Acta vet. scand. 1982, 23, 313—324.

From the National Food Administration, Nutrition Laboratory and Toxicology Laboratory, Uppsala, Sweden.

DETERMINATION AND HEALTH-RISK EVALUATION OF NITROXYNIL RESIDUES IN THE EDIBLE TISSUE OF CATTLE

By

Lars-Gösta Ekström and *Premysl Slanina*

EKSTRÖM, LARS-GÖSTA and PREMYSL SLANINA: *Determination and health-risk evaluation of nitroxynil residues in the edible tissues of cattle.* Acta vet. scand. 1982, 23, 313—324. — A specific polarographic method with a sensitivity of $\geq$ 2 µg/kg (ppb) has been used to determine the plasma and tissue concentrations of nitroxynil (NTX), which is used against Parafilaria bovicola in cattle. After treatment with the therapeutic dose on NTX (2 × 20 mg/kg b.w., s.c.), there was an initial rapid decrease in the plasma concentration followed by a slower elimination phase. The plasma levels of NTX were 8 mg/kg (ppm) and 3 mg/kg after 6 weeks and 2 months, respectively. The muscle and other edible tissue from treated cattle contained 0.1—0.3 mg/kg NTX after 2 months, and µg/kg amounts were still detectable 3 months after the injection. Based on available pharmacological and toxicological data, a 3-months withdrawal time for NTX in cattle is proposed.

nitroxynil-residues; cattle; Parafilaria bovicola treatment; polarography.

In the last 3 years increasing number of cattle in certain regions of Sweden have been infected with a previously undetected parasite — Parafilaria bovicola (*Hugoson* 1980).

The parasitosis (known to occur in e.g. South Africa and several other countries) leads to a significant degrading of the meat quality of the carcasses, resulting in substantial economic losses (*Wellington* 1980).

Nitroxynil (NTX, Trodax®) was found to be effective against the parasite and is currently being used to control the spread of the disease. It is estimated that in 1980 alone some 1 200 bullcalves were treated with the preparation in Sweden (*Wahlgren*

1980). Although the drug is known to have a very narrow therapeutic index, there is a scarcity of published data in its toxicology and on residue levels in animal tissues.

In the present investigation a sensitive polarographic method has been used to monitor the concentration of NTX in the plasma, muscle and other tissues of animals treated with the drug. A discussion of tentative withdrawal times for meat-producing cattle is also presented.

MATERIALS AND METHODS

Chemicals

Nitroxynil (4-cyano-2-iodo-6-nitrophenol) was obtained as Trodax® (56 % w/v solution of the N-methylglucamine salt containing 34 % w/v active substance) from May & Baker Ltd., England. All the chemicals for the analytical procedure were standard commercial products (p.a. grade, Merck) and were used without any further purification.

Extraction procedures

The tissue samples were homogenized for 3—5 min in a Moulinex homogenizer. In some cases, when the amount of material available was limited (0.3—0.4 g biopsies) the tissue was mixed with an equal volume of sea sand and homogenized with a glass rod directly in a test tube.

The extraction of the samples was carried out essentially according to *Parnell* (1970) and *Takeshita et al.* (1980), with some modifications as shown in Fig. 1. In the case of the liver, kidney, and boiled and fried meat samples, the extraction procedure had to be repeated in order to remove contaminants which interfered with the polarographic determinations.

Differential pulse polarography

The polarographic measurements were performed on a PAR polarograph (174A) equipped with Hg-drop timer (172A) and an Ommigraphic XY-recorder (model 2 000) (for principles of the technique see e.g. *Bond* 1969). The reference and counter electrodes were a Ag/AgCl and Pt-wire, respectively. Traces of O_2 in the protecting gas (argon) were removed in a chromium reductor prepared according to *Meites* (1965).

