EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C3 - Management of scientific committees II; scientific co-operation and networks

Opinion of the Scientific Committee for Animal Nutrition on the use of Dimetridazole in animal feedingstuffs.

(expressed on 12 September 2000)

TERMS OF REFERENCE (June 1996, extended 11 November 1997)

The Scientific Committee for Animal Nutrition (SCAN) is requested to answer the following questions

1. Is it possible

a. to assess the toxicological potential of DMZ (suspected of having genotoxic and carcinogenic properties, namely on the basis of recent test results concerning the structurally analogous compound metronidazole) or DMZ-OH or of its bound residues on the basis of the available data ?

b. that, by using DMZ (E-754) in the feedingstuffs for turkeys, according to the conditions set up by Council Directive 70/524/EEC, both the parent substance (DMZ) and the metabolite 2-hydroxymethyl-1-methyl-5-nitroimidazole (DMZ-OH) can still be found in animal tissues after the statutory withdrawal period of 6 days ?

2. In view of the above, is the use of DMZ as feed additive in turkeys of toxicological concern for the consumer ?

BACKGROUND

In accordance with the provisions of Council Directive 70/524/EEC, the use of dimetridazole (E-754) 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, Section D: Coccidiostats and other medicinal substances

Additive	Species or type of animal	Maximum age	Minimum and maximum content in (mg / kg) of complete feedingstuffs		Other provisions
E-754 Dimetridazole	Turkeys	-	100	200	Use prohibited from laying age onwards and at least 6 days before slaughter respectively
	Guinea-fowl		125	150	Use prohibited from laying age onwards and at least 6 days before slaughter respectively

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

   - whether the products, as authorised, did show in the experiments any mutagenic or carcinogenic effects;
   - whether the use of these products as additives in feedingstuffs could result in the presence of residues under the authorised conditions of use;
   - whether these residues could be harmful to the consumer;
   - whether 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 report of 5 October 1977 "*On the use of Nitroimidazole derivatives in feedingstuffs*"[1].

   At that occasion the SCAN observed:

   a) 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.

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1 Reports of the Scientific Committee for Animal Nutrition, First Series (1979) Catalogue Nº CB-28-79-277-EN-, Page 11. Concerns Dimetridazole (Turkey, Guinea Fowl & Swine), Ipronidazole (Turkeys & Guinea Fowl), Ronidazole (Turkey & Swine).

b) 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.

c) 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.

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

Therefore the Committee was of the opinion 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.

2. On 07 August 1997, the communication from the Federal Republic of Germany was made to the Commission "The following explains the reasons for their recommendation that the authorisation of DMZ should be suspended :

- DMZ (1,2-dimethyl-5-nitroimidazol, DMZ) is used as a food additive to protect against histomoniasis in turkeys (dose: 100-200 mg/kg of feed) and guinea fowl (dose: 125-150 mg/kg of feed). A withdrawal period of 6 days is compulsory.

- The use of DMZ as a veterinary medicine for animals used for the production of food is no longer authorised since it was included (by Regulation (EC) No 1798/95) in Annex IV to Regulation (EEC) No 2377/90. This was because of doubts about its safety in health terms, which it was not possible to eliminate because the necessary studies had not been carried out; these doubts have been reinforced by new findings about the effects of a structurally analogous compound.

- It is suspected that DMZ is genotoxic and carcinogenic and the conditions governing its use currently in force do not offer adequate safety guarantees, since, despite earlier findings on this subject, it has now been established that residues persist beyond the legally required withdrawal period; the exact length of the period for which they persist cannot be established.

3. On 22 April 1999, the Swedish Government took a decision prohibiting the use of DMZ as an additive in feedingstuffs pursuant to the same article 11 of Council Directive 70/524/EEC on the following scientific grounds :

- Scientific research has shown that dimetridazole may be a genotoxic and carcinogenic substance. The risks to the consumers cannot be assessed with any certainty. For this reason DMZ is not approved as a drug in the EU. In addition to the risks to consumers, DMZ represents a risk from the point of view of safety, hygiene and health at work.

- Furthermore, Sweden pointed out the risks associated with the use of DMZ in a report entitled Antimicrobial Feed Additives (SOU [Official Reports Series] 1997:132, Chapter 7, page 181-185), which was presented to the Commission in February 1998. This documentation indicates that the use of DMZ represents a risk to human and animal health.

- Other documentation comprised :

  – the scientific report from Germany (see 2.),

  – scientific references and an opinion from Swedish expert authorities : Medicinal Products Agency, National Veterinary Agency and the National Food Administration,

  – scientific references and an opinion from the Swedish National Food Administration concerning an opinion on DMZ from Denmark 1997,

  – Recent scientific references and an evaluation from the Swedish National Food Administration 1999.

Opinion of the Committee

In answering Question 87 by the Commission, the SCAN has considered it necessary first to answer the notification of the health authorities of Germany and Sweden (Part A) and then to answer the Commission's Questions (Part B).

Part A

Consolidated response to the list of argued points from the communication of the Federal Republic of Germany and Sweden to the Commission of the European Communities, 07 August 1997 and 22 April 1999 respectively, concerning the use of DMZ (E 754) in feedingstuffs for turkeys. The other opinions and references supplied by the Swedish authorities, and dealing with the same three items, have been taken into account by SCAN in these comments.

In this document the arguments put forward by the German Authorities and referred to by the Swedish authorities are reproduced in **bold type** (see Annex, statement of position concerning DMZ by the Federal Institute for the Protection of Consumer Health and Veterinary Medicine). The comments of the Scientific Committee on Animal Nutrition (SCAN) follow in normal type. In line with the German document the response is divided into sections dealing with mutagenicity, carcinogenicity and residues.

