voor het langdurige gebruik van monolayer cultures veelal worden beperkt door de achteruitgang van de

activiteit van bepaalde bij de biotransformatie betrokken enzymen (Hoofdstuk 1). Daarom is bij varkenshepatocyten ook aandacht besteed aan het bestaan en de mogelijke consequenties van dit verschijnsel, door bestudering van de biotransformatie van eerder genoemde stoffen. Er werd een achteruitgang waargenomen in de absolute gehaltes van het cytochroom P-450 en de daaraan gerelateerde deëthylering van 7-ethoxy-coumarine. In het geval van de N^4-acetylering van sulfadimidine, werd er een toenemende activiteit gevonden, die mogelijk verklaard kan worden uit de achteruitgang in de tegengestelde reactie, namelijk de deacetylering van de N^4-acetyl metaboliet. Furazolidon werd relatief langzaam omgezet gedurende de eerste 24 uur, in vergelijking met de volgende 72 uur, waar nauwelijks enige variatie werd waargenomen. Samenvattend kan gezegd worden, dat het effect van het ouder worden van de cultures varieert per stof, hetgeen ervoor pleit om zo mogelijk vers geïsoleerde cellen te gebruiken. Echter, het inlassen van een zekere wachtperiode biedt de cellen de gelegenheid om zich te herstellen van de isolatie. Zo is in deze studie gebleken dat het intracellulaire gehalte van het gereduceerde glutathion (GSH) in vers geïsoleerde cellen veelal erg laag was en vervolgens sterk toenam gedurende de eerste 24 tot 48 uur na het in kweek brengen van de cellen (Hoofdstukken 3, 5 en 7). Gezien de belangrijke rol van GSH bij de bescherming van de cel tegen de schadelijke effecten van vele xenobiotica, waaronder furazolidon (zie Hoofdstuk 7), zouden vers geïsoleerde cellen weleens overgevoelig kunnen zijn voor zulke stoffen. Daarom werd ervoor gekozen om experimenten uit te voeren met 24 uur oude monolayer cultures.

Bij studies met het anabole steroïd hormoon ß-nortestosteron, werd o.i.v. de leeftijd van de cultures, pas een wezenlijke verandering in het metabolietpatroon waargenomen na 72 uur (Hoofdstuk 4). Deze stof werd door de cellen relatief snel geoxideerd tot het norandrosteendion, dat vervolgens werd gehydroxyleerd en geglucuronideerd op de C-15 plaats. Er werden geen verschillen waargenomen tussen cellen geïsoleerd van levers van zeugen en borgen, geheel in overeenstemming met het zogenaamde "feminizing" effekt van castratie. Omdat de voor validatie benodigde informatie omtrent de biotransformatie van ß-nortestosteron bij varkens ontbrak, werden drie dieren behandeld met deze stof. Het feit dat de twee hoofdmetabolieten vervolgens konden worden aangetoond in de urine van deze dieren bevestigt de waarde van dit model bij het opsporen en identificeren van belangrijke metabolieten.

Mede dankzij de hoge levensvatbaarheid van de cellen bleek het model uitermate geschikt te zijn voor toxiciteitsstudies. In de experimenten beschreven in Hoofdstuk 5 werden een aantal functies van de cellen uitgekozen, waarmee vervolgens de effekten van vijf verschillende nitrofuranen werden bekeken en vergeleken. Deze eindpunten, namelijk de lekkage van het cytosol enzym lactaat-dehydrogenase (LDH) door beschadiging van de celmembraan, de inbouw van ^{14}C-leucine in nieuw gesynthetiseerde eiwitten, de intracellulaire concentraties van gereduceerd en geoxideerd glutathion en het pyruvaat metabolisme, konden allen op eenvoudige en reproduceerbare wijze worden