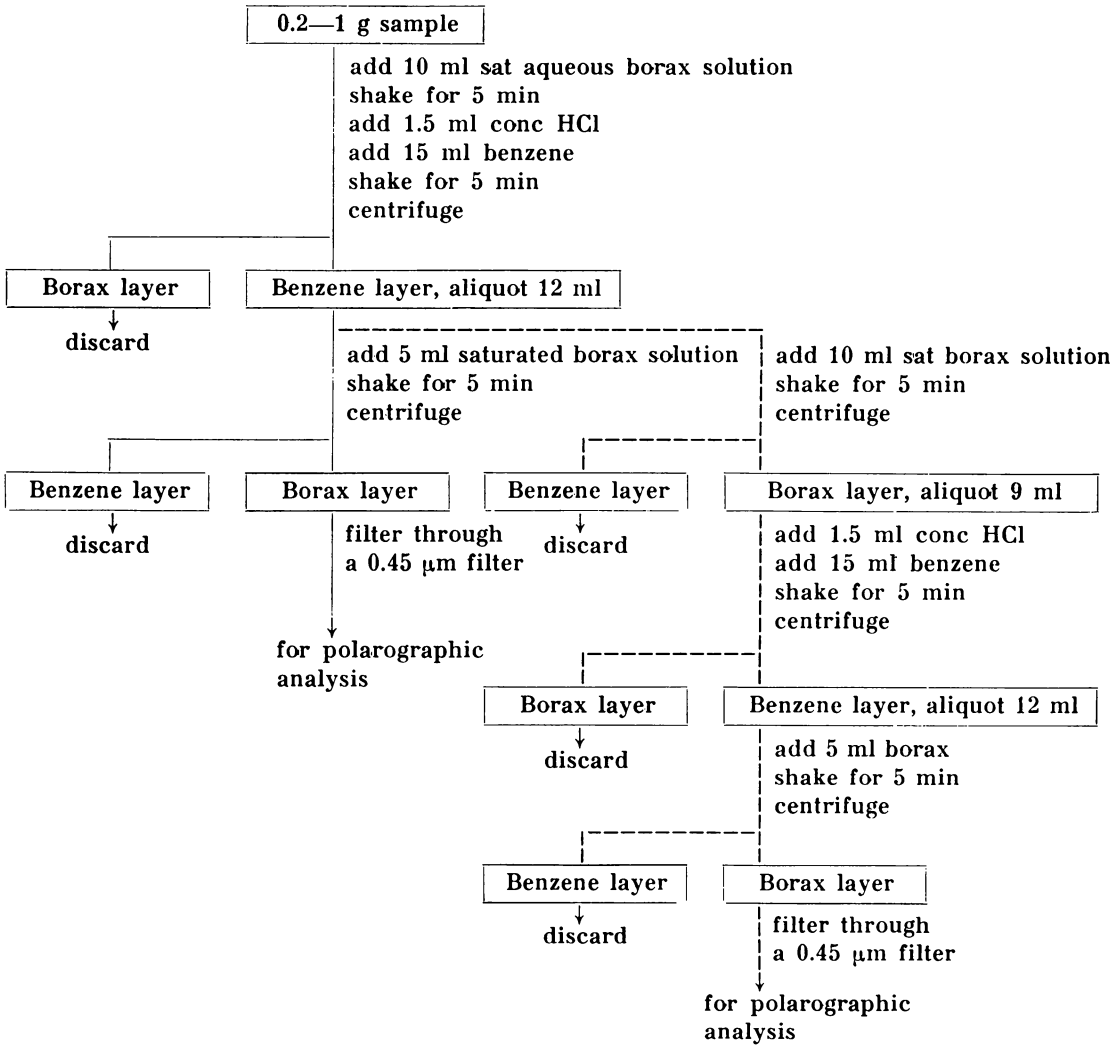
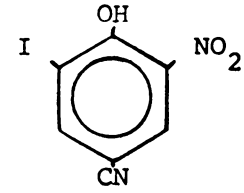


Figure 1. Extraction scheme.

The polarographic determination of NTX is based upon the reduction of the nitro group in the NTX molecule to hydroxylamine at a dropping electrode.