The document from the German authorities (as translated by the Commission's services) states as follows;

"The following explains again the reasons for our recommendation that the authorisation of DMZ should be suspended:

- **DMZ (1.2-dimethyl-5-nitroimidazol, DMZ) is used as a food additive to protect against histomoniasis in turkeys (dose: 100-200 mg/kg of feed) and guinea fowl (dose: 125-150 mg/kg of feed). A withdrawal period of 6 days is compulsory.**
- **The use of DMZ as a veterinary medicine for animals used for the production of food is no longer authorised since it was included (by Regulation (EC) No 1798/95) in Annex IV to Regulation (EEC) No 2377/90. This was because of doubts about its safety in health terms, which it was not possible to eliminate because the necessary studies had not been carried out; these doubts have been reinforced by new findings about the effects of a structurally analogous compound.**
- **It is suspected that DMZ is genotoxic and carcinogenic and the conditions governing its use currently in force do not offer adequate safety guarantees, since, despite earlier findings on this subject, it has now been established that residues persist beyond the legally required withdrawal period; the exact length of the period for which they persist cannot be established.**

Current scientific knowledge concerning these aspects is as follows :

A1. Mutagenicity Studies : A number of microbial test systems (the Ames test with a variety of *Salmonella typhimurium* strains, Luria-Delbrück fluctuation tests with *Klebsiella pneumonia*, *Escherichia coli* K12HfrH and *Citrobacter freudii* 425, mitotic gene conversion tests with *Saccharomyces cerevisiae* D4) have shown that DMZ is mutagenetically active. These results were achieved with and without metabolic,

activation. In these studies the mutagenic properties were bound to the nitro-reductase activity of the test strains.

In in vivo and in vitro test systems using mammalian cells, the results for DMZ were negative (dominant, lethal, CHO/HGPRT and micronucleus test, unscheduled DNA synthesis test in vivo and in vitro) or marginal (recessive lethal test).

No definite conclusions can be drawn from the results of these tests about the importance of the mutagenic properties for human health. Since there are no more recent studies available on the mutagenicity of DMZ, the new assessment requested has drawn on data on a structural analogue of DMZ: metronidazole.

In the case of metronidazole an anaphase-telephase test in vitro with CHO cell cultures, a chromosome aberration test with human lymphocytes and a micro-nucleus test with human lymphocytes were carried out in vitro. In the mutagenicity test with CHO cell cultures, the frequency of abnormal anaphases increased significantly in the case of each dose tested. The chromosome aberration test with lymphocyte cultures showed a significant increase in chromosome aberrations in the case of each dose tested. In this test there was also a linear correlation between the increase in the dose and the increase in the chromosome aberration frequency. In the micronucleus test with lymphocytes the frequency of micronuclei in bi-nuclear cells increased. In this case there was no linear correlation with dose and effect.

An in vivo bone marrow micronucleus test was carried out using CFW mice. It showed an increase of micronuclei in polychromatic erythrocytes in the case of all the dose size tested. Again, a linear correlation was found between the dose and micronuclear induction. (Mudry *et al.*, 1994).

Assessment of the above information should be qualified by noting that the source of the metronidazole was not cited for any of the tests, so that there is also a possibility that the increased mutation rate was caused by impurities in the compound. Furthermore, the strain/variety of mice used for the bone marrow micronucleus test has a high spontaneous mutation rate.

The occurrence of gene mutations in the HPRT locus in peripheral sheep lymphocytes after the administration of metronidazole in therapeutic doses has been studied. The average values of the results showed no significant induction of mutations. However, individual results showed a connection between steady-state metronidazole concentrations in the plasma and an increase in the HPRT variant frequency. Animals with the slowest elimination rate (first-order-elimination rate constant) showed the highest frequency of HPRT variants . In three of the nine sheep a significant increase in the frequency of variants in the HPRT locus was established. These were also the animals with the slowest elimination and highest steady-state concentration in the plasma (Ostrosky-Wegmann et al. 1994).

A study was made of the frequency of chromosome aberrations in peripheral lymphocytes from 10 human patients before and after metronidazole therapy. It was found that there was a significant increase in the frequency of chromatid breaks and isochromatid breaks. However, there was no correlation between that frequency and the plasma level of metronidazole. In these studies both the increase in cells with chromosome aberrations and the increase in chromosome aberrations per cell were significant (Elizondo et al. 1996).

A number of studies on the mutagenicity of 5-nitroimidazoles found a link between the redox potential of individual 5-nitroimidazoles and mutagenicity. Substances with a higher level of electron affinity are more toxic and produce more DNA damage that substances with low electron affinity (Adams et al. 1976, Chin et al., 1981; Olive 198l). The redox potential of DMZ and metronidazole is comparable (Declerck and Ranter 1986; Knox et al. 1981) as is the substitution pattern in the imidazole ring. In ring/ring position 2, metronidazole has a hydroxyethyl group and DMZ has a methyl group. A number of mutagenicity tests on the two substances have produced similar results (Voogd et al. 1974; Voogd et al. 1992).

It therefore seems justifiable to consider DMZ and metronidazole to be analogous: both have comparable mutagenic properties.

Extract of the summary assessment :

The anti-parasitic effectiveness, but also the genotoxic effect, of 5-nitroimidazoles and so of DMZ depends on the formation of reactive metabolites through the reduction of the nitro-group. These reductive conditions are present in bacteria with nitro-reductase activity and/or in an anaerobic environment (Elhard et al, 1988; Carlier et al 1997). In the mammalian organisms these conditions are provided by the intestinal bacteria; in various organs of the organism (including the liver), areas with low oxygen tension or periodically occurring hypoxic conditions can be found. It must therefore be concluded that human intake of DMZ residues may entail lead the formation of genotoxic metabolism.

The following SCAN response has been drafted on the basis of the data available as in references and taking into account the RHÔNE-POULENC SANTE and BASF files.