gemeten. Bij de effekten van de diverse nitrofuranen op deze eindpunten konden zowel overeenkomsten als verschillen worden waargenomen. Bij de hoogste concentraties was er een kleine maar significante verhoging in de LDH-lekkage meetbaar, vooral in het geval van nitrofurantoïne. Bij alle stoffen ging dit gepaard met verlaagde LDH-activiteit in de cellen, die echter niet volledig kon worden toegeschreven aan het verlies naar het medium. Dit zou ten dele verklaard kunnen worden door de verlaagde eiwit-synthese, die bij blootstelling aan alle nitrofuranen werd waargenomen. Overigens was het remmend effekt vooral duidelijk wanneer gekeken werd naar eiwitten die werden uitgescheiden in het medium. Na blootstelling aan alle nitrofuranen werd er een verhoging van de intracellulaire gehaltes aan geoxideerd glutathion (GSSG) gemeten, geheel overeenkomstig het feit dat de reductie van de nitrogroep van dit soort stoffen veelal leidt tot een zogenaamde redox-cycling en daaraan gepaard de vorming van waterstofperoxyde (Fig. 7.1). Daarmee in tegenspraak lijkt de waarneming dat er, met uitzondering van nitrofurantoïne, geen verlaging van de GSH niveaus optrad. De meest gevoelige en wellicht meest specifieke parameter was de remming van het pyruvaat metabolisme door alle nitrofuranen, behalve nitrovin, zoals afgeleid uit de ophoping van pyruvaat en lactaat in het medium. In een meer gedetailleerde studie met alleen furazolidon, bleek dat de cellen zich slechts langzaam herstelden van dit effect, hetgeen uit de literatuur ook bekend was uit *in vivo* studies. Dit effekt zou evenals sommige van de andere gemeten effekten ten grondslag kunnen liggen aan een aantal van de vele bijwerkingen die zijn waargenomen bij zowel mensen als dieren na behandeling met nitrofuranen, zoals de verhoogde plasma niveaus van pyruvaat en lactaat. Echter, over het algemeen was die informatie te beperkt om het model volledig te valideren en verdere *in vivo* studies zouden hiervoor moeten worden uitgevoerd. Desalniettemin bevestigt deze studie de waarde van het model voor het bestuderen van de toxiciteit van stoffen en de onderliggende werkingsmechanismen.

In Hoofdstuk 6 werd een ander specifiek effect van furazolidon bestudeerd, namelijk de remming van het enzym monoamine oxidase (MAO), zoals waargenomen bij mensen en dieren. Bij deze studie werden voor het meten van de MAO-remming intakte cellen gebruikt in plaats van weefselhomogenaten, hetgeen het voordeel biedt dat er een volledig metaboliserend systeem aanwezig is. Met gebruik van p-tyramine als substraat voor MAO-enzym(en), kon de remming door bekende remmers als clorgyline en iproniazide eenvoudig worden gemeten. Incubatie van cellen met furazolidon leidde tot een tot nog toe onbekende volledig omkeerbare MAO-remming. Echter, het irreversibele type remming, zoals beschreven *in vivo*, werd alleen waargenomen na blootstelling van de cellen aan 3-amino-2-oxazolidinon (AOZ), de zijketen van furazolidon en ß-hydroxyethylhydrazine, een veronderstelde metaboliet bij dit proces (Fig. 6.1). De tijdsafhankelijke toename in de MAO-remming door 3-amino-2-oxazolidinon, suggereerde dat deze verbinding eerst moet worden omgezet in een metaboliet die in staat is om de MAO-enzymen irreversibel te remmen, mogelijk door covalente binding. Daarmee

ondersteunen de resultaten van dit onderzoek de hypothese van Stern *et al.* (Fig. 6.1), waarin de zijketen van furazolidon verantwoordelijk wordt gehouden voor het irreversibele type MAO-remming.

De grote voordelen van het model voor residu-toxicologisch onderzoek werden verder aangetoond in Hoofdstuk 7 en 8, waar de diverse mogelijkheden van het model werden benut om meer inzicht te verkrijgen in de identiteit en eigenschappen van eiwit-gebonden metabolieten van furazolidon. Als vervolg op de microsomale studies uitgevoerd door Vroomen *et al.*, werd in eerste instantie gekeken naar de invloed van glutathion op de biotransformatie en toxiciteit van furazolidon (Hoofdstuk 7). Met behulp van ^{14}C gelabeld furazolidon kon worden aangetoond dat de afbraak van de stof door de cellen resulteert in de vorming van de cyano metaboliet, een groot aantal meer polaire metabolieten en eiwit-gebonden metabolieten. Deze resultaten zijn geheel in overeenstemming met die van *in vivo* studies en bevestigen nogmaals de experimentele waarde van het model.

