



Report of the Scientific Committee for Animal Nutrition on the Use of Ronidazole in Feedingstuffs for Turkeys

(Opinion expressed, 26 September 1997; Text consolidated: 5 November 1997)

TERMS OF REFERENCE (June 1996)

The Scientific Committee for Animal Nutrition (SCAN) is requested to give an opinion on the following questions:

1. Is it possible that, by using Ronidazole (E-759) in the feedingstuffs for turkeys, according to the conditions set up by Council Directive 70/524/EEC (1) (See background), residues with the intact nitroimidazole structures may still be present, beyond the six-day pre slaughter withdrawal period?
2. In view of (1) above, may the consumer be exposed to these residues ? and would these residues be of toxicological concern ?

(1) Concerning additives in feedingstuffs (OJ No L270, 14.12.70 p.1.) as amended by Directives 84/58/EEC (O.J. No. L319 8/12/84 p 13) and 93/114/EC (OJ L334 31.12.93, p. 24)

BACKGROUND

In Accordance with the provisions of Council Directive 70/524/EEC, the use of ronidazole (E-759) is authorised at Community level in the Annex I, Section D (Coccidiostats and other medicinal substances) as follows (See Table I)

Table I. Annex I, Part D (Coccidiostats). Ronidazole

Species or category of animal	Maximum age	Minimum / Maximum content mg/kg of complete feedingstuff		Other provisions
Turkeys	-	60	90	Use prohibited from laying age onwards and at least 6 days before slaughter respectively.

In 1976 the Commission asked the SCAN a question on the use of nitroimidazole derivatives (Dimetridazole, Iprnidazole, Ronidazole) in feedingstuffs, concerning:

- if the products, as authorised, did show in the experiments any mutagenic or carcinogenic effects,
- if the use of these products as additives in feedingstuffs could result in the presence of residues under the authorised conditions of use,
- if these residues could be harmful to the consumer, and
- if in view of the answers to the above-mentioned questions, the use as additives in feedingstuffs of the products concerned or of some of them be prohibited in Member States or their conditions of use should be modified?

In answer to this question, the Scientific Committee for Animal Nutrition expressed its favourable opinion in its Reports of 5 October 1977 " *On the use of Nitroimidazole derivatives in feedingstuffs*" (2).

(2) Reports of the Scientific Committee for Animal Nutrition, First Series (1979) Catalogue N° CB-28-79-277-EN-, Page 11. Concerns Dimetridazole (Turkey, Guinea Fowl & Swine), Ipronidazole (Turkeys & Guinea Fowl), Ronidazole (Turkey & Swine).

At that occasion the SCAN observed:

1. An increase in the number of benign and malignant mammary tumours and also mutagenic effects were observed in laboratory animals, in particular in rats, to which high doses of dimetridazole, ronidazole and ipronidazole were administered orally over their lifetime.
2. The sensitivity and specificity of analytical methods and also knowledge of the metabolism of these products enable a precise evaluation of their residues to be made. These are made up of the initial compound and oxidation products, which undergo rapid breakdown in animal products after withdrawal of the additive in the diet.

Under the conditions of use for these additives (in 1976) and, in particular, for withdrawal periods varying between 3 to 5 days, it may be stated that, at the lower limit of analytical determination (0.002 mg/kg) there are no significant residues in the edible products.

Possible traces in muscle or skin are broken down through cooking and during cold storage.

Therefore the Committee was of the opinion of that there was no reason to prohibit the use of dimetridazole, ronidazole or ipronidazole as additives in feedingstuffs, and that in order to ensure the absence of residues in products of animal origin and taking into account the similarity of their metabolism, the withdrawal periods before slaughter should be standardised, with an additional safety factor. To this effect, a withdrawal period of at least 6 days was recommended for each of these additives.

On the other hand, and taking account of their efficacy for the various animal species, minimum and maximum dose-levels were recommended.

Later to this, the Commission requested the SCAN to discuss the safety of use of Nitroimidazoles and at the request of the Federal Republic of Germany, and of the United Kingdom, at the 90th, 91st and 93rd meetings.