SCAN comments:

A battery of tests (*in vitro* and *in vivo*) on mutations in bacteria and chromosomal damage in mammalian cells was examined. Dimetridazole (DMZ) and its main metabolite 2-hydroxymethyl-1-methyl-5-nitroimidazole (DMZ-OH) proved to be mutagenic in a reverse mutation assay on TA1535, TA1537, TA98 and TA100NR+ (nitroreductase positive) *Salmonella typhimurium* strains and *Saccharomyces cerevisiae* D4 (also nitroreductase positive) (Benazet and Cartier, 1977; Voogd, 1981). This mutagenic potential of the compound is dependent on the reduction of the 5-nitro group. On the TA100NR- (nitroreductase negative) strain DMZ was not mutagenic even after S9 fraction metabolic activation. This finding suggests that rat hepatic microsomal enzymes are unable to produce DMZ mutagenic metabolites *in vitro*. This conclusion emerged also from *in vivo* tests where neither mammalian liver nor intestinal flora showed any capability in producing DMZ mutagenic metabolites in urine (Cordier and Bonneau, 1987).

DMZ proved to be devoid of genotoxic activity in a test for unscheduled DNA synthesis *in vitro* in Chinese hamster lung fibroblasts even after metabolic activation. It was not mutagenic in a gene mutation CHO/HGPRT Chinese hamster test with and without metabolic activation.

DMZ proved to be not clastogenic in the chromosome aberration test of Chinese hamster ovary cells *in vitro* and did not induce micronuclei in an *in vivo* micronucleus test in CD_1 mice. Also when administered at 1000 mg/kg bw/day for 5 consecutive days to CDA

male mice (oral route) DMZ did not induce dominant lethal effects. A similar result was obtained when DMZ was administered intraperitoneally at 220 mg/kg body weight/day.

The positive results with DMZ in a sex-linked recessive lethal test in *Drosophila melanogaster* (Kramers 1982) were of doubtful significance. These authors admitted that their results were not reproduced in replicate experiments and for this reason they were refuted by Cordier (Report SP 3648.14; pp. 6-13).

In conclusion DMZ and DMZ-OH showed mutagenic activity only in prokaryotes and with *Saccharomyces cerevisiae*, being micro-organisms with high nitroreductase activity. DMZ did not show genotoxic activity in *in vitro* or *in vivo* mammalian systems. It is therefore not considered as a genotoxic compound.

Another argument provided by the German and Swedish authorities is that DMZ could be reduced by the intestinal flora. Furthermore it is claimed (but not substantiated by literature references) that direct reduction of the absorbed fraction of DMZ by mammalian cells, preferably in the hypoxic state, may occur. The possibility of nitroimidazoles to be reduced by bacteria or eukaryotic cells depends on the respective redox potentials of the chemicals and cells, as described in Edwards review (1993). *"All anaerobes possess redox mechanisms of c. - 430 to - 460 mV, the value typical of ferredoxin, whereas metronidazole has a reduction potential of - 415 mV* [DMZ redox potential is in the same range as metronidazole, as established by Knox et al, 1981 (note of the SCAN)]. *Consequently, the drug is a more efficient electron acceptor [than the cell]. The most negative redox potentials in aerobes are those of the NAD/NADH couple (-320 mV) and NADP/NADPH (- 324 mV), redox potentials which are more positive than the 5-nitroimidazoles. The latter are not reduced in aerobes and consequently inactive. The reduction of the nitro group might occur as the drug enters the intestinal cell or further tissue cell, but in the presence of oxygen ("oxic" cells), damage would be very limited or absent because oxygen is the best biological electron acceptor known and would rapidly remove the electron from the nitro radical anion, reforming the original drug and superoxide, a process known as "futile cycling". In hypoxic tumours, however, the redox potential is believed to be significantly lower than in normal, healthy, aerobic cells, but not as negative as in anaerobes."*

When the production within the intestinal flora (and the implicitly suggested subsequent toxicity for the host) of DMZ reactive metabolites is concerned, it must be noted that the lifetime of these reactive metabolites depends on the reduction potential of the parent molecule; the lower that potential the longer is the lifetime (about 12 seconds for 5-nitroimidazoles) and the more limited is the damage (cytotoxicity)(also Edwards review, 1993). The absence in long term studies with 5-nitroimidazole compounds of intestinal tumours is consistent with these observations.

It should be noted that the overall evaluation of the genotoxic potential of DMZ in eukaryotic cells is based on a sufficient number of tests in compliance with the requirements of Council Directive 87/153/EEC[1] fixing guidelines for the assessment of additives in animal nutrition as last amended by Council Directive 95/11/EC[1]. Even

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1 OJ L64 of 7.3.1987, p.19

though these studies were not carried out under GLP-compliance (which were not compulsory at that time), their quality and the conclusions are still acceptable.

In the arguments provided by the German and Swedish authorities, reference is made to metronidazole, another structurally close 5-nitroimidazole widely used drug in human medicine. It is indeed recognized that metronidazole has shown genotoxic effects on different *in vitro* and *in vivo* mammalian (including human) systems (Korbelik and Horval, 1980 ; Reitz *et al*., 1991; Mudry *et al*., 1994; Ostrosky-Wegmann *et al*., 1994). The chemical similarity of both compounds is presented as a strong presumption that DMZ could be genotoxic also, notwithstanding the fact that the DMZ genotoxicity tests performed on eucaryotic cells were all negative. However, the extrapolation of data obtained with metronidazole to DMZ needs further and thorough analysis.