Nitroxynil (R-NO_2)



electrode reaction $R\text{-}NO_2 + 4\bar{e} + 3H^+ \rightarrow R\text{-}NHOH + OH^-$

From the polarographic point of view it is very favourable that four electrons are transformed per molecule NTX, as the size of the signal is proportional to the current. With the chosen differential pulse polarographic technique a linear relationship is obtained between the signal (peak height) and the concentration of NTX over the range 1—10^5 µg/kg. The quantitation of NTX in tissue and blod samples has been carried out by the method of standard addition, to avoid the influence of matrix effects.

The following parameters were chosen for the polarographic analysis:

Initial potential	—0.25 V
Scan rate	0.001 V/s
Modulation amplitude	0.05 V
Drop time	1 drop/s
Low pass filter	0.3

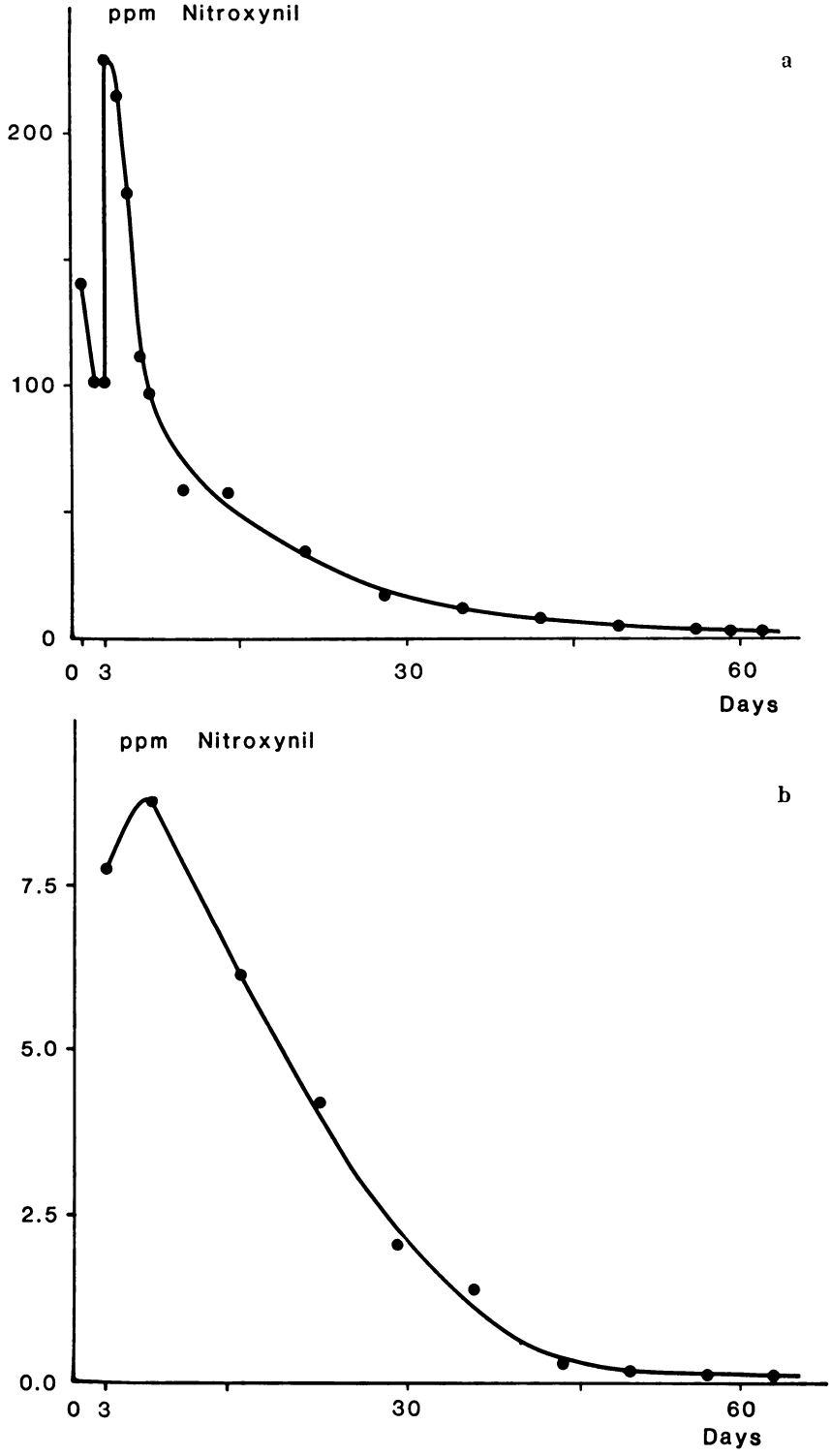
NTX is reduced at —0.392 V under these conditions, but the peak is slightly shifted to a more negative potential at higher concentrations.