- At the 90th meeting (29 September 1994) a note from the DE delegation concerning ronidazole as additive was discussed. In this regard previous information (SCAN/94/34, SCAN/94/35) was also circulated.
- At the 91st meeting, and at the request of the UK delegation, document SCAN/94/104 concerning resistances was distributed for information.
- At the 93rd meeting (23-24 March 1995) information on Codex Committees on Residues were given

Since 1994, other information distributed by the Commission are shown at the end.

In January 1996, a communication from the Federal Republic of Germany was made to the Commission according to which,

'Since the parent substance Ronidazole is mutagenic and carcinogenic, when judging its safety, the bound residues must also be taken into consideration since it cannot be ruled out that these residues retain the intact nitroimidazole structure or that the breakdown of protein adducts will again release the nitroimidazole structure.'

The withdrawal period of 6 days laid down in the case of turkeys assumes the elimination of the parent substance and the assumes that the animal edible-products is free of residues detection limit of the method: 2 mg). There are problems with this method when persistent residues occur, which, because of their properties, are very difficult to detect.

With the knowledge available to us today, it is essential that these residues are taken into consideration since the more recent tests on Metronidazole indicate that the genotoxic potential of Ronidazole needs to be reassessed, and, at the

same time, there is the possibility of that residues, with the intact nitroimidazole structures may still be present beyond the six-day period. Therefore, consumer exposure to these residues cannot be ruled out".

A file, with the information in support of the FRG's view, have been submitted.

OPINION OF THE COMMITTEE

In answering Question 84 by the Commission, the SCAN has considered necessary first to answer the notification of the health authorities of Germany (Part 1) and then to answer the Commission's Question.

PART 1:

CONSOLIDATED RESPONSE TO THE LIST OF ARGUED POINTS FROM THE NOTIFICATION OF GERMANY EXERCISING THE CLAUSE OF SAFEGUARD PROVISION IN ARTICLE 11 OF DIRECTIVE 70/524/EEC CONCERNING THE USE OF RONIDAZOLE IN FEEDINGSTUFFS FOR TURKEY

(The following SCAN's consolidated response has been done on the basis of the data available and taking into account the Merk Sharp & Dohme dossier.

Part II : Ronidazole, Preliminary Remarks :

II .A.I. *The German Delegation first pointed out in 1992 in the meetings of the expert committee "additives" that health effects of veterinary medicine containing nitroimidazole, including ronidazole, were to be evaluated anew; and requested a review of the authorization of the additive ronidazole. This was due to the so-called "negative monographies" of the (then) Bundesgesundheitsamt, carefully examining the veterinary medicines dimetridazole, metronidazole, and ronidazole. The "negative monographs" concluded that, because of serious health concerns, the use of the above substances as veterinary medicines in food-producing animals could no longer be justified. Inter alia, the following is said about ronidazole :*

II .A.I.I. *"In animals treated with ronidazole the drug leads to bound tissue residues with persisting imidazole structure, the safety of which (with regard to toxicity) can currently not be confirmed with absolute safety. Carcasses usually contain these residues.*

SCAN comments:

The metabolic fate of ronidazole has been established 20 years ago in the turkey and pig. Taking into account the methods and techniques available at that time, these studies were correctly assessed. However certain data are lacking, such as the nature of the fecal metabolites that could highlight the role of the intestinal flora, in the reduction of the nitro group.

Another weakness is the lack of precise quantification of the distribution of the different metabolites in the urine and tissues. Additional studies concerning the appearance of ronidazole bound residues in the pig have been published.

A central point that deserves attention is the fact that chemical treatment (oxidation in acid medium) of bound residues in pig muscle generates a minor (1%) quantity of 1-methyl-5-nitro-imidazole (Wolf et al., 1983; Journal of Agriculture and Food Chemistry 31, 559-564), i.e. a molecule harbouring a 5-nitro group which is potentially genotoxic. However, the unequivocal *in vitro* and *in vivo* characterization of the metabolic pathway leading to the formation of these bound residues in the pig (Alvaro et al., 1992; Chemical-Biological Interactions 82, 21-30), involving an initial 4e- reduction of the nitro group, rule out the possibility of this transformation.