Firstly, the genotoxic potential of metronidazole in mammalians remains questionable as other authors results were negative (Lambert *et al*., 1979a,b; Hartley-Asp, 1979; Lambert and Lindblad, 1980; Mitelman *et al*., 1980; Mahood and Wilson, 1981 ; Dayan *et al*., 1982 ; Neal and Probost, 1984 ; but also review from IARC 1987) or inconclusive (LaRusso *et al*.,1978 ; Probst *et al*.,1981 ; Martelli *et al*., 1990 ; Elizondo *et al*., 1996). Recently, Ré *et al.* (1997) have shown, using the comet assay, that metronidazole as well as DMZ induce DNA damage *in vitro* in human lymphocytes, but that the effect is related to the production of hydroxy radicals through the oxidative « futile cycle » instead of the $4e^-$ reduction of the nitrogroup to hydroxylamine that would lead to DNA adducts. Consistently, these authors have established that the effects were dose-dependent and sensitive to the antioxidant status of the cell. Other authors (Fahrig and Engelke, 1997) could not confirm the induction of DNA damage in peripheral lymphocytes of human beings exposed to therapeutic doses of metronidazole.

Secondly, the close structural similarity of both molecules does not rule out differences in physico-chemical and biological properties. It is well established that the electron affinity (or one-electron redox potential) of nitroimidazole compounds correlates positively with aerobic cytotoxicity (Adams *et al*., 1979), mutagenicity (Chin *et al*., 1978) and DNA binding *in vitro* (Knight *et al*., 1978 ; Ludlum *et al*., 1988), even if many other factors (e.g. half-life of the one electron nitro radical anion, pH and nature of the medium) may substantially affect this relationship (Edwards, 1993). Indeed, the mutagenic activity of DMZ and metronidazole measured on prokariotic cells is very similar (De Méo *et al*., 1992), that correlates well with the close redox potential of both compounds. However, the corresponding and structurally close C2 hydroxy metabolites that appear as the major products formed in mammalian systems and are suspected to be the ultimate reactive metabolites (Martelli *et al*., 1990 ; Elizondo *et al*., 1996), exhibit different mutagenic potential in prokaryotic cells. When tested on different *Salmonella typhimurium* strains, 2-hydroxy-metronidazole exhibits a ten-fold higher mutagenic potential than metronidazole (Connor *et al*., 1977), while DMZ-OH has the same mutagenic potency as DMZ (Benazet and Cartier, 1977).

This very different behaviour of close compounds indicates the limitations of extrapolations based on structural considerations. Therefore, any extrapolation to DMZ of data concerning metronidazole genotoxicity remains purely speculative.

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[1] OJ L106 of 11.5.1995, p.

SCAN Conclusions on mutagenicity:

- DMZ and DMZ-OH induced gene mutations in prokaryotes and *Saccharomyces cerevisiae* characterised by high nitroreductase activity.

- DMZ did not show evidence of genotoxic activity in *in vitro* or *in vivo* mammalian systems, except for a single *in vitro* comet assay.

- Direct extrapolation of (inconsistent) results obtained in genotoxicity tests from metronidazole to DMZ is not scientifically justified.

On balance, the weight of evidence indicates that DMZ should not be considered as a genotoxic compound in mammals.[1]

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[1] Minority opinions of Dr D. Anderson and Mr D. Renshaw:

We are concerned that dimetridazole may be genotoxic. The dimetridazole molecule contains a structural alert: the 5-nitro ring. Several other compounds with a 5-nitro ring have been convincingly shown to be genotoxic *in vivo*. The results of genotoxicity testing of dimetridazole suggest two possible mechanisms by which dimetridazole may be genotoxic:

- In some assays in bacteria and in yeast, dimetridazole was nitroreduced to a reactive substance that caused gene mutations. Such nitroreduction may also occur in gut bacteria and in some mammalian tissues.

- The results of the *in vitro* comet assay show that dimetridazole, under certain conditions, can damage the DNA in mammalian cells by a mechanism involving production of active oxygen species. We can not exclude the possibility that dimetridazole may have similar genotoxicity *in vivo*.

We expect that the exposure of human consumers to dimetridazole will be very low, but we can not identify a safe level of exposure.

A2. Carcinogenicity studies: In assessing the carcinogenic qualities of DMZ, the following studies were consulted:

0.2% DMZ was administered to 35 Sprague Dawley rats in their feed over 46 weeks after which the rats were fed a control feed over 20 weeks. Another group was fed only the control feed for 66 weeks. After 66 weeks the animals which had been treated with DMZ had a considerably higher increase in benign mammary tumours than the control group. The average value of the number of tumours per rat had also increased significantly. Malignant tumours were not found (Cohen 1973).

It should be noted that the test was carried out over an unusual length of time and that this variety of rats is characterised by a high rate of spontaneous mammary tumours

In another study DMZ was administered in feed to a group of 50 female and 50 male CFY rats in doses of 0, 100, 400 and 2000 ppm. The average daily intake was 0; 3.8; 15.1 and 77.7 mg/kg bw/day. Where the highest doses were administered, there was an increase in benign mammary tumours (adenomas, fibroadenomas, fibromas) in both sexes. At a dose of 400 ppm the increase was somewhat smaller in the female rats. In the highest and medium dosage groups, in the case of females the number of tumours per rat also increased. No malignant tumours were found and no tumours of any kind were found in the other tissues tested.

In another study with CFY rats in which doses of 0 and 10 ppm were administered in feed over 128 weeks, no significant increase in benign or malignant tumours of the mammary glands were found (Lowe et al. 1976).

To investigate the possible influence of hormonal changes on carcinogenesis, DMZ was administered to rats in feed over two months in doses of 100, 2000 and 5000 mg/kg. In the female animals a significant increase was observed in progesterone levels where the two highest doses were administered. Evidence of a significant increase was not found in the females treated with the 100 ppm dosage and was not found at all in the male animals.

However, this test does not appear to provide adequate evidence of a non-genotoxlc mechanism of carcinogenesis, since there was also a significant increase in mammary tumours in the male animals without any corresponding rise in progesterone levels.

All studies of the carcinogenicity of DMZ carried out so far are flawed by contemporary standards. In particular there is a lack of studies on a second species of animal. Nor are the studies carried out so far adequate to allow a genotoxic mechanism of tumour genesis to be ruled out.