The detection limit for samples spiked with NTX was found to be about 2 µg/kg. The recovery of NTX after the extraction procedure was 95 % for pure standard solutions, but decreased to 80—90 % for spiked tissues samples, depending on the concentration used.

Animal samples

One bull-calf (A) Swedish pulled cattle breed, 3-months-old was injected subcutaneously (s.c) with the recommended dose (20 mg NTX/kg b.w.) and the injection was repeated after 72 h. An untreated animal of the same breed and age was used as a control.

Figure 2a and b. Nitroxynil concentrations in blood plasma (a) and biopsy muscle (m. semitendinosus) samples (b) from a calf at various times after the s.c. administration of a therapeutic dose (2 × 20 mg/kg b.w.) of the drug (Trodax®). The 2 injections were given on days 0 and 3, respectively. The first blood sample was taken 24 h after the first injection (day 1). On day 3 one blood sample was taken just before the second injection, and a second blood sample 3 h later. The first biopsy sample was taken 3 h after the last injection (day 3, for details see Materials and Methods).


ppm Nitroxynil
a
200
100
0
0 3
30
60
Days
ppm Nitroxynil
b
7.5
5.0
2.5
0.0
0 3
30
60
Days

The blood samples were drawn every 24 h during the first 7 days after the first injection of NTX and once a week during the following 7 weeks. An aliquot of the blood sample taken during the 4th week was used for serum protein fractionation. Serial biopsy samples of m. semitendinosus (2 × 0.3—0.4 g) were taken under local anesthesia (Xylocaine®) weekly for 7 weeks, starting 3 h after the second injection. Immediately after the biopsy, the tissue was frozen in liquid N_2 to avoid water loss, weighed and kept frozen until analyzed. At the end of the experiment, 9 weeks after the first injection, the animal was slaughtered and samples from several muscles, as well as from liver, kidney and lung, were taken for determination of NTX. Muscle tissue from the injection site (neck) was divided into 9 squares (approx. 5 × 5 cm each) and each part was analyzed separately. To determine if cooking of the meat can result in NTX degradation, portions of muscle tissues were subjected to boiling or frying before being assayed for NTX.

Samples from a further 2 animals (B and C) were obtained through local sanitary slaughter-houses 4 weeks and 3 months respectively after the last injection of NTX. The samples of liver, kidney and muscle (including the injection site) were immediately removed and stored frozen until analysed.

Separation of serum protein fractions

A 2 ml sample of serum taken on day 28 (animal A) was fractionated on a Sephacryl 300 column (Ø = 2.5 cm, length 80 cm). The buffer used was a 0.1 mol/l Tris-HCl, containing 0.5 mol/l NaCl (pH 8.0).*

RESULTS

The plasma concentration of NTX in the treated calf (A) measured by the mentioned polarographic method was 140 mg/kg 24 h after the first s.c. injection and reached a peak of 228 mg/kg 3 h after the second injection (Fig. 2 a). Subsequently, the concentration decreased rapidly during the first 10 days and then decreased as a slower rate to 8 mg/kg at 42 days. The drug was still present (3 mg/kg) in the plasma 63 days after the first injection, suggesting a binding to some plasma component(s).

* The fractionation has been kindly performed by Dr. Christer Wahlter at the National Veterinary Institute in Stockholm.

