IN VITRO STUDY OF DIMINAZENE ACETURATE COMPLEX WITH B-CYCLODEXTRIN FOR *ICHTHYOPHTHIRIUS MULTIFILIIS*

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Abstract

The histophagous ciliate Ichthyophthyrius multifiliis can cause lethality in farmed carp brood (Cyprinus carpio) as well as other representatives. In the present study, an antiparasitic substance (diminazene aceturate) and its complex with a cyclodextrin were tested for its activity against this pathogen in vitro. The purpose of this paper is to highlight the therapeutic potential of diminazene and the enhancement by the β-cyclodextrin. Of these, the complex proved to be more effective (i.e., killed all parasites in a test period of 6-8 hours). Administration in filtered water suggests that these compounds can not be effective in bathing. In view of these findings, we will discuss the potential utility of chemotherapy as a strategy for controlling ciliatosis in farmed fish.

Keywords*: DSC, β-ciclodextrine, diminazene aceturate, complex*

Introduction

Diminazene is an aromatic diamidine of synthetic origin linked to a triazene bridge. The experimental procedure for the synthesis of this compound and other diazoaminobenzenes has been reported by Hochst (Hochst, 1954) with the primary objective of controlling diseases caused by blood transmissible protozoan parasites such as *Trypanosoma sp*. and *Babesia sp*. Diminazene is also known as diminazene aceturate when it is a pharmaceutical formulation containing two salt acetates (Atsriku et al., 2002). The experimental procedure for the synthesis of diminazene aceturate derives from the physicochemical characteristics of diminazene, such as instability and insolubility in aqueous solutions.

Since diminazene aceturate has been used for decades in the treatment of animal tripanosomiasis, some species of the genus *Trypanosoma*, such as *T. congolense*, developped tolerance and resistance (Moti et al., 2015). For this reason, several studies have investigated alternatives to improve success in tripanosomal treatment using new drugs in combination with the chemotherapy agent diminazene aceturate (Mbaya, 2009; Tonin, 2011), as well as the search for new pharmacological applications.

Despite the existing scientific literature that has been addressing this topic for over 50 years, several review articles have discussed in detail the chemical/pharmacological activities of diminazene aceturate. Two articles of comprehensive review (Peregrine, 1993) highlighted its pharmacological functions, the most recent being that of Kuriakose et al. (Kuriakose, 2014), which reports the pharmacological importance for modulation of the immune system.

Therefore, diminazene aceturate is still the target of studies on its therapeutic potential and therefore attracted great interest in the development of new research. Thus, given the pharmacological potential of diminazene, the aim of this study was to develop the systemic and pharmacological effects by including it in a cyclodextrin (β-cyclodextrin) and the use of the complex against aquatic protozoans such as ciliate *Ichthyophthirius multifiliis*.

Ichthyophthirius multifiliis is a protozoan that causes "white spot" disease and is a major burden for farmers and aquarists around the world. The infected stage of the parasite invades the

skin and gills of the fish, penetrates into the epidermis and is located above the basal lamae (Ventura, 1985). Here it turns into trophont stage feeding on fish tissues until it reaches a size of 0.5-1.0 mm and is macroscopically visible as a white spot (Buchmann, 2001). The mature trophont comes out of the fish and turns into a tomont, looking for bottom surfaces for encysting in a tomocyst where asexuous reproduction occurs. When the trophont leaves the fish host, it disrupts the epidermis and the epithelium, which can perturb osmoreglation and may leave fish sensitive to secondary infections (Matthews, 2005).

Various treatments have been used to combat the parasite with treatment regimens that change according to the new legislation on toxicity and carcinogenicity of the applied substances. The trophont status of the parasite, which is protected from the epidermis of the fish, is generally more resistant to treatments than the previous stage but requires intensive effort and repetitive treatments to eliminate the infection by targeting the frontal phase. A search for new effective and safe compounds for the treatment of the disease is in progress and some new candidate drugs seem promising.

In this article we proposed to include diminazene aceturate in β-cyclodextrin to obtain a favorable *in vitro* effect against the protozoan *Ichthyophthirius multifiliis*.

Material and method

The substances used, diminazene aceturate (purity> 97%) and β-Cyclodextrin (purity> 98%) were purchased from Sigma-Aldrich. All other reagents were of analytical quality. The water used was double distilled and deionized.