SCAN comments:

In the German and Swedish clause of safeguard, reference is made to the chronic (carcinogenicity) studies in rats. These studies have already been analysed on several occasions (SCAN (1979), JECFA (1989), CVMP (1989, 1994)). Points of interest are:

- The essential finding was an increase in benign mammary tumours in CFY rats fed 400 and 2000 mg DMZ/kg feed (females) and 2000 mg DMZ/kg feed (males), for 122

weeks (Lowe *et al*, 1976), while no increase in malignant tumours was reported. A corresponding finding was published in the Cohen and coworkers study (1973) (Sprague Dawley rats), although the design of this study fell short of a standard carcinogenicity study.

- The studies do not comply with present quality (GLP) standards, however, this does not imply that they are scientifically unsound. One of the consequences is that the study by Lowe and coworkers was of longer duration than the current standard (122 weeks *versus* 104 weeks), which leads to a relative overestimation of mortality rate and tumour yield compared with the current standards.

- In order to support a non-genotoxic mechanism of DMZ tumorigenicity via endocrine disturbance, various hormones were measured in CD^{TM}Crl:CD^{TM}(SD)BR rats after short term exposure (0, 100, 2000 and 5000 ppm for 2 months) (Belin et al, 1991). A dose-related and statistically significant increase in progesterone blood levels was seen in females from 2000 ppm upwards but not at any feed level in males.

- The carcinogenicity studies show a pattern of increase in benign mammary tumours in male and female rats at high doses tested. Such a pattern is considered of doubtful relevance with respect to prediction of human cancer risk, since the rat strains used in these studies are known for their high incidences of such tumours, while no increase in malignant tumours was seen. It should also be noted that such a pattern (single organ, benign tumours, no acceleration) is uncommon for known genotoxic carcinogens.

- A carcinogenicity study in a second species as recommended but not mandatory in Directive 87/153/EEC has not been performed. However, the available rat studies show induction of benign tumours in a single organ that has a high background incidence of such tumours. This together with the negative mammalian genotoxicity findings indicates a non-genotoxic mechanism of action to be the most probable explanation of this effect. Use of a second species study is not considered likely to fundamentally alter this conclusion. This justifies a threshold approach for risk assessment.

SCAN conclusion on carcinogenicity:

On the basis of the present data, DMZ is not considered as a genotoxic tumorigen.[1]

[1] Minority opinions, same as expressed in Conclusion of part A.1 (page 10).

A3. <u>Residue studies</u> : New residue studies have been submitted under the procedure for establishing maximum levels of residues of veterinary medication under Regulation (EEC) No 2377/90. The following picture may be derived from them as regards residues of DMZ in animal tissue.

A single dose of 50 mg/kg KGW of radioactively marked DMZ was administered orally to a turkey. After 15 days measurements showed that there were still residues in various tissues. The highest concentrations, expressed in μg equivalents/kg were in the liver (558), the kidneys (534) and the leg muscle (443). Of these residues, the following percentages were proven to be non-extractable residues: liver (45,91%), kidneys (47.46%) and leg muscle (62.17%). In the 15-day study period these proportions were relatively constant in these tissues. On the other hand, in the fat there was evidence of a decrease of the bound proportion over this period. This suggests a release of the bound residues from the tissue.

The test was carried out on one animal only. Neither the bound residues nor the metabolites that formed were identified or studied in detail.

In another residue study DMZ was administered to turkeys in their drinking water at a dosage of 553 mg/l. Twelve days after the last dose was administered, evidence of 0.3 μg/kg of DMZ or 0.6 μg/kg of 2-hydroxymethyl-1-methyl-5-nitroimidazole could still be found in the muscle. Higher concentrations were found in the skin/subcutaneous fat higher (39.3 μg/kg and 28.1 μg/kg respectively).

It is not possible to compare this study with the radioactive study since the dosage, duration of administration and different ways of cleaning the samples (means of extraction) were used.

It is not therefore possible to state the proportions of DMZ and 2-hydroxymethyl-1-methyl-5-nitroimidazole in the total residue. In this study the liver and kidneys, which also contained high concentrations of residues in the radioactive study, were not investigated.

SCAN comments :

The data available concerning the metabolic fate and residues of DMZ in turkeys have been analysed by JECFA (1989). ^{14}C-DMZ is largely absorbed (88%) and excreted within three days (79.4% with urine, 8.0% with faeces and 1.2% with expired air respectively). The predominant metabolite in the excreta was DMZ-OH, free and conjugated as glucuronide and sulphate (main compound), accompanied by 1-methyl-5-nitroimidazole-2-carboxylic acid. DMZ-OH was also the main metabolite in tissues. Moreover, the metabolism of DMZ leads to reduction products of the 5-nitro group, cleavage of the imidazole ring and formation of covalently bound residues. A lack of data was mentioned by JECFA concerning residue depletion and characterisation of bound-residues. It must be noticed that although the identification on bound residues is not explicitly required in Directive 87/153/EEC fixing Guidelines for the Assessment of Additives in Animal Nutrition, it may be requested if needed, e.g. when doubts exist concerning the safety.