It may be concluded that the identified intermediary metabolites and final bound residues do not induce genotoxic effects in different *in vitro* systems (Wislocki and Lu, 1990; Drug Metabolism Reviews 22 (6-8), 649-661). This is in agreement with the metabolic rationale. A technical artifact, such as ronidazole contamination in the treatment of sample, or a limited chemical re-oxidation of the 5-amino group of the free residue, might explain the detection of a

small quantity of that nitroimidazole derivative in the Wolf study.

II.A.1.2. Ronidazole proved to be embryo-toxic in mice, rats, and rabbits, and proved to be carcinogenic in mice and rats. The International Agency for Research on Cancer (IARC) evaluated the structural analogue metronidazole as possibly carcinogenic to humans and classified it under group 2Ab.

SCAN comments:

In relation to the reproductive toxicity studies, a three-generation reproduction study was carried out in male and female Charles River CD male rats. Ronidazole was administered in the diet at concentrations of 0.0%, 0.02%, 0.04% and 0.08%, seven days a week, 70 days before the first mating and throughout the production on three generations. These dietary concentrations resulted in approximate dosage levels of 0, 15, 30, and 60 mg/kg/day. Two litters were produced with each generation of parents. The second litter was used to produce the succeeding generation and the first litter was examined and then discarded at weaning. There were neither alterations in behaviour nor in appearance, body weight or mean food consumption values. No compound-related abnormalities were noted in the pups at any dietary level of ronidazole. During each of the six whelping phases, the fertility, pregnancy period, viability and lactation indices were comparable for the control and treated groups.

There were no adverse effects on the average body weight of pups at birth. There were significantly fewer numbers of pups per litter at the 0.08% concentration (60 mg/kg/day) than at the control or lower dietary levels. There were no compound-related gross or microscopic changes in any of the treated pups of the second litter of the third generation.

Teratology studies were performed in mice, rats and rabbits. Ronidazole was administered orally to pregnant CF1 mice from the 6th through the 15th days of pregnancy at dosage levels of 50, 100, and 200 mg/kg/day, and to pregnant New Zealand rabbits at 3, 10, and 30 mg/kg/day from the 7th through the 15th day of pregnancy. No teratogenic, embryotoxic, or foetotoxic effects attributable to ronidazole treatment were observed in mice at any of the doses studied or in rabbits at 3 and 10 mg/kg/day. In mice given 50 and 100 mg/kg/day or in rabbits at 3 and 10 mg/kg/day, average maternal weight gain was unaffected, although at 200 and 30 mg/kg/day in mice and rabbits, respectively, a significant decrease in weight gain occurred. In rabbits given 30 mg/kg/day of ronidazole, no embryotoxicity occurred, but average foetal weight was significantly decreased. In rabbits given 30 mg/kg/day, occasional foetuses with malformations involving the heart and great vessels were observed although these quoted malformations have been observed to occur spontaneously in rabbits.

Ronidazole was also administered orally to pregnant rats at dosage levels of 50, 100, and 200 mg/kg/day and in a second study at dosage levels of 100, 150, and 200 mg/kg/day. No compound-related embryotoxicity occurred at 50, 100, or 150 mg/kg/day of ronidazole.

Average fetal weight per litter was unaffected at a dosage level of 50 mg/kg/day of ronidazole. At 100 mg/kg/day and above (150 and 200 mg/kg/day) decreases in average fetal weight per litter were observed. At dosage levels of 150 or 200 mg/kg/day, average maternal weight gain was significantly retarded. No death occurred in any of the compound-treated groups from either study. In conclusion both maternal and foetal weights were reduced at the highest doses but there was no statistically significant teratogenicity.

With respect to carcinogenic activity, a chronic toxicity study (22 months) was carried out. Ronidazole was administered to both male and female rats at doses of approximately 10, 20 and 40 mg/kg/day. These doses would correspond approximately to 250, 500 and 1000 ppm in the complete feed. During the first 12 months, no sign of tumorigenic activity attributable to the compound was shown in the treated animals, but during the second year of the experiment the females developed subcutaneous masses and at the end of the experiment an increased incidence of mammary fibroadenomas were scored in all groups of treated animals compared to the control one. The figures relative to the increased incidence of benign mammary tumors were + 86% , + 200% and + 171% for the 10, 20 and 40 mg/kg/day groups respectively. The male showed an increase of the same type of mammary tumors only at the 40 mg/kg/day level of treatment (5 animal out of 32). The incidence of malignant mammary tumors both in female and male rats was low and moreover not clearly dose-related.