All experiments and handling steps were performed at 22°C. The fish were taken from a breeder in Iasi who owned carp fish, carp "mirror" and Japanese carp (koi) recently purchased from Israel. Carp were infested naturally with *I. multifiliis* and anesthetized in 100 mg / L MS-222, and mucus samples were collected by scraping the skin. The mucus was dispersed in a Petri dish containing dechlorinated water. Under a stereomicroscope, live trophonts (Figure 1) were taken up with a Pasteur pipette and placed in another Petri dish containing dechlorinated water. This step was repeated twice to separate the trophonts from the surrounding mucus.

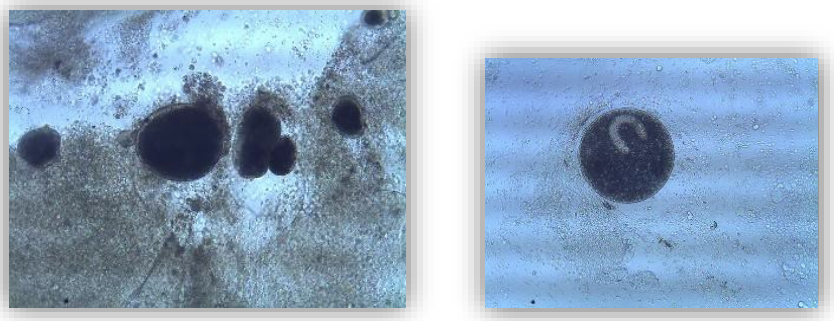

Figure 1 Trophonts of *I. multifiliis* (x20)

Using diminazene aceturate and β-CD-Diminazene aceturate, we prepared a stock solution of 100 mg/ml in dimethyl sulfoxide (DMSO) and diluted to the required concentrations using dechlorinated water. The pH of the dechlorinated water was not affected by the addition of the substances and was in the range of 7.5-8.0. For *in vitro* tests, 2 ml of the test solution was placed

in the wells of the microtiter plates, and a total of 100 trophonts was added. The controls were made in dechlorinated water containing appropriate concentrations of DMSO. After 6 hours, viable and non-viable trophonts were counted under a stereomicroscope, and movement and intact structure were used as viability criteria. The microtiter plates were re-incubated for a further 12 hours. Trophonts were counted on the MOTIC 40X microscope in the Bürker Türk counting room.

Compounds or suspensions of powder compounds (the tablets were previously sprayed) in distilled water or dimethylsulfoxide (DMSO) were prepared from the compounds. The resulting stocks or commercially purchased solutions were diluted in phosphate-based physiological saline solution (PBS, pH 7.2) or in water at the final concentrations used in the screening. Tests in filtered water allow automatic exclusion of the possibility of partial or total inactivation of the test substance under the given conditions. A substance considered to be effective *in vitro* by this procedure can be expected to be effective in administrating in the bath or medicated feed to infected fish.

The ciliates in the late exponential phase or in the early plateau phase of culture were concentrated by centrifugation at 650 x g for 5 minutes and then resuspended in PBS or filtered water. After counting into a hemocytometer, 10 µl of ciliate suspension containing ~100 ciliates in each well of 96-well microtiter plates containing 90 µl of the candidate antiprotozoal at the required dose in PBS or filtered water. The final doses tested were: 100, 50, 25, 12.5, 6.2, 3.1, 1.5 and 0.8 ppm for the remaining test substances. Each determination was carried out in duplicates. To exclude the possible effects of the solvent in the compounds dissolved in DMSO, duplicate wells with PBS or filtered water containing the highest DMSO concentration used (up to 2.5%) were included. Plates were incubated at 18°C for 24 hours. Ciliary motility after incubation was checked using a phase contrast illumination microscope. Prior to scanning, each culture plate was gently rotated to ensure a uniform distribution of the ciliate in the medium. The minimal lethal concentration (CML) for each drug was defined as the highest drug dilution at which 100% of the ciliate were lysed or non-motile (there was no evidence of ciliary motion using a 40x objective).

Thermogravimetric analysis was determined by thermal desorption of diethylamine on a DuPont Instruments Thermal Analyst 2000/2100 coupled with a 951 thermogravimetric analyzer. Differential Scanning Calorimetry (DSC) measurements were performed using a DSC 823 (Mettler Toledo). The sample was packed in aluminum cans placed in the DSC cell and then heated at a rate of 10°C/min from room temperature to 200°C, maintained for 2 minutes at 200°C, then cooled to ambient temperature.