Zoals eerder waargenomen (Hoofdstuk 5), had furazolidon ogenschijnlijk geen invloed op de intracellulaire GSH concentratie. Door remming van de GSH-synthese alsmede door labeling van GSH met ^{35}S, kon echter worden aangetoond dat blootstelling van cellen aan furazolidon wel degelijk leidde tot een verlies van GSH, maar dat dit verlies door de cellen werd gecompenseerd middels een verhoogde synthese van GSH. Het verhoogde verlies van GSH ging gepaard met verhoogde medium concentraties van GSSG en een tweede stof, mogelijk gevormd na uitscheiding van GSH door de cellen. Echter, de vorming en uitscheiding van het glutathion-conjugaat dat eerder werd gevonden bij microsomale incubaties, kon niet worden bevestigd. Zo werd er ook geen enkel bewijs gevonden voor de aanwezigheid van instabiele eiwitgebonden thiol-conjugaten. Daardoor lijkt het onwaarschijnlijk dat dit soort metabolieten verantwoordelijk is voor de eerder waargenomen hoge biologische beschikbaarheid van eiwit-gebonden metabolieten in ratten, die waren gevoerd met vlees van een met furazolidon behandeld big, alsmede de vorming van gebonden metabolieten in die ratten.

Op grond van de resultaten beschreven in Hoofdstuk 8 werd er een alternatieve verklaring opgesteld voor bovengenoemde waarneming. Daarin werd allereerst een dosis- en tijdsafhankelijke vorming van eiwit-gebonden metabolieten van furazolidon gevonden. Daarbij werden er door gebruik te maken van twee verschillend gelabelde preparaten van furazolidon (^{14}C-label in nitrofuraan- of in oxazolidinonring) duidelijke aanwijzingen verkregen voor de aanwezigheid van beide gedeelten van de moederstof in dit type metabolieten. Naar aanleiding hiervan, werd er een nieuwe methode ontwikkeld voor het aantonen van gebonden metabolieten van furazolidon, gebaseerd op het vrijmaken en detecteren van de 3-amino-2-oxazolidinon zijketen. Met behulp van deze relatief eenvoudige methode kon vervolgens worden aangetoond dat tenminste 75% van de metabolieten die gebonden waren aan het eiwit van hepatocyten, deze zijketen bevatte, waarmee voor de eerste keer werd aangetoond dat in elk geval een deel van de gebonden metabolieten een zekere mate van verwantschap vertoont met de moederstof.

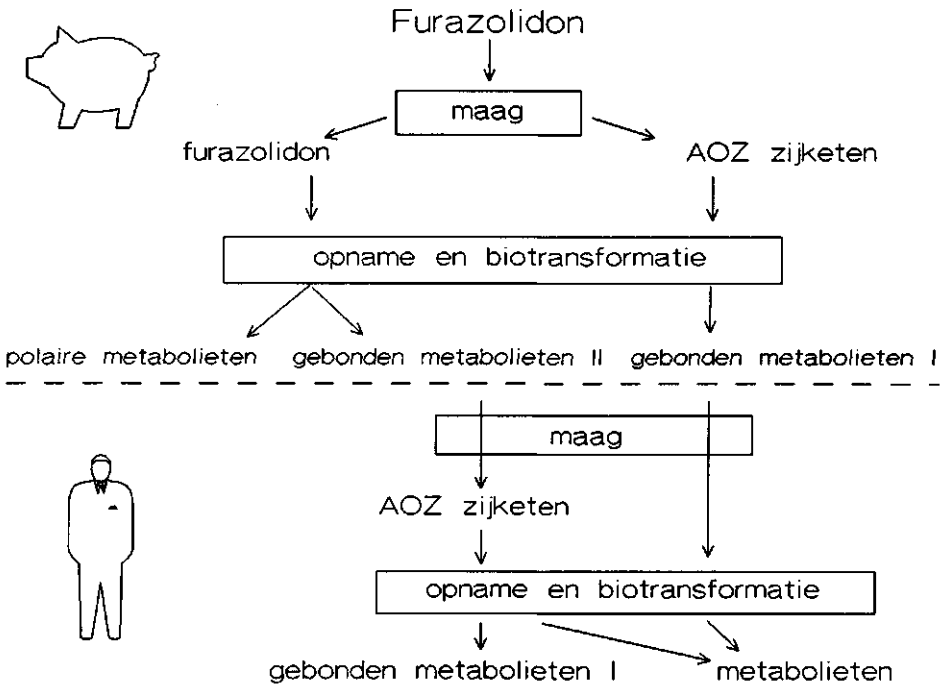

Fig. 10.1 Schematische weergave van het mechanisme dat mogelijk ten grondslag ligt aan de vorming van gebonden residuen van furazolidon in het doeldier en de mogelijke lotgevallen van gebonden residuen na opname door de consument.

Met diezelfde methode kon de zijketen ook worden vrijgemaakt van 15-25% van de gebonden metabolieten in levermonsters van biggen, behandeld met furazolidon, zelfs na opslag gedurende zeven jaar bij -40 °C.