From this study no definitive explanation for this tumorigenic effect of ronidazole could be inferred owing to the incidence of spontaneous mammary tumors in the strain of rat used. A possible correlation with an estrogenic activity of ronidazole was considered but it was found that this nitroimidazole does not exert any estrogenic effect even at doses as high as 200 mg/kg/day as verified in ad hoc experiments.

A supplemental 104 weeks oral toxicity study on rats fed with diets containing ronidazole at concentration providing doses of 0.5 and 5 mg/kg/day, confirmed neither significant changes in organ weight nor macroscopic and microscopic anatomical damages related to the treatment.

In dogs administered daily with oral ronidazole in gelatine capsules seven days each week for 104 weeks at dosage levels of 10, 20, and 30 mg/kg (40 mg/kg for the first month), slight signs of occasional intolerance (tremors and dehydration) were observed in the low dosage treated group. The animals treated with the higher doses exhibited alterations of the nervous system, cardiovascular system and particularly testicular toxicity, but no tumours.

This finding was confirmed in another supplemental 105 weeks chronic toxicity study in dogs treated with 0.5 and 5 mg/kg/day. In this study no relevant compound-induced changes were detected in these animals, following histomorphologic examination of their organs.

Carcinogenic studies were performed in rats receiving dietary concentrations of ronidazole corresponding to 5, 10 or 20 mg/kg/daily for 104 weeks. *Post-mortem* examination revealed increased incidence of benign fibro-epithelial mammary tumors in males treated with high dosages and in females treated with mid- and high dosages.

Mice submitted to a comparable experimental design also showed an increase in pulmonary neoplasms. These were more frequent in males than in females and proved to be adenomas rather than carcinomas. The global tumor incidence reached significant values only in animals treated with the highest dose level (i.e. 20 mg/kg/day).

It may be concluded that ronidazole increased the incidence of benign mammary tumors in rats and of both benign and malignant pulmonary tumors in mice but only at 20 mg/kg b.w./day and above.

In the German Delegation communication, continuous reference is made to metronidazole. The SCAN notes that metronidazole is not authorized as a feed additive and although structural similarities exist to ronidazole, the results of the experimental data, and the conclusions derived from it cannot be extrapolated indiscriminately

It must be noted that metronidazole is approved as human medicinal product and widely used..

II.A.I.3. *There can be no doubt about mutagenic effects in various bacteria and in drosophila in a multitude of the tests' terminal points. Only in organisms deficient in nitro-reductase can negative test results be observed. However, there are insufficient overall data on the mutagenicity of ronidazole.*

SCAN comments:

In vitro, ronidazole increased the number of revertants in histidine mutants of TA 1530 and TA 1535 strains of *Salmonella typhimurium* when tested at concentrations starting from 1-3 mg/plate up to a maximum of 50 mg/plate. Higher concentrations of ronidazole could not be tested because of a clear antibacterial activity. The results were not affected by the presence of rat liver microsomes sub-fraction, i.e. an *in vitro* metabolizing system (see German monograph).

Also in the sex-linked recessive lethal test in *Drosophila melanogaster* ronidazole provided positive results for mutagenicity. By contrast, in a dominant lethal test in mice given 50-200 mg/kg/day ronidazole neither lethal effects nor adverse effects on mating performances were observed.

In an *in vivo* micronucleus test performed in mice administered with two or five daily set of doses from 50 to 200 mg/kg/day ronidazole no mutagenic properties were seen.

Cytogenetic studies on mouse bone marrow were performed in singly dosed or 5 days dosed animals. The dosage

regimens examined were : 50, 100 and 200 mg/kg b.w. The multiple dose regimen was performed to better understand some increases in chromosome aberrations observed in singly dosed animals. This was thought to be due to an abnormally low frequency of these cytogenic abnormalities recorded in the control animals. From the multiple dosing experiment no effect was observed in the 50 and the 200 mg/kg/day groups while a significant increase in the number of cells bearing broken chromosomes was recorded in the 100 mg/kg/day group. Owing to the dose-independence of the event the findings should not be considered as reliable evidence of genotoxicity.