Results and discussion

Although the effects on cell morphology were different, most active substances induced cell rounding and vacuolization changes before the eventual lysis. In all β-CD-diminazene aceturate tests, all ciliates died after 6 hours at doses of 100 and, respectively 80 ppm. In similar *in vitro* studies, diminazene aceturate have been shown to be effective against other fish pathogens, such as *Gyrodactylus spp*. This compound is not commonly used in aquaculture to control ectoparasite diseases. However, we consider that diminazene is suitable for the treatment of this disease, given the ectoparasite location of *Ichthyophthirius multifiliis*. When a ciliatosis outbreak due to *Ichthyophthirius multifiliis* is diagnosed for the first time in a large farm, many fish in the affected reservoir already have an infection characterized by the presence of numerous ciliate in the tegument and in the gills. In such cases, the respiratory capacity of fish will be seriously compromised, and treatment will not only be ineffective but may even accelerate death by reducing

oxygen availability. However, given its efficacy for killing free ciliate forms, we may consider future chemotherapeutic use in aquaculture that could be used for prophylactic purposes.

The chosen substance was diminazene aceturate or Berenil. This was a drug widely used in tripanosomiasis and babesiosis. Although the compound is on the market since 1955, the mechanisms of action are poorly understood. While early reports show that Berenil possesses tripanolytic and tripanostatic properties, some studies show that it can affect the immune system of the host. Recent studies show that treatment with Berenil reduces the production of proinflammatory cytokines (IL-6, IL-12 and TNF) *in vivo* and *in vitro*. Berenil's ability to disrupt major intracellular signaling pathways leading to the production of proinflammatory cytokines indicates that it can be used in the treatment of diseases that produce excess proinflammatory cytokines (Shiby K., 2014).

Thermogravimetric analysis of the complex

The analysis of diminazene aceturate with β-cyclodextrin was carried out at the National Research and Development Institute for Chemistry and Petrochemistry (ICECHIM) in Bucharest.

After confirming and characterizing complex formation in solution, we continued with the preparation of the complex in solid state. For this purpose, we used the lyophilization method, which usually offers good yields over other methods, but which has industrial scale applications, given its simplicity.

In addition, the characteristics of the mixture by lyophilization means that it could easily be incorporated during the manufacture of the feed.

As stated, the β-Cyclodextrin inclusion complex with diminazene aceturate was obtained by the lyophilization method (coevaporation). To determine the efficacy of complexing with diminazene aceturate, they were mixed in various proportions using a mixture of water and 50:50 glacial acetic acid as a wetting agent. Preliminary studies have indicated the need to reduce the pH of the wetting agent to facilitate partial dissolution of the drug and thus improve complex formation; Indeed, formation of the complex does not occur if the wetting agent is only water. Acetic acid has been chosen in view of its high volatility, so it is rapidly eliminated from the complex, minimizing toxicity problems.

For the preparation of inclusion complexes, diminazene aceturate and β-cyclodextrin were mixed in appropriate proportions and then milled. Subsequently, the wetting agent was added. The obtained paste was dried in an oven at 40°C for 24 hours and the 200-500 μm fraction was obtained and used for subsequent tests. Using this method, mixtures of diminazene aceturate and β-cyclodextrin were prepared in molar ratios of 1:1.

The stability of the inclusion complex was determined both in the presence of inert matter and in the presence of air. Fig. 2 shows both thermogravimetric analysis for diminazene/β-cyclodextrin complex. The air and inert mass loss curves are similar, demonstrating a high stability to the oxidation complex inclusion. There is a mass loss of about 4% at temperatures up to 160°C, which corresponds to evaporation of the solvents, a mass loss of about 15% at temperatures up to 250°C and a weight loss of over 40% at 350°C. Mass loss occurring in the temperature range of 200-250°C is probably due to dehydration of cyclodextrin and at temperatures of 250-350°C, probably due to dextrin degradation.