Additional studies were carried out to support the establishment of Maximum Residue Limits as required under Council Regulation (EEC) No. 2377/90 laying down a community procedure for the establishment of maximum residue limits (MRLs) for

veterinary medicinal products in foodstuffs of animal origin (Johnston and Weir, 1991; Gibson, 1990). Consequently a therapeutic regime was used (e.g. about 5-12 times higher than allowed as feed additive respectively), which makes the extrapolation of the results questionable. Moreover, and as mentioned by the German and Swedish authorities, the dosage regime (dose, interval and duration) and analytical methodology were different. However, one pertinent observation concerns the analysis of bound residues, e.g. tissue residual radioactivity resistant to thorough extraction procedures, which represented 40-60 % of the total radioactivity in most target tissues and for the whole residue depletion period following withdrawal of the compound. Considering the fact that DMZ ring degradation occurs, that $^{14}C0_2$ is exhaled (even though to a limited extent), and that labelled products of the intermediary metabolism such as amino-acids, fatty acids or purine and pyrimidine bases, have been identified in the tissues of the pig and rat dosed with ^{14}C-DMZ, it is reasonable to assess that at least a fraction (if not all) of the bound residues results from the incorporation of labelled carbon fragments namely via a glycine/serine hydroxymethylation (May and Baker report RG/1612). Should DMZ-derived covalently bound residues be formed *in vivo*, which a recent study (Hoogenboom *et al*., 1997) using primary cultures of pig hepatocytes tends to suggest, then it should be referred to the genesis of 5-nitroimidazole bound residues established for ronidazole (Wislocki and Lu, 1990). This involves the initial reduction of the 5-nitro group as the critical key reaction which implies that bound residues would not contain the intact 5-nitro group which is associated with the (nitroreductase dependent) mutagenic potential of these compounds. Consequently, these covalently bound residues could be considered as being of no toxicological concern.

A second observation in the turkey using a single dose of 50 mg/kg bw (Johnston and Weir, 1991) was that the extractable radioactivity, i.e. DMZ and DMZ-OH as the main components, in liver, kidney, muscle and fat decreased rapidly following very similar kinetics all along the withdrawal period, the residual concentration in liver and kidneys being on average three times that measured in muscles after seven days. The Gibson study (1990) indicated that DMZ and DMZ-OH were both eliminated from muscle and skin/subcutaneous fat concomitantly following very similar kinetics. Six days after withdrawal of treatment with therapeutic dosage (553 mg/l drinking water for 15 days), mean residue concentrations in muscle were 0.0005 and 0.002 mg/kg for DMZ and DMZ-OH respectively. The corresponding residue concentration for skin/subcutaneous fat were 0.034 and 0.021 mg/kg. However, these values appeared to be excessively low when compared to the data obtained after 9- and 12-day withdrawal, due to the fact that only a limited number of samples (3 out of 10) were analysable, i.e. available in sufficient quantities and freed from interfering substances. Therefore calculated values based on regression equations established from the available data covering the all withdrawal period were used instead. The resulting figures were 485 ng DMZ and 454 ng DMZ-OH per g skin + fat. Due to analytical difficulties (*i.e.* biological degradation of DMZ during the extraction phase and interfering substances), no data were presented concerning liver and kidney.

In the absence of new quantitative data obtained under conditions of feed additive application of DMZ to turkeys, the figures on residues to be referred to are those mentioned in the reports of SCAN (1979) and JECFA (1989). They indicated that the concentration of residues of only DMZ, as determined by polarography or high performance liquid chromatography, decreases to less than 0.002 mg/kg (limit of detection of both methods) in turkey edible tissues after a withdrawal period of 6 days. Considering that DMZ and DMZ-OH are the main residual compounds in the tissues,

sensitive analytical methods (Carignan *et al.*, 1988a,b; Mallinson *et al*., 1992) have been developed (limit of detection : 0.001 mg/kg) which enable both residual compounds to be separately and simultaneously determined in tissues. The only and limited data available concerning DMZ-OH residues in turkey muscle following a 7-day administration of DMZ at a 150 mg/kg feed level (feed additive conditions) (Mallinson *et al*., 1992) showed a decrease to the limit of detection (0.001 mg/kg) after 24 hours withdrawal.

SCAN conclusions on residues :

- Total residues (measured as ^{14}C-activity) in liver, kidney, muscle and skin/subcutaneous fat deplete rapidly and in parallel following ^{14}C-DMZ withdrawal. Residue levels in liver and kidney are about three times higher than in muscle.

- Non-extractable residues resulting either from the re-utilisation of DMZ fragments or bound metabolites are considered of no toxicological concern.

- Data show that the depletion of both DMZ and its main metabolite DMZ-OH occurs rapidly and in parallel from liver, kidney, muscle and skin/subcutaneous fat. Residue levels are very low and of the same order of magnitude in muscle and subcutaneous fat from 6-day withdrawal time onwards.

- The quantitative data obtained using DMZ at therapeutic levels overestimate the residual status in tissues that would result from its use as feed additive and cannot be considered as applicable.

SCAN overall conclusions :

The data reviewed in the safeguard clause correctly represent the results of the studies available. However, the SCAN is of the opinion that their interpretation by the German and Swedish authorities deserves further and even different analysis. The SCAN reaches the conclusion that DMZ should not be considered as a genotoxic compound for mammals[1]. Concerns arising from the comparison of DMZ with other 5-nitroimidazoles (JECFA) or more specifically metronidazole (CVMP) are not substantiated.

No new data have been provided that may give rise to concern for carcinogenicity. The benign tumours reported in rats are considered to be the result of a non-genotoxic mechanism.

No new relevant quantitative data concerning residues in turkey tissues have been produced since the SCAN (1979) and JECFA (1989) opinions were delivered. Complementary studies performed using DMZ at therapeutic levels allow the conclusion that non-extractable residues including bound-residues are of no toxicological concern. DMZ and its main metabolite DMZ-OH behave similarly and disappear totally or reach very low levels within the 6-day compulsory withdrawal time.

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1 Minority opinions, same as expressed in Conclusion of part A.1 (page 10).

Part B

Response to Question 87 of the European Commission

1. Is it possible

> **a.** to assess the toxicological potential of DMZ (suspected of having genotoxicity and carcinogenicity, namely on the basis of recent test results concerning the structurally analogous compound metronidazole) or DMZ-OH or of its bound residues on the basis of the available data ?
>
> **b.** that, by using DMZ (E-754) in the feedingstuffs for turkeys, according to the conditions set up by Council Directive 70/524/EEC, both the parent substance (DMZ) and the metabolite 2-hydroxymethyl-1-methyl-5-nitroimidazole (DMZ-OH) can still be found in animal tissues after the statutory withdrawal period of 6 days ?