In conclusion, ronidazole revealed mutagenic activity in bacterial tests (i.e. on prokaryotic cells). In eukaryotic species with the exception of the sex-linked recessive lethal test in *Drosophila melanogaster* there were no consistent findings of genotoxicity using a variety of tests. This is consistent with the antibacterial effects of ronidazole and the other nitroimidazole derivatives which depend on damage to bacterial DNA following reduction of the nitro group.

II.A.1.4. Mechanistic studies, showing a direct interaction of reactive ronidazole metabolites with DNA have been confirmed in recent examinations proving the clastogenic (mice) and genotoxic (humans) effects of metronidazole. These findings suggest a genotoxic mode of action also for ronidazole in the organisms of mammals and humans.

It is absolutely necessary to make further studies of genotoxicity, carcinogenesis and metabolism of bound residues in order to be able to make a final evaluation of ronidazole.

SCAN comments:

Due to the complex biotransformation undergone by ronidazole in animals and despite its ready clearance in treated animals, small amounts of residues which are not extractable still persist for long time. Such residues bound to body tissues could indicate possible concern for the consumer.

Through the use of ¹⁴C-labeled ronidazole, a conclusion has been reached that part of the residual material is of no toxicological interest because it is the result of incorporation of ¹⁴C-label into one- and two- carbon fragments produced following ronidazole degradation through normal intermediary metabolic pathways.

We note that the negative results obtained in the Ames tests with a range of hypothesized and/or identified ronidazole metabolites extracted from muscle of treated pigs were considered by JEFCA (34th Meeting) valid in assessing genotoxicity of ronidazole.

Additional bound residues have been however detected which comprises compounds partially retaining the structure of the parent compound. To investigate the mutagenicity of these residues (and following the demonstration that a comparable metabolism is performed by rat hepatocyte microsomes *in vitro* and by pigs *in vivo*) metabolites of ronidazole obtained by incubation with rat microsomes *in vitro* were submitted to the Ames test, which had given positive results for the parent compound.

Three types of materials were tested i.e. (a) compounds structurally related to the bound residues resulting from *in vitro* incubations, namely the acetylamino derivative resulting from a sequential process of decarboxylation and nitro-reduction of ronidazole, the mono-cysteine-ronidazole adduct and the dithionite reduction product of ronidazole bound residues, (b) free and bound microsomal metabolites of ronidazole including soluble cysteine- and protein-bound adducts and (c) bound residues previously digested with an enzymatic process mimicking the human gastrointestinal digestion (37°C and combined treatments by pepsin, chymotrypsin and trypsin followed by carboxy- and leucylamino-peptidase). All these materials showed very low or no mutagenic activity when compared to the parent compound (Wislocki and Lu, 1990; Drug Metabolism Reviews 22(6-8), 649-661).

A structure-activity relationship analysis for the mutagenicity of these compounds revealed the key roles of the carbamate group and the methyl group at 4 position in the ronidazole molecule. As a matter of fact dimetridazole (devoid of the carbamate group and methylated at C-4 position) showed 10% and 1% residual mutagenic activity respectively when compared with ronidazole.

II.A.I.5. The veterinary medicinal product ronidazol was included in Annex IV to Regulation No. 2377/90/EEC

through Commission Regulation No. 3426 No. 3426/93/EC, adopted at the end of 1993. Pursuant to Article 5 of the above Regulation, this Annex includes substance for which it is not possible fix a maximum level for residues of a substance which is contained in veterinary medicinal products and which is pharmacologically effective, since this substance's residues in foodstuffs of animal origin represent a risk to the consumers' health regardless of its concentration. There is an EU-wide ban on administering the substances listed in Annex IV to animals used for food production.

Because of the current EU-wide ban on using the veterinary medicinal product ronidazole in food-producing animals the Federal Government asked the Commission repeatedly to adopt measures to withdraw the authorization of the feed additive ronidazole. The Federal government underlined this request stating that, due to consumer protection, it could no longer be justified to authorize ronidazole as a feed additive, since even if the prescribe 6-day waiting period was complied with there would still be the danger of exposing consumers to residues which might cause health problems

SCAN comments:

This question has already been answered in this report.