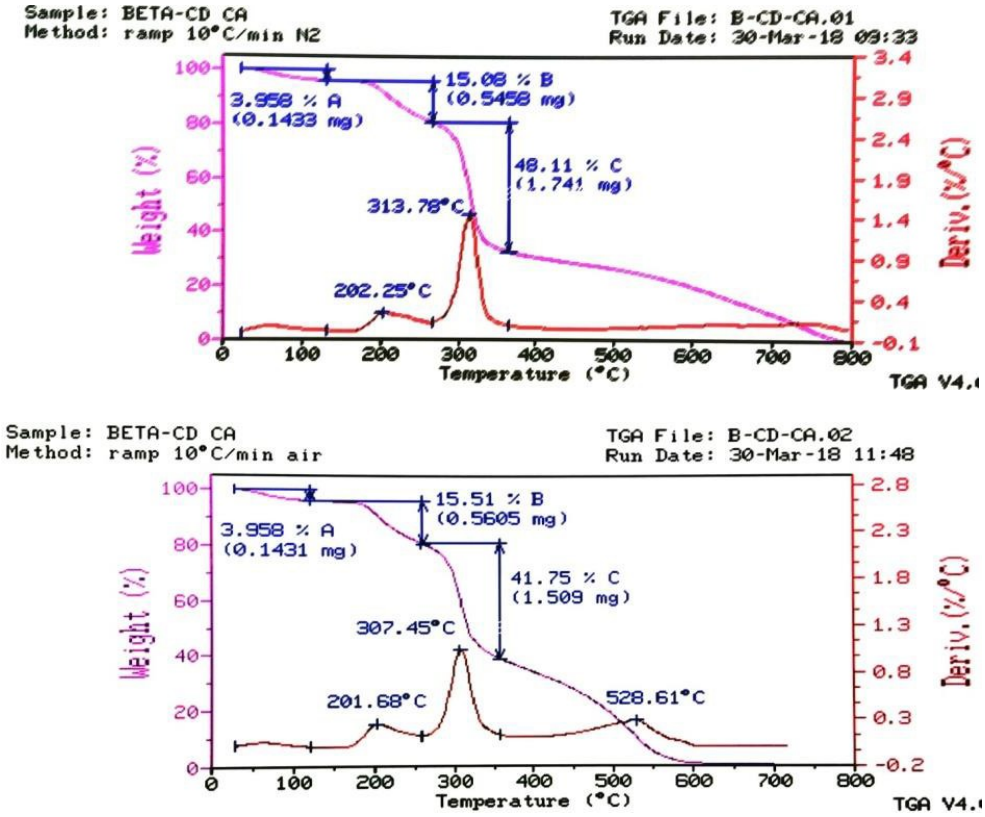

Figure 2 Termogravimetric analysis in inert gas and in synthetic air for diminazene aceturate/ β-cyclodextrin complex

Differential Scanning Calorimetry analysis of the complex

Thermal curves obtained by DSC provide information on the stability of the complex and, implicitly, the degradation temperature of one component (Figure 3). We noticed the presence of exothermic phenomena at temperatures up to 146.4°C as well as the occurrence of stronger interactions between the two components of the inclusion compound, whereas from about 160°C, endothermic processes such as phase changes or even degradation occur.

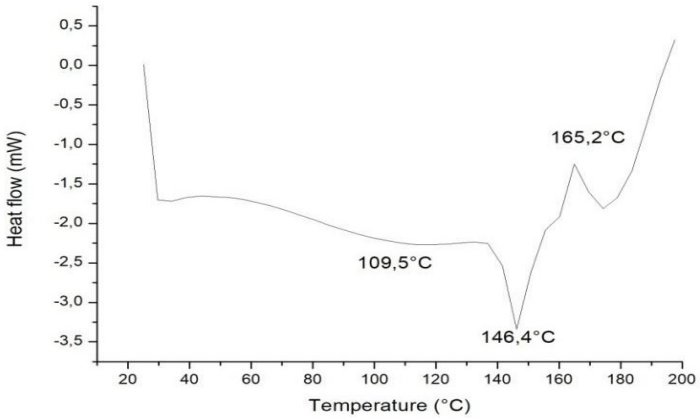

Figure 3 DSC analysis of diminazene aceturate/ β-cyclodextrin complex

In vitro analysis of the complex for I. multifiliis

The β-CD-diminazene aceturate complex was the one that showed activity against *Ichthyophthirius multifiliis* (MLC = 80 ppm). This product inhibits the transport of electrons associated with oxidative phosphorylation and thus deprives the cell of its energy source. β-CD-diminazene aceturate also has lethal *in vitro* activity against *Tetrahymena pyriformis*, another pathogenic ciliate. However, despite its *in vitro* efficacy, a number of factors should be considered in conducting tests to determine *in vivo* therapeutic capacity. First, bathing should be done cautiously, as this compound is very toxic in this way (Schmahl et al., 1989). Secondly, diminazene aceturate exhibit a lower intestinal absorption compared to β-CD-Diminazene aceturate (Swan, 1999), so that oral tolerance in food can be greater than the tolerance of bathing administration.