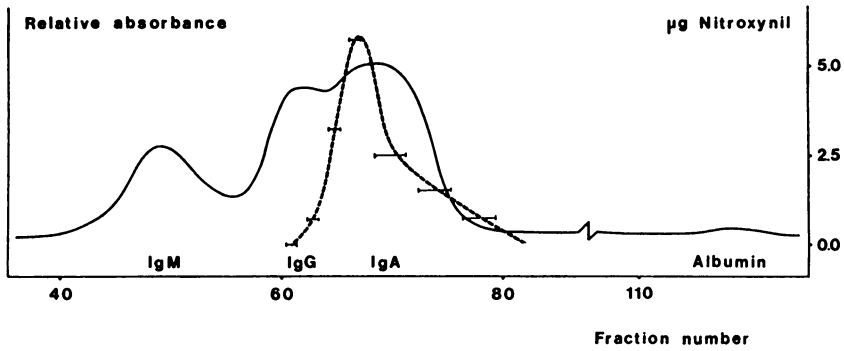


Figure 3. Distribution of NTX (-----) in the serum protein fractions (————) from a treated calf (see Fig. 2a). The blood sample was taken on day 28 after the last injection. Relative absorbance was measured at 280 nm.

A subsequent separation of plasma protein fractions revealed that almost-all the NTX was associated with the γ-globulins, no substance being detectabel in the albumin fraction.

From the total amount of sample containing 37.4 µg NTX applied to the column, 36 µg was recovered, all found in the IgA peak (Fig. 3).

The NTX residues in the muscle biopsy material (calf A) and the tissue samples from the slaughtered animals (A, B, C) reflected the elimination pattern observed in the plasma. There was a rapid decrease in NTX levels between 7 and 42 days after the treatment, followed by a slower disappearance of the substance, 0.11 mg/kg being present at 63 days. 5 µg/kg of NTX was still present in the muscle 90 days after the last injection (Fig. 2 b and Table 1).

Table 1. Nitroxynil concentrations in the tissues of cattle slaughtered various times after the injection of a therapeutic dose of the drug (Trodax®).

Animal	Time after the last injection (days)	Nitroxynil concentration (mg/kg)			
		Injection site	Muscle	Liver	Kidney
B	28	1.50	0.60	0.20	1.10
A	63	0.21	0.11	0.25	0.15
C	90	0.006	0.005	—*	—*

* not analysed

Table 2. Effect of cooking on the nitroxynil concentrations in the edible tissues from a calf (A) slaughtered 9 weeks after the administration of a therapeutic dose of the drug (Trodax®).

Tissue	Cooking	Nitroxynil (ppm)
Loin	—	0.09
	frying (60°C)[1]	0.09
	frying (77°C)[2]	0.04
Chuck	—	0.11
	boiling[3]	0.002
Tongue	—	0.34

[1, 2] Temperature inside the sample.
[3] Boiling time 2 h in salted water.

After 63 days (calf A) the concentrations of NTX in the tounge and at the injection site were three times and twice respectively those in skeletal muscle (Tables 2 and 3). Boiling of the meat sample resulted in a complete loss of the substance (< 2 μg/kg remained), while fried samples showed only a slight decrease, depending on the temperature reached in the centre of the samples during frying (Table 2).

The residues of NTX in the kidney (1.5 mg/kg) were found to be higher than those in the muscle and the liver 28 days after the injection (calf B). On the other hand, 63 days after the treatment (calf A) the liver showed a somewhat higher concentration (0.25 mg/kg) than the muscle (0.10 mg/kg) and kidney (0.15 mg/kg), respectively (Table 2).

A 0.19	B 0.17	C 0.32
D 0.16	E 0.21	F 0.18
G 0.24	H 0.22	I 0.21

Table 3. Distribution of nitroxynil around the site of injection in a calf (A) 9 weeks after the last injection of the drug (Trodax®). Each square represents an approximately 5 × 5 cm piece from the left lower third of neck musculature around the site of injection (E). NTX concentration (mg/kg) is given inside each square.

DISCUSSION

The polarographic method used in the present study is highly specific for NTX. Theoretically, any metabolite possessing an intact nitro-group, and having a favourable water-solvent partition would also be detected providing that the half way potential for the metabolite is situated in the tested potential range. However, there is at present no evidence that such metabolites are present in plasma or tissues of domestic animals treated with NTX (*Parnell* 1970, *Douch & Buchanan* 1979).

