Malachite green in freshwater fish in the Czech Republic

Simona Haldová[1], Jiří Drápal[1], Alena Honzlová[2]

[1]Central Veterinary Administration of the State Veterinary Administration
Prague, Czech Republic
[2]State Veterinary Institute
Jihlava, Czech Republic

Abstract

This paper presents the results of testing for residues of malachite green (MG) and its leucoform (LMG) in freshwater fish in the Czech Republic in the years 2012 – 2014 and a comparison with other EU states (results for 2012). A total of 260 samples of fish were tested. Thirty-two samples (12.31%) had a measurable concentration above the LOQ (the CC-alpha for MG and LMG is 0.3 $\mu g \cdot kg^{-1}$). Twelve samples (4.62%) failed to satisfy the minimum required performance limit of 2 $\mu g \cdot kg^{-1}$. Almost ninety-one percent of all of the samples in which a measurable concentration of MG/LMG was found were samples of fish from the Salmonid family (*Salmonidae*).

Freshwater fish, leucomalachite green, malachite green

Introduction

Malachite green is a triphenylmethane dye. It is a dark-green crystalline substance that has been used as a fungicide and anti-parasitic agent on fish farms all over the world since 1936 (Srivastava 2004). It is widely used as a dye for leather, wool, cotton and jute and in the paper industry. It is also used as a pH indicator and for the detection of blood in forensic medicine. It is said that around 10 – 15% of all dyestuffs end up in wastewater during the dyeing process (BfR 2007; Arnold 2008). It is an extremely effective substance in the treatment of mycoses in fish and fish eggs and in the treatment of bacterial infections and parasitoses. In this country, it has been used primarily on the rainbow trout (*Oncorhynchus mykiss)* and the brook trout or char (*Salvelinus fontinalis*). It is often applied in the form of short or long baths (BfR 2007), with immersion baths for a period of 10 – 30 seconds at a concentration of up to 100 $mg \cdot l^{-1}$ used to treat mycoses, short baths of 60 – 90 minutes at a concentration of 6.7 $mg \cdot l^{-1}$, and long six-day baths at an MG concentration of 0.15 $mg \cdot l^{-1}$ for Salmonids (*Salmonidae*) and 0.5 $mg \cdot l^{-1}$ for Cyprinids (*Cyprinidae*). This therapy is used primarily to treat freshwater white spot disease (caused by the ectoparasite *Ichthyophthirius multifiliis*). The concentration of MG may be reduced if a multi-component bath is used, e.g. in combination with formaldehyde (Arnold 2008). MG therapy can have numerous undesirable effects on the fish and fish eggs treated. Temperature is an important factor determining therapeutic and toxic effects (Arnold 2008). Lower weight gain, higher mortality and anaemia have all been observed as negative side effects. The formation of tumours in the stomach, intestine and liver has been reported in sea trout. The lethal concentrations of certain types of MG are extremely close to the therapeutic concentrations. The course of poisoning is extremely rapid, with the clinical description including great agitation, uncoordinated movement around the bath, fish keeping to the upper half of the tank, leaping above the surface, and gasping in carp, followed by a loss of balance, apathy, agony and death (Máchová 2001).

In 2005, a report from the National Toxicology Programme was published which concluded that MG may cause tumours in the thyroid glands, liver and mammary glands in female rats and cancer of the liver in female mice (NTP 2005). It may, then, be a potentially carcinogenic chemical in man. Further clinical and experimental observations describe MG as a multi-organ toxin causing renal changes in rabbits, reduced food intake, reduced

Address for correspondence:
MVDr. Simona Haldová
Central Veterinary Administration of the State Veterinary Administration
Slezská 7/100, 120 56 Praha 2, Czech Republic
Phone: +420 227 010 397
Email: s.haldova@svscr.cz
www.maso-international.cz

growth and fertility, and damage to the liver, spleen, kidneys and heart, and also causing lesions on the skin, eyes, lungs and bones and having teratogenic and mutagenic effects in rats (Srivastava 2004). An MG is highly cumulative in nature; its content in the bodies of treated fish increases with the number of repeated baths and the level of the concentration used. It has been shown that as much as 90% of the ingested MG is accumulated in the fish muscle as leucobase. While the coloured form is quickly excreted, the leucobase is excreted only extremely slowly. It has been shown that analysed samples of muscle, liver and skin were not negative until 12 months after an MG bath, while the period of decline of MG is extended still further in the case of repeated baths (Máchová 2001).