Part A: *Health evaluation of the feed additive ronidazole, bases on data which are currently available.*

Part A(a): *Mutagenic and carcinogenic qualities of the parent substance ronidazole.*

A. A(a).1: *The parent substance ronidazole has mutagenic and carcinogenic qualities. It has not been clarified how ronidazole's dose-related, carcinogenic effect comes about. A genotoxic mechanism of the carcinogenesis cannot be ruled out.*

A. A(a).2: *The results of mutagenicity studies with ronidazole carried out to date do not permit a clear interpretation. This is the reason why it is necessary to draw analogous conclusions from studies with other 5-nitroimidazoles, e.g. metronidazole. In two recent studies (Reitz et al., 1991b; Reitz et al., 1991a) the use of metronidazole induced DNA single-strand breaks.*

It is true that one assumes that the damage is eliminated by natural DNA repair processes (Reitz et al., 1991a), but in patients with impaired repair mechanisms one has to assume, due to the above studies, that metronidazole might damage the genotype -and, by analogy, that this could also apply to the structurally related ronidazole.

SCAN comments:

As expressed earlier the toxicological data obtained with metronidazole cannot with certainty be extrapolated to ronidazole. Although the two substances belong to the same 5-nitroimidazole family, their chemical structure and therefore their reactivity towards living organisms is different. The nature of the leaving group at position 3 as well as the unsubstituted C-4 position are essential in this respect. From a metabolic point of view, metronidazole undergoes an oxidation at the N-3 position (N-oxyde) which does not occurs with ronidazole. This supports the view that it is innappropriate to use data from metronidazole studies to clarify the toxicological properties of ronidazole.

Part A(b) *Residue behaviour of ronidazole*

A.A(b).1. *In an evaluation whether ronidazole is safe or not as regards its residue behaviour, bound residues have to be taken into consideration as well, since they show an intact nitroimidazole structure, as proven in a residue study of swine. Therefore it is possible that the nitroimidazole structure (comparable to nitrofurans) is released once again when the bound molecules are decomposing, e.g. via hydrolysis or a metabolic activation of the gastro-intestinal tract.*

SCAN comments:

The existence of an intact and releasable 5-nitroimidazole structure in the bound residues is questionable due to the

limitations of the methodology used by Wolf et al. (1983)(Journal of Agriculture and Food Chemistry 31, 559-564). The fact that the digestion of these bound residues in simulated intestinal fluids do not release genotoxic material is an additional and strong argument against the existence of intact 5 nitroimidazole bound residues.

A.A(b).2. *The waiting period for the use of ronidazole as feed additive in a dose of 60 to 90 mg/kg was to be six days due to the assumption that the parent substance ronidazole is completely eliminated and thus that the food produced is residue-free (detection limit of method : 2 ppb).*

However, the assumption that it is residue-free, made on the basis of the most sensitive method and taking account of a safety period, only applies to the parent substance ronidazole; possible persistent residues are not taken into consideration.

Part A(c) *Evaluation of health data*

A.A(c).1. *According to the present findings it is absolutely necessary to also take account of the persistent residues, since :*

- 1. *Apart from the proven carcinogenic qualities of ronidazole, recent studies of metronidazole (Reitz et al., 1991a, Reitz et al., 1991b) also indicated a genotoxic potential of ronidazole.***
- 2. *Examinations of swine (Wolf et al., 1983) showed that there are residues with an intact nitroimidazole structure going far beyond the 6-day period. Similar results cannot be ruled out as regards the use of ronidazole in turkeys.***

SCAN comments:

These latter questions are not relevant to ronidazole because they concern specifically metronidazole.

A.A(c).2. *Compared to the data available at the time when ronidazole has been authorized this has considerably broadened the knowledge on the occurrence and the structure of bound residues. This shows that it is absolutely necessary to evaluate anew the situation regarding residues. In doing so, one has to keep in mind that the bound residues can hardly be detected and that there is no marker helping to draw conclusions concerning the quantity of these residues.*

A.A(c).3. *The present studies of the toxicity of these bound residues do not suffice for a final evaluation of their relevance.*

However, against the background of the mutagenic and carcinogenic qualities of the parent substance ronidazole and a possible renewed release of the nitroimidazole structure of the parent substance from bound residues one has to expect risks to consumers' health due to toxicologically relevant substances caused by the use of ronidazole as a feed additive, even if the prescribes 6-day waiting period is adhered to.