Table 1.

Efficacy of the complexed drug in different doses (mortalities in 6 h)

Dosage(ppm)	**Control**	**DA**	***β*-CD-DA**
1,5	0	12	1
3,1	1	19	5
6,2	1	25	8
12,5	1	32	17
25	1	35	30
50	1	34	80
100	32	87	100

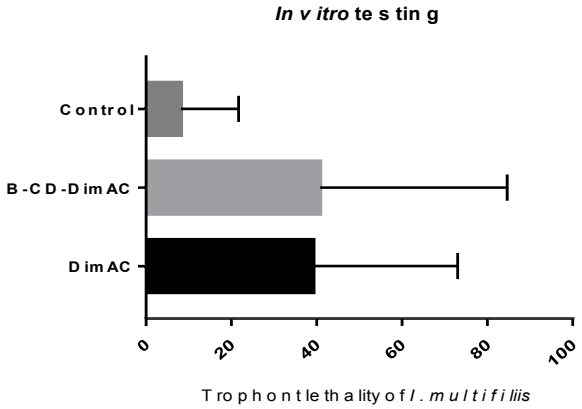

Figure 5 The effectiveness of the substances in 6h

Furthermore, β-CD-diminazene aceturate can be a good candidate for controlling ciliatoses caused not only by *Ichthyophthirius multifiliis* but also against other protozoans living in tissues (Schmahl et al., 1989). The mechanism of action against these parasites is not clear, although it has been suggested that they can act on enzymes, affecting the synthesis of pyrimidine.

Despite the fact that it has been shown that bathing is highly effective against certain intracellular or tissular parasites of freshwater fish, we can assume that its efficacy would be more significant in medicated feed. Therefore, oral administration should not be excluded in future evaluations of *in vivo* efficacy. In the present study, high efficacy when administered in water argues its future use *in vivo* for the treatment of ciliatoses.

Conclusions

We chose only diminazene aceturate and complex β-CD-diminazene aceturate for their proven antiprotozoan activity that killed the ciliates in 6-8 hours at quite high doses (CLM = 80 ppm in both cases). Numerous authors have reported the effectiveness of the bath for the treatment of ectoparasites and subepidermic protozoan infections of fish. Moreover, continued administration of diminazene in fish feed may be effective in eliminating the *Ichthyophthirius multifiliis* ciliate trophonts that parasite into the skin of the fish. In this case, the uncomplexed compound causes rupture of the external alveolar membrane and alteration of intracellular digestion. The mechanism of the antiprotozoan substance is not precisely known and appears to differ from one organism to another. However, in other protozoa, diminazene aceturate due to its chemical structure containing two identical cationic groups (dicyclic diamidine) has a high affinity for the adenine-thymine base pair sequence in cDNA, resulting in non-covalent interactions (electrostatic interactions and hydrogen bonds). In the mitochondrial genome, diminazene aceturate interacts strongly with the minor helix of the double helix so that this diamidine compromises essential replication processes and induces changes in ribosomes, mitochondrial membranes and amino acid transport (Kuriakose et al 2012, Sow et al., 2012, Caramelo-Nunes C., 2011). Due to the ability of this compound to produce *in vitro* mortality for *Ichthyophthirius multifiliis*, the possible effects on cell division in similar protozoa suggest that their efficacy *in vivo* should be analyzed independently.

The stability of the inclusion complex was determined both in the presence of the inert matter as in the presence of air. The thermogravimetric analysis for the diminazene aceturate-β-

cyclodextrin complex, in air and inert matter, is similar, demonstrating a high stability to the oxidation of the inclusion complex.

In conclusion, the candidate inclusion complex tested proved to be effective against *Ichthyophthirius multifiliis in vitro*. In view of these results, the efficacy of this compound *in vivo* clearly deserves attention. However, it should be kept in mind that various factors can be expected to influence the success of chemotherapeutic measures on ciliate in farmed carp or other fish. First of all, *Ichthyophthirius multifiliis* is a highly virulent species that breaks rapidly (by binary division) and migrates through tissues as well. Secondly, only a few chemotherapeutic agents have been accepted for use in aquaculture by legislative organs in different countries, making selection of medicines difficult for the treatment of infectious fish diseases, including ciliatosis.

Finally, the fact that this inclusion complex from the current study showed increased *in vitro* activity could mean that it can also be effective enough in oral administration (in a medicated feed), but very difficult to achieve in fish at an advanced stage of disease.

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