2. In view of the above, is the use of DMZ as feed additive of toxicological concern for the consumer ?

1.a. Toxicological potential

In assessing the toxicological potential of DMZ, the following points are of importance :

1) Genotoxicity aspect

- DMZ and DMZ-OH induced gene mutations in prokaryotes and *Saccharomyces cerevisiae* characterised by high nitroreductase activity.

- DMZ did not show evidence of genotoxic activity in *in vitro* or *in vivo* mammalian systems, except for a single *in vitro* comet assay. Since DMZ-OH is a conversion product of DMZ in mammalian systems, these findings are implicitly applicable to DMZ-OH. Direct extrapolation of results obtained in genotoxicity tests from metronidazole to DMZ is not scientifically justified. Moreover genotoxicity findings on metronidazole are not consistent.

- The pre-requisite for DMZ genotoxicity is the reduction of the 5-nitro group which is easily performed by anaerobic bacteria with high nitroreductase activity. DMZ covalently bound residues that may eventually explain part of the observed non-extractable residues in tissues, would result from the compulsory initial reduction of the 5-nitro group. Therefore none of these bound residues bearing the resulting 5-amino group would generate a stable potentially genotoxic compound following human consumption.

2) Carcinogenicity aspect

- The essential finding was an increase in benign mammary tumours in CFY rats fed 400 and 2000 mg DMZ/kg feed (females) and 2000 mg DMZ/kg feed (males) for 122 weeks (Lowe *et al*, 1976), while no increase in malignant tumours was reported. A

corresponding finding was published in the Cohen and coworkers study (1973) (Sprague Dawley rats), although the design of this study fell short of a standard carcinogenicity study. The studies do not comply with present quality (GLP) standards. However, this does not imply that they are scientifically unsound. For instance, the study by Lowe and coworkers was of longer duration than the current standard (122 weeks *versus* 104 weeks), which leads to a relative overestimation of mortality rate and tumour yield compared with the current standards.

- In order to support a non-genotoxic mechanism of DMZ tumorigenicity via endocrine disturbance, various hormones were measured in CD^{TM}Crl:CD^{TM}(SD)BR rats after short term exposure (0, 100, 2000 and 5000 ppm for 2 months).A high relationship between DMZ levels in feed and increases of progesteronemia was seen in females but not in males.

- The carcinogenicity studies show a pattern of increase in benign mammary tumours in male and female rats at high doses tested. Such a pattern is considered of doubtful relevance with respect to prediction of human cancer risk, since the rat strains used in these studies are known for their high incidences of such tumours, while no increase in malignant tumours was seen. It should also be noted that such a pattern (single organ, benign tumours, no acceleration) is uncommon for known genotoxic carcinogens.

- A carcinogenicity study in a second species as recommended but not mandatory in Directive 87/153/EEC has not been performed. However, the available rat studies show induction of benign tumours in a single organ that has a high background incidence of such tumours. This together with the negative mammalian genotoxicity findings indicates a non-genotoxic mechanism of action to be the most probable explanation of this effect. Use of a second species study is not considered likely to fundamentally alter this conclusion. This justifies a threshold approach for risk assessment.

Conclusion

On the basis of the above analysis, it is possible to assess the toxicological potential of DMZ and its metabolites. SCAN concludes that DMZ is a non genotoxic tumorigen. [1]

1.b. Residues

Since the SCAN, CVMP and JECFA opinions have been issued new data have been generated concerning the metabolism and residues of DMZ in turkeys.

(1) Therapeutic dosages (respectively 5 and 12 times those used for feed additives) were applied in two studies.

- In the Johnston and Weir study (1991), it was observed that total residues deplete in parallel in all edible tissues, those in liver and kidney (the highest) being 3 times higher than in muscle all along the withdrawal

[1] Minority opinions, same as expressed in Conclusion of part A.1 (page 10).

period. Moreover DMZ and DMZ-OH residues depleted following very close kinetics.

- In the Gibson study (1990), despite the applied therapeutic dosage overestimates the residual status of DMZ when used as feed additive, the data provided indications that DMZ and DMZ-OH residue concentrations were as low as 0.0005 mg/kg and 0.002 mg/kg in muscle respectively, after 6 days withdrawal. Corresponding calculated values for skin/subcutaneous fat were 0.485 and 0.454 mg/kg respectively.

Taking into account the fact that DMZ and DMZ-OH residues follow a very close kinetic depletion, and refering to the figures indicated above concerning residue ratios between liver or kidney and muscle, it can be anticipated that DMZ-OH residues in liver and kidney are not higher than 0.006 mg/kg after 6-day withdrawal (*e.g.* three times the level in muscle).

(2) In another study where DMZ was administered at feed additive level, DMZ-OH residues were less than 0.001 mg/kg in muscle after 24 hours withdrawal.

Conclusion

When DMZ is administered at feed additive level:

- DMZ residues are below 0.002 mg/kg in all edible tissues of turkeys after 6 days withdrawal (see SCAN, CVMP and JECFA opinions).

- DMZ-OH residues disappear (0.001 mg/kg, limit of detection) after 24 hours withdrawal in muscle. In the absence of experimental data for the other tissues, a worse case scenario leads to the following figures: 0.006 mg/kg for liver and kidney and 0.454 mg/kg in skin/subcutaneous fat after 6-day withdrawal.