De verschillen tussen *in vivo* en *in vitro* studies, voor wat betreft de fracties gebonden metabolieten waarvan de zijketen kan worden vrijgemaakt, kunnen wellicht worden toegeschreven aan de rol van de maag bij de biotransformatie van furazolidon in het dier; door zure hydrolyse van de azomethine band in het furazolidon-molecule zou 3-amino-2-oxazolidinon kunnen worden gevormd (Fig. 10.1), dat vervolgens wordt geabsorbeerd en omgezet, mogelijk via het ß-hydroxyethylhydrazine (Fig. 6.1), in een stof die zich kan binden aan eiwitten (type I). Zoals beschreven in Hoofdstuk 6 zijn er sterke aanwijzingen dat dit mechanisme ten grondslag ligt aan de irreversibele MAO-

remming. De gebonden metabolieten *in vivo* zouden derhalve deels kunnen worden toegeschreven aan een reactieve verbinding afkomstig van de zijketen (type I) en deels aan reactieve verbindingen gevormd door reductie van de nitro-groep (type II), waarbij dus de zijketen intakt blijft (zie Fig. 7.1). In het geval van incubaties van furazolidon met microsomen en hepatocyten bij pH 7, lijkt het waarschijnlijk dat er slechts sprake is van de vorming van het laatste type gebonden metabolieten (type II). Aangezien alleen van deze metabolieten de AOZ zijketen kan worden vrijgemaakt, zou hiermee het verschil met *in vivo* verklaard kunnen worden. Het kan niet worden uitgesloten dat een soortgelijk mechanisme, namelijk de zure hydrolyse van de azomethine band en het activeren van het daarbij vrijkomende AOZ, verantwoordelijk is voor de hoge biologische beschikbaarheid van gebonden residuen en de hernieuwde vorming van gebonden metabolieten in ratten, gevoerd met vlees van een met furazolidon behandelde big.

Dit is de eerste keer dat aan de hand van concrete resultaten aannemelijk kan worden gemaakt dat gebonden metabolieten van een stof nadelige effecten kunnen veroorzaken na opname door de consument. Derhalve is het van groot belang om de aanwezigheid van dergelijke residuen zoveel mogelijk te beperken en om analytische detectie methoden te ontwikkelen voor de controle hierop, zoals beschreven in dit proefschrift. Daarbij moet erop gewezen worden dat de potentiële toxiciciteit van gebonden residuen van andere stoffen sterk afhankelijk zal zijn van de structuur en eigenschappen van die stoffen en derhalve van geval tot geval bekeken dient te worden. Met deze studie wordt wel nog eens het belang van het betrekken van metabolieten in de risico-beoordeling van in de landbouw gebruikte stoffen onderstreept.

Samenvattend kan gesteld worden dat de resultaten van dit onderzoek de vele mogelijk-heden laten zien van *in vitro* modellen met hepatocyten van grote landbouw-huisdieren. Gezien de hoge kosten en speciale voorzieningen die verbonden zijn aan studies met grotere dieren, zal het gebruik van dit soort *in vitro* modellen niet alleen leiden tot een reductie van het aantal voor experimenten gebruikte dieren, maar ook de mogelijkheden vergroten om de gedetailleerde gegevens te verzamelen omtrent de voor dit soort dieren specifieke biotransformatie en toxiciteit van stoffen, zoals die meer en meer gevraagd wordt door overheidsinstanties. In het algemeen kunnen dergelijke modellen bijdragen tot de ontwikkeling van nieuwe middelen met een minimaal risico voor de consument en weinig bijwerkingen bij het doeldier.

Zoals verder uit deze studie blijkt, kan een beter inzicht in de identiteit en eigenschappen van metabolieten, tevens leiden tot de ontwikkeling van nieuwe analytische methoden voor de controle op een goede toepassing van hulpstoffen in de dierlijke produktiesector.

CURRICULUM VITAE

Ron (L.A.P.) Hoogenboom werd geboren op 4 november 1959 te Maastricht, en behaalde in 1978 het diploma gymnasium ß aan het Bisschoppelijk College St. Jozef te Sittard. In dat jaar begon hij zijn studie aan de Landbouwhogeschool te Wageningen, waar hij in 1985 afstudeerde in de richting Voeding, met Toxicologie als verzwaard hoofdvak.

Sinds september 1985 is hij werkzaam bij de afdeling Toxicologie van het Rijks-Kwaliteitsinstituut voor Land- en Tuinbouwprodukten (RIKILT) te Wageningen. Vanaf september 1986 is hij daar actief met het onderzoek naar de mogelijkheden van *in vitro* modellen met hepatocyten van landbouwhuisdieren.

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