Due to financial constraints only a very limited material could be analysed in the present study. Therefore it is not possible to evaluate the significance of individual differences, age, health and nutritional status on NTX residues in the edible tissues from the treated animals. Nevertheless, both the NTX residues measured in the serial biopsy tissue material and those from the tissues of the slaughtered animals (after treatment with the recommended dose of the drug) are in a good agreement with the concentrations reported earlier in more extensive studies in calves and sheep (*Parnell* 1970). Therefore, based upon those and our results it is plausible to expect the drug to be present in meat at a level of about 0.1 mg/kg 2 months after the injection, and that detectable amounts (μg/kg range) are still present at least 90 days after the end of treatment.

Our results also suggest that there is an almost complete loss of NTX residues in boiled meat, while the residues would diminish only slightly after conventional frying. Analysis of the injection sites shows that 4 weeks after the injection the levels of NTX there are significantly higher than those in the muscles examined, but the difference diminishes after 9 weeks. The relatively high level of NTX in the tongue observed in the present investigation is difficult to explain and more experiments are necessary to verify this finding.

In agreement with the results of *Parnell* (1970) a retention of NTX was found in plasma, with about 3.0 mg/kg still present 2 months after treatment. From the results of their in vitro experiments *Berelta & Licatelli* (1967) suggested that NTX is bound to serum albumin. Under the present experimental conditions in vivo virtually all of the NTX was found to be associated with the γ-globulin fraction (IgA) and no drug was detectable in the serum albumin from the treated animal. The reason for this discrepancy is not clear at present and both the

practical implication for, for example, immunological functions as well as the further evaluation of this binding require further studies.

NTX was originally introduced as an antifasciolitic drug in the late 1960s (*Davis et al.* 1966). The relatively high acute toxicity of NTX observed in food-producing animals (*Lucas* 1967, *Guilhon* 1968) is probably connected to an uncoupling of oxidative phosphorylation and an increase in metabolic rate caused by this class of chemical substances (*Miert & Groeneveld* 1969). However, to our knowledge, no systematic toxicological study in warm-blooded animals has been published to enable the assessment of any potential health-risk to the consumer from the drug residues present in edible tissues.

Limited acute and subacute toxicological data from studies in laboratory animals are available from the producer (May & Baker Ltd.). Using these data we have calculated a tentative acceptable daily intake (ADI) for NTX of 0.001 mg/kg b.w., using a safety factor of 2 000 when extrapolating the "no effect dose" from animals to man. Such a safety factor has been recommended for veterinary drugs in the absence of long-term toxicological and teratogenic studies (*Perez* 1977) to compensate for possible differences in, for example, absorption, metabolism and sensitivity between the animal species and man (*FAO/WHO* 1974).

The estimated ADI for NTX should be compared with the concentration of 3.0 mg/kg of the drug which persists in the blood and the 0.1—0.3 mg/kg present in the edible tissues 2 months after the treatment. From this it can be concluded that a withdrawal time of 3 months for NTX in the edible tissues of cattle seems to be warranted in order to minimize the possible health risk to the consumer. Since a period of at least 2 months from the last NTX injection is necessary for the morphological changes in the meat of the infested animals to recede (*Wahlgren* 1980), the withdrawal time should not be impractical from the producers point of view. Withdrawal times exceeding 2 months are also prescribed in other countries, e.g. South Africa, where NTX is used to control Parafilaria b. NTX concentrations of up to 1.0 mg/kg after 24 h and 0.5 mg/kg after 48 h with detectable levels present up to 45 days post injection, have recently been reported in the milk of treated cows by *Takeshita et al.* (1980). Therefore the use of NTX is not to be recommended in lactating animals.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Prof. Göran Hugoson (National Veterinary Institute, Uppsala) for putting calves at our disposal. We are also indebted to Doc. Dan Christenson (National Veterinary Institute) for collecting the blood samples and for valuable discussions, and to Prof. Arne Lindholm (Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala) for taking the biopsy material. Dr. Wahlgren was most helpful with the slaughter material. Skillful technical assistance of Mrs. Ingegerd Bergström och Mr. Jorma Kuivinen is also appreciated.