2. Conclusion for the consumers

The SCAN considers that the weight of evidence indicates that DMZ should not be considered as a genotoxic compound for mammals and is not a genotoxic tumorigen[1]. Therefore a threshold approach can be applied for DMZ risk assessment. A No Observed Effect Level (NOEL) of 4.6 mg/kg bw/day can be established based on the 122-week carcinogenicity study in CFY rats. Applying a safety factor of 1000 which takes into account the fact that this study was not GLP-compliant and the hormonal data for males was not consistent with the proposed tumorigenic mechanism, a toxicological Acceptable Daily Intake (ADI) of 0.0046 mg/kg bw (0.28 mg/day for a 60 kg bw person) is calculated.

No quantitative information on DMZ residue kinetics following feed additive administration to turkeys is available. SCAN (1979) and JECFA (1989) indicated that after a withdrawal period of 6 days, DMZ concentration in turkey edible tissues decreased to less than 0.002 mg/kg (limit of detection of polarographic or HPLC methods).

[1] Minority opinions, same as expressed in Conclusion of part A.1 (page 10).

Considering residue levels of DMZ plus DMZ-OH measured at the compulsory 6-day withdrawal period following a therapeutic treatment (in order to consider the worst case scenario), the theoretical maximum daily intake for a consumer would be 0.086 mg/person. This figure takes into account a daily consumption of 300 g muscle, assumes that the residual concentration in 100 g liver and 10 g kidney is three times higher than that measured in muscle, and considers for the 90 g skin/subcutaneous fat the concentrations calculated from the regression equations established from the available data covering the all withdrawal period. The maximum daily intake of total residues, which overestimates the residues that would result from DMZ used as feed additive, represents 30.7% of the ADI as calculated above.[1]

Conclusion

SCAN concludes therefore that, when used as feed additive for turkeys in accordance with the current regulation (establishing a statutory 6-day withdrawal period), dimetridazole is of no concern for the consumer health.

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[1] Results from toxicological studies

A toxicological ADI of 0.0046 mg/kg bw (0.28 mg/day for a 60 kg bw person) based on the NOEL of 4.6 mg/kg bw/day from the 122-week carcinogenicity study in CFY rats and applying a safety factor of 1000 could be established.

Results from residue studies

On the basis of the residue levels of DMZ + DMZ-OH calculated after a 6-day withdrawal period following a therapeutic treatment of DMZ (553 mg/l drinking water for 2 weeks)(see Table 1), the theoretical maximum daily intake (TMDI) of residues for the consumer would be 0.086 mg/person (see Table 2). This figure takes into account the measurement of the residual amounts resulting from the daily consumption of 300 g muscle, an evaluation of the residual contribution of 100 g liver and 10 g kidney assuming that the concentration in these tissues would be three times higher than that measured in the muscle, and the residual contribution of 90 g skin/subcutaneous fat calculated from the regression equations established from the available data covering the all withdrawal period. The theoretical maximum daily intake of residues (0.086 mg/person) (see Table 2) will represent 30.7% of the ADI.

Table 1. Tissue residue levels (ng/g) of dimetridazole (DMZ) and its main metabolite 2-hydroxymethyl-1-methyl-5-nitroimidazole (DMZ-OH) in the turkey, following the administration of DMZ (Emtryl) (553 mg/l drinking water per day, for 15 days).

Withdrawal Period	**Muscle (ng/g)**		**Skin/subcutaneous fat (ng/g)**	
	DMZ	**DMZ-OH**	**DMZ**	**DMZ-OH**
2 hours	5260 ± 4140 (10/10)[1]	8320 ± 9070 (10/10)[1]	12000 ± 4340 (8/10)[1]	87400 ± 18500 (8/10)[1]
1-day	87.4 ± 124 (6/10)[1]	198 ± 432 (10/10)[1]	1610 ± 1470 (10/10)[1]	5200 ± 7770 (10/10)[1]
2-day	11.5 ± 11 (10/10)[1]	6.6 ± 6.3 (10/10)[1]	1220 ± 522 (5/10)[1]	2160 ± 1020 (5/10)[1]
3-day	4.1 ± 4 (8/10)[1]	2.0 ± 2.1 (9/10)[1]	1750 ± 986 (10/10)[1]	1460 ± 631 (3/10)[1]
6-day	0.5 ± 0.4 (8/10)[1]	1.9 ± 1.5 (10/10)[1]	33.7 ± 21.3 (3/10)[1] **485** [2]	20.6 ± 1.7 (3/10)[1] **454** [2]
9-day	0.7 ± 1.0 (6/10)[1]	1.2 ± 0.7 (10/10)[1]	115 ± 186 (7/10)[1]	93.5 ± 153.5 (7/10)[1]
12-day	0.3 ± 0.2 (4/10)[1]	0.6 ± 0.2 (10/10)[1]	39.3 ± 24.9 (8/10)[1]	28.1 ± 13.7 (9/10)[1]
Control	< 0.2 (10/10)[1]	< 0.2 (10/10)[1]	< 8.31 ± 14.2 (10/10)[1]	0.3 ± 0.4 (10/10)[1]
Control	< 0.2 (10/10)[1]	< 0.2 (10/10)[1]	<0.7 (10/10)[1]	<0.2 (10/10)[1]

[1] (…/…): number of samples analysed/total number of samples available (one sample per bird)

[2] values calculated from the regression equations established from the data of the all withdrawal period

Table 2. Theoretical Maximum Daily Intake (TMDI) of DMZ residues at 6-day withdrawal.

	Tissue residue levels (ng/g) (DMZ + DMZ-OH)	**Daily intake (g)**	**Total residues (ng)**
Muscle	0.5 + 1.9 = 2.4	300	720
Skin + fat	485 + 454 = 939 [1]	90	84510
Liver	2.4 x 3 = 7.2 [2]	100	720
Kidney	2.4 x 3 = 7.2 [2]	10	72
			TMDI =86022

[1] values calculated from the regression equations established from the available data covering the all withdrawal period

[2] values calculated on the basis of 3 times the residual levels measured in muscle

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