In view of the accumulation ability of MG/LMG and the negative effects found, the use of this pharmacologically effective substance is currently possible only on aquarium fish and other fish that are not destined for human consumption. Its use on animals intended for the production of food is not permitted in the European Union (EU). No maximum residue limit (MRL) is stipulated for this substance in Commission Regulation (EU) No. 37/2010. The EU Commission has called on member states both to respect the value of the harmonised minimum required performance limit (MRPL) which is, *de facto*, the stipulated lowest value to be achieved by analytic methods during the determination of residues of MG and its metabolic form (leucobase – LMG), and to use it as the decisive limit for releasing or not releasing fish onto the market. An MRPL of 2 $\mu g \cdot kg^{-1}$ for the sum of MG and LMG is stipulated for aquaculture products (Commission Decision No. 2002/657/EC).

The aim of this work was to assess the number of findings of residues of malachite green or its leucoform in freshwater fish in the Czech Republic in the years 2012 – 2014 and to compare this finding with similar results from other EU member states.

Materials and Methods

Test results were obtained within the framework of the National Plan for Monitoring Residues and Contaminants in accordance with Council Directive No. 96/23/EC and associated legislation. Minimum numbers of samples were determined according to the methodology in the given directive. Each sample was comprised of the muscle tissue with skin (fillets) of five fish of the same species (trout, char, carp and other freshwater fish) to a total weight of 0.5 kg. Individual fillets were wrapped in aluminium foil or in an opaque safe collection bag. Samples were taken from fish destined for the market, no more than two months and no less than two weeks before their intended release onto the market. Samples were taken by veterinary inspectors at random in the Czech Republic from 2012 to 2014. Analysis was performed in a liquid chromatograph with a mass spectrometer operating on the triple quadrupole principle (LC-MS/MS). The sample of fish was thoroughly homogenised before analysis. Muscle tissue with a natural proportion of skin was processed. Following the weighing of the sample and the addition of hydroxylamine, the MG/LMG was extracted from the sample with acetonitrile. Quantification was performed by the internal standard method (malachite green D5 and leucomalachite green D5 are used as internal standards). The chromatographic analysis itself was performed by gradient elution (0.1 M ammonium acetate pH = 4.5/acetonitrile) in a Symmetry C18 column (150 mm x 3.9 mm, 5 µm) with a Symmetry C18 precolumn (20 mm x 3.9 mm, 5 µm). The method is validated in accordance with Commission Decision No. 2002/657/EC. The CC-alpha for MG and LMG is 0.3 $\mu g \cdot kg^{-1}$.

Results and Discussion

Between 2012 and 2014, totally 260 samples of fish were taken in the Czech Republic for MG/LMG content testing. The largest proportion of samples (73.5%) was taken from fish from the Salmonid family (trout, char, whitefish) in view of the fact that MG is used most frequently in the rearing of these fish. The remaining 69 samples (26.5%) were fish from the Cyprinid family and other fish (catfish and pike).

Thirty-two samples (12.31%) had a measurable concentration above the CC-alpha, of which 20 samples (7.69%) had a concentration beneath the MRPL. Twelve samples (4.62%) did not satisfy the MRPL value of 2 $\mu g \cdot kg^{-1}$. This means that the residues exceeded the decisive criterion and the fish could not be released onto the market for human

consumption. The number of samples with values above the MRPL fell from 2012 to 2014 by 33.33%. In 2013, the number of samples attaining a measurable concentration of LMG/MG also fell, this by a half over 2012. The 20% increase in the number of samples in 2014 was not reflected in an increase in the number of unsatisfactory findings. Of all the samples in which MG/LMG was found at a measurable concentration, 30 samples (93.8%) were from the Salmonid family (trout, whitefish, char), of which 10 samples (33.3%) did not satisfy the MRPL value of 2 $\mu g \cdot kg^{-1}$. The remaining two samples (6.2%) were of fish from the carp family (carp, bream); the residues in these samples exceeded the MRPL of 2 $\mu g \cdot kg^{-1}$ for MG/LMG. Of the 32 samples of fish in which LMG was found at a measurable concentration, 3 samples also contained MG, which is evidence of the recent application of MG. The highest concentration of LMG found was 28.1 $\mu g \cdot kg^{-1}$, this in a sample of trout in which an MG concentration of 0.76 $\mu g \cdot kg^{-1}$ was also found. An overview of tested samples according to the sum concentration of LMG and MG is given in Table 1.