SCAN comments (paragraphs A.A(b).2., A.A(c).2. and A.A(c).3.):

The long lasting radioactive residues found in tissues correspond mainly to the metabolic incorporation of ronidazole carbon fragments into normal tissue components as a result of the biotransformation of the molecule. This is without toxicological significance. Only a very limited fraction (1%) of the radioactivity was attributed to a 5-nitro-derivative. Taking into account the metabolic rationale leading to the formation of bound residues, and the fact that metabolites and products released by digestion of the tissues are not genotoxic, it can be considered that these residues are of no toxicological concern either. Therefore ronidazole should be considered as the marker residue.

Part B : *Data on the veterinary Medicine ronidazole*

B.A.1. *To complement Part A, Part B of this dossier presents expert opinions and monographies which, regarding the evaluation of the veterinary medicinal product ronidazole, represented the basis for including the substance in Annex IV of Regulation No. 2377/90/EEC.*

Pursuant to Article 5, the inclusion of a veterinary medicinal product in Annex IV of the above' Regulation requires that no maximum level can be fixed, since residues of the relevant substance in foodstuffs of animal origin represent a risk to consumers' health regardless of its concentration.

B.A.2. *The fact that ronidazole is being used under conditions (dose, indication, animal species) comparable with the use of the former veterinary medicinal product, and the fact that its use as a veterinary medicinal product has been prohibited with regard to consumer protection, automatically entail a request to also withdraw its authorization as a feed additive.*

SCAN comments:

The SCAN is not in a position to comment about the reports made by the CVMP but notes that its range of actions is constrained by the specifications of the 4 annexes into which it may place the veterinary drugs which it assesses.

Conclusions of the Federal Government

C.A.1. *If ronidazole is used as feed additive, residues are found in the animal's tissue, even if the prescribed 6-day waiting period has been observed. Thus, with regard to the mutagenic and carcinogenic qualities of the parent substance ronidazole and a possible renewed release of the nitromidazole structure of the parent substance from the bound residues, a risk to consumers' health caused by the use of ronidazole as a feed additive cannot be ruled out, even if it is used as prescribed. From the point of view of precautionary protection of consumers' health, the Federal Government considers that this risk cannot be justified.*

C.A.2. *Similar considerations were also decisive for the ban imposed in the sector of veterinary medicine, for which Directorate-General III was responsible. The clear characterization of the ronidazole residues concerned, i.e. by integrating them in Annex IV of Regulation No. 2377/90/EEC ("a risk to consumers' health regardless of its concentration"), leads to the conclusion that thus the requirement of Article 7 paragraph 2 letter A sub-paragraph b of Directive 70/524/EEC on additives in animal nutrition is no longer met.*

C.A.3. *Therefore, the Federal Government asks the Commission to introduce as quickly as possible the necessary steps aiming at an EU-wide ban on the feed additive ronidazole.*

SCAN conclusions:

1. Ronidazole clearly exhibits mutagenic activity against prokaryotic cells and the sex-linked recessive lethal test in *Drosophila melanogaster*. The positive responses are most likely related to nitro-reductase activity although this has not been unequivocally demonstrated. All tests performed on mammalian cells (which do not express nitro-reductase activity) were negative.
2. Mammary neoplasms in female and male rats, as well as pulmonary neoplasms in male and female mice have been observed. As the raw data were not available, it is not possible to analyze further important parameters such as the historical aspects of the spontaneous tumors of the animal strains used or indications for a possible epigenetic origin of the tumors. Therefore a conclusive evaluation of the mechanism of carcinogenicity and consequently human risk assessment, cannot be made.
3. The extrapolation of data on the mutagenicity and carcinogenicity of metronidazole to ronidazole cannot be accepted as chemical compounds belonging to the same family may behave entirely differently from a pharmacokinetic, metabolic and subsequently toxicological point of view.
4. Some data on the metabolic fate of ronidazole in turkeys, such as the nature of fecal metabolites or ronidazole metabolic distribution in the tissues along the withdrawal period are lacking. However a substantial amount of sound data have been produced for the pig. This could reasonably be expected to apply to the turkey, although it would require justification. However, the discrepancy observed between the very well documented ronidazole metabolic pathway leading to bound residues, and the limited data on the occurrence of trace quantities of a nitroimidazole compound chemically released from these residues, strongly suggests the possibility of an analytic artefact.
5. Notwithstanding the fact that many of the scientific arguments addressed by DE to support the ban of ronidazole