REFERENCES

Beretta, C. & A. Locatelli: Anthelmintic and diffusion properties of 2-iso-4-cyano-6-nitrophenol affected by blood serum or by serum albumin solutions in vitro. Brit. vet. J. 1969, *125*, 483—491.

Bond, A. M.: Modern Polarographic Methods in Analytical Chemistry. M. Dekker. Inc., New York and Basel 1980, p. 1—518.

Davis, M., J. M. S. Lucas, J. Rosenbaum & D. E. Wright: 4-cyano-2-iodo-6-nitrophenol: a new fasciolide. Nature 1966, *211*, 882—883.

Douch, P. G. C. & L. L. Buchanan: The metabolism of nitrophenolic and 5-arylazorhodamine anthelmintics by Ascaris suum, Moniezia expansa and by mouse- and sheep-liver enzymes. Xenobiotica 1979, *9*, 467—474.

FAO/WHO: Toxicological evaluation of certain food additives with a review of general principles and of specifications. Seventh Report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Tech. Report Series 1974, No 539, 8—92.

Guilhon, J.: Tolérance du mouton an iodo-3 hydroxy-4 nitro-5 benzonitrile administré par voie parentérale. (Tolerance of sheep against parenteral application of iodo-3 hydroxy-4-nitro-5 benzonitrile). Bull. Acad. Vet. 1968, *41*, 37—40.

Hugoson, G.: Personal communication.

Lucas, J. M. S.: 4-cyano-2-iodo-6-nitrophenol, M & B 10.755. I: Activity against experimental fascioliasis in rabbits, sheep and calves. Brit. vet. J. 1967, *123*, 198—211.

Meites, L.: Polarographic Techniques. 2nd ed. J. Wiley & Sons Inc., New York 1965, p. 89.

van Miert, A. S. J. & H. W. Groeneveld: Anthelmintics used for the treatment of fasciolasis as uncouplers of oxidative phosphorylation in warm blooded animals. Europ. J. Pharmacol. 1969, *8*, 385—388.

Parnell, M. J.: Determination of nitroxynil residues in sheep and calves. Pestic. Sci. 1970, *1*, 138—143.

Perez, M. K.: Human safety data collection and evaluation for the approval of new animal drugs. J. Tox. environ. Health 1977, *3*, 837—857.

Takeshita, J., Kishi, M. Seki, K. Fujiyama, O. Otsaka & K. Ahiko: Analysis of nitroxynil (Fasciolicide) in milk and dairy products. Milchwissenschaft 1980, *35*, 133—135.

Wahlgren, T.: Personal communication.

Wellington, A. C.: Parafilaria bovicola in cattle and its control. J. S. Afr. vet. med. Ass. 1980, *51*, 243—244.

SAMMANFATTNING

Bestämning och hälsoriskvärdering av nitroxynilrester i ätbare vävnader från nötkreatur.

En specifik polarografisk metod, med en känslighet ≥ 2 µg/kg, har använts för att bestämma resthalter nitroxynil (NTX) som användes mot Parafilaria bovicola hos boskap i plasma och vävnad. Efter behandling med den terapeutiska dosen NTX (2 × 20 mg/kg kroppsvikt, s.c) erhölls initialt en snabb minskning av NTX i blodet följt av en långsammare nedgång med en retardation av 8 mg/kg NTX efter 6 veckor och 3 mg/kg NTX efter 3 månader. Muskel och andra ätbara vävnader från boskap slaktade vid olika tidpunkter efter behandlingen, innehöll 0.1—0.3 mg/kg NTX efter 2 månader och fortfarande detekterbara spårmängder (µg/kg området) 3 månader efter injektionerna. Med hänsyn till tillgängliga farmakologiska och toxikologiska data föreslås en karenstid av 3 månader för NTX behandlad boskap.

(Received April 22, 1982).

Reprints may be requested from: Lars-Gösta Ekström, the National Food Administration, Nutrition Laboratory, Box 622, S-751 26 Uppsala, Sweden.