Table 1. Number of samples by sum concentration of LMG and MG in the Salmonid family, the carp family and other fish in the Czech Republic in 2012 – 2014

Year	2012				2013				2014			
Fish	Salmonid	Carp	Other	Total	Salmonid	Carp	Other	Total	Salmonid	Carp	Other	Total
n	67	13	0	80	63	16	1	80	64	34	2	100
< CCα	51	13	0	64	56	15	1	72	57	33	2	93
> CCα	16	0	0	16	7	1	0	8	7	1	0	8
< MRPL												
> CCα	10	0	0	10	4	0	0	4	6	0	0	6
> MRPL	6	0	0	6	3	1	0	4	1	1	0	2

MRPL - minimum required performance limit; n – number of samples

In spite of the fact that the use of MG is not permitted in the EU, it is clear from the analysis results that it is a substance that is still in use, primarily on fish in the Salmonid family. This is, in all probability, the result of the lack of a similarly effective and favourably priced alternative. It is, nevertheless, possible to use, for example, a solution of formaldehyde, iodine detergent preparations, a solution of sodium chloride, peracetic acid, a veterinary formulation with the active substance Bronopol, and other formulations, an overview of which has been published in the article "Therapeutic Possibilities on Fish Farms in the Czech Republic – An Overview" (Kolářová and Nepejchalová 2014).

The EFSA technical report for 2012 shows the most frequent detection of MG/LMG residues in the Czech Republic (16.3% of 80 samples). A significant number of positive findings was also seen in Slovakia (5% of 60 samples) and Poland (3.3% of 152 samples), and also in Bulgaria (2.6% of 39 samples), Belgium (3.9% of 76 samples) and Austria (1.1% of 90 samples) (EFSA 2014). The large proportion of MG/LMG residues found in the Czech Republic in comparison with other EU member states can be put down either to the greater frequency of actual illicit use of MG, particularly on Salmonid fish farms, in the Czech Republic, or to the fact that MG is still used by fishing clubs in rearing Salmonid fish that are released into watercourses for sports angling and thereby contaminate the water in our rivers. Industrial wastewater must also not be neglected as a possible source of contamination.

Conclusions

From the viewpoint of assessment of the health safety of fish muscle in the Czech Republic according to the decisive limit for MG/LMG given in the legislation, fish (and in particular fish of the Salmonid family) have represented a problem in the Czech Republic in the past, and continue to do so. There has, however, been significant improvement to the situation in the last two years. A possible reason for this is more intensive veterinary inspection of farms rearing fish destined for the market and the sanctions imposed when an unsatisfactory finding of MG/LMG in fish tissue is made. The primary emphasis on fish farms should be placed on preventive measures, i.e. on the rearing of fish in optimal conditions (the quality of hatching material, water temperature, adequate nutrition and the chemical parameters of the water).

References

Arnold D, LeBizec B, Ellis R 2008: Malachite Green. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs **6**: 63-108

BfR 2007: Collection and pre-selection of available data to used for the risk collection and pre-selection of available data to be used for the risk assessment. Updated BfR Expert Opinion No. 007/2008

EFSA 2014: Report for 2012 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products. Available online: http://www.efsa.europa.eu/fr/supporting/doc/540e.pdf

Kolářová J, Nepejchalová L 2014: Terapeutické možnosti v chovech ryb v ČR – přehled. Veterinářství **7**: 533-538 (In Czech)

Máchová J, Svobodová Z, Svobodník J 2001: Rizika používání malachitové zeleně v rybářství. Veterinářství **51**: 132-135 (In Czech)

Commission Regulation (EU) No. 37/2010 of 22nd December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of the European Union L 15/1: 72 p

National Institutes of Health 2005: NTP Tecnical report on the toxicology and carcinogenesis studies of malachite green chloride and leucomalachite green. National toxicology program. Available online: http://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr527.pdf

Commission Decision No. 2002/657/EC of 12th August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance) (notified under document number C(2002) 3044). Official Journal of the European Communities, L 221/8: 29 p

Srivastava S, Sinha R, Roy D 2004: Toxicological effects of malachite green. Aquatice Toxicil **66**: 319-329