cannot be fully accepted, several important issues remains unclear, namely data on possible genotoxic effect on eukaryotic cells, carcinogenicity, metabolic fate and residues in turkeys. Therefore an ADI cannot be established at present.

Part 2

Response to Question 84 by the Commission

1. The critical issues regarding the safety of ronidazole residues in food for human consumption are the mutagenic and carcinogenic potential of the parent compound and/or its biotransformation products.

Limited data are available on the metabolic fate and residue kinetics of ronidazole in turkeys. However many studies performed in pig show that ronidazole undergoes a rapid depletion (Wolf et al., 1983; Journal of Agriculture and Food Chemistry 31,559-564).from tissues even if more persistent residues (bound residues) are detectable. Among these bound residues those maintaining an 5-nitroimidazole structure are of special concern because ronidazole reactive metabolites originate from the nitro group reduction. Based on the isolation of ^{14}C -labelled methionine from hydrolyzed tissues of pigs treated with N- $^{14}\text{CH}_3$ -labelled ronidazole, it was assessed that some of the nonextractable residues are ^{14}C -labelled endogenous products arising from the incorporation of ^{14}C into one- and two-carbon fragments coming from the pathways of normal intermediary metabolism. These endogenous products are of no toxicological concern.

However, some residues are compound-related bound-residues as evidenced by the formation of ^{14}C -methylamine and ^{14}C -oxalic acid from the hydrolysis of tissues from swine treated with N- $^{14}\text{CH}_3$ -ronidazole and 4,5- ^{14}C ronidazole, respectively.

Comparative metabolic studies of ronidazole in vivo (pigs and rats) and in vitro (rate hepatocyte microsomal preparations) provided results which enabled to clarify the mechanism of formation and the nature of the bound residues. From these studies it appears that ronidazole undergoes an initial 4e-reduction leading to the hydroxylamine intermediate and then to the amine. This reaction is coupled to a sequential addition of an unknown nucleophile to carbon 4, followed by the split of carbamate and deprotonation. The resulting 5-aminoimidazo reactive intermediate binds to protein cystein thiol residues at the 2-methylene carbon or possibly the 4-position, yielding the protein-bound adduct. The detection of a very limited amount (1%) of 1-methyl-5-nitroimidazole following the severe acidic treatment of pig liver bound residues is contradictory to the metabolic rationale of the genesis of these residues, possibly being due to a methodological artifact.

These metabolic data show that the mutagenic effect of ronidazole residues in pig tissues should be ascribed to the parent compound only. Conclusively ronidazole should be considered as the market residue.

The Ames test revealed mutagenicity of ronidazole at 1-3 mg/plate level of Salmonella strains. Mutagenicity studies carried out with either compounds structurally related to the bound residues or compounds derived from microsomal metabolism (including bound residues) or digests of proteins from treated pigs, indicated that the protein bound ronidazole materials are not mutagenic.

Under these considerations the bound residues of ronidazole detectable in the tissues of pigs at the proposed time of slaughter (i.e. 6 hours ronidazole withdrawal) dot not pose hazard for the consumer.

Even if it seems reasonable to anticipate that a similar ronidazole behaviour would occur also in the turkey, this should be verified experimentally.

The overall conclusion it that up to date in ADI cannot be proposed.

2. On the basis of the above, a conclusive evaluation of ronidazole cannot presently be made for the following reasons :

- lack of data on possible mutagenic risk for eukaryotic cells;

- absence of the raw data from the carcinogenicity experiments (i.e. mammary adenoma in rats and the pulmonary tumours in mice)
- limited data on the metabolic fate and residues (bound residues and residue depletion) of ronidazole in the turkey.

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