

Original Article

Biodynamic parameters of micellar diminazene in sheep erythrocytes and blood plasma

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In this work, we used a preparation of diminazene, which belongs to the group of aromatic diamidines. This compound acts on the causative agents of blood protozoan diseases produced by both flagellated protozoa (*Trypanosoma*) and members of the class Piroplasmida (*Babesia*, *Theileria*, and *Cytauxzoon*) in various domestic and wild animals, and it is widely used in veterinary medicine. We examined the behavior of water-disperse diminazene (immobilized in Tween 80 micelles) at the cellular and organismal levels. We assessed the interaction of an aqueous and a water-disperse preparation with cells of the reticuloendothelial system. We compared the kinetic parameters of aqueous and water-disperse diminazene in sheep erythrocytes and plasma. The therapeutic properties of these two preparations were also compared. We found that the surface-active substances improved intracellular penetration of the active substance through interaction with the cell membrane. In sheep blood erythrocytes, micellar diminazene accumulated more than its aqueous analog. This form was also more effective therapeutically than the aqueous analog. Our findings demonstrate that use of micellar diminazene allows the injection dose to be reduced by 30%.

Keywords: babesia, biodynamic, diminazene, kinetic, micelles

Introduction

Increasing the effectiveness of targeted drug delivery is an important issue in biomedicine and is an area of active and intense research [10]. In drug manufacturing, polymers and surface-active substances (SASs) are extensively used as auxiliary components (*e.g.*, substances that prolong drug action and emulsifiers). In the past few

years, research has shown that these may serve as a basis for the development of effective targeted delivery systems for high- and low-molecular weight compounds [1,3,5,9, 15].

Currently, one of the most active areas of research in veterinary medicine is the construction of drugs based on vesicular (micelles and liposomes) or similar (dendrimers and fullerenes) nanosystems. Micellar and liposomal corpuscular systems are similar in structure and delivery properties and permit intraorganismal and intracellular delivery of markedly hydrophobic drugs [11]. At times, the introduction of an active substance into such a system enhances drug bioavailability; this subsequently increases the therapeutic activity and allows the use of lower drug doses. The resulting preparations are often less toxic and have greater therapeutic effectiveness.

Micelles are aggregates formed by long-chain diphilic molecules or SAS ions. These aggregates form spontaneously in solutions containing these molecules when a specific concentration, called the critical concentration of micelle formation (CCMF), is reached. The CCMF is primarily dependent on the nature of the polar group and on the length of the molecular chain [14]. At SAS concentrations close to the concentration termed CCMF1, micellar particles are formed in which the inner areas are created by the hydrophobic portions of SAS molecules, with hydrophilic portions concentrated at the surface. As the SAS concentration increases, the micelles become polymorphic (globular, cylindrical, or hexagonal) and form more complex shapes [17].

Among the wide variety of anti-parasitic veterinary drugs, anti-piroplasm agents deserve particular attention. Piroplasms are protozoa that destroy blood cells (primarily erythrocytes), thereby causing oxygen starvation in the affected organisms. These microorganisms are extremely damaging in animal husbandry and are the causative agent of piroplasmosis, a major disease in tropical and sub-

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tropical regions that threatens more than 600 million animals.

Piroplasms are transmitted by ticks and include the genera *Babesia* and *Theileria* (syn. *Cytauxzoon*). The asexual reproduction of these microorganisms occurs in blood stem cells (*Babesia*) and lymphoid cells (*Theileria*). Sexual reproduction and sporulation is carried out in the body of the transporting host (tick). Piroplasms also are transmitted transovarially.

Occasional cases of human piroplasm infection in the United States and Europe are associated with *Babesia microti* and *Babesia divergens*, which are transmitted by *Ixodes* ticks [16]. In the southern regions of Russia, three outbreaks of cattle piroplasmosis and francisellosis as well as horse nutalliosis occur, usually from April until October. In southwestern Russia, cases of cattle babesiosis have been recorded in the spring. This occurrence can be explained by inadequate prophylactic measures for the control of transmitter ticks, and also by increases in tick populations caused by sudden changes in solar activity.

The anti-piroplasm reagents most commonly administered in veterinary medicine are diminazene aceturate [2,6,7] and berenil (a mixture of diminazene aceturate and antipyrine). In 1958, diminazene was resynthesized in the USSR and named azidine. The purpose of our study was to examine the biodynamics of micellar diminazene. We assessed the effectiveness of this form of the drug in terms of intracellular drug localization and selective accumulation at the sites of infectious agent aggregation.

Materials and Methods

Materials

Trizma base and 3-sn-phosphatidylcholine were purchased from Fluka Chemie AG (Switzerland). RPMI 1640 medium and fetal calf serum were obtained from Sigma (USA). Diminazene aceturate was purchased from Transchem Ltd. (India). Neozidin M (ZAO Nita-Farm, Russia) was used as a micellar diminazene preparation.

Methods

In this study, aqueous and micellar forms of diminazene were used. Aqueous diminazene was administered to experimental animals at a therapeutic dose (3.5 mg/kg body weight) as recommended by the manufacturer. Micellar diminazene was prepared in the following solution: 5% antipyrine, 5% diminazene benzoate, 50% dimethylacetamide, 17% Tween 80, 5% polyvinylpyrrolidone (PVP) 12PF, 1% benzyl alcohol, and 17% distilled water. Detailed descriptions of the procedure used to prepare the stable water-disperse form of diminazene benzoate [18] and to assess its effectiveness have been given previously [19]. Experimental animals chosen by the analogy principle, consisting in choosing animals of the same sex,

age, and weight that were reared under the same conditions. Experimental animals (Stavropol Merino mongrel sheep) were divided into two groups of three sheep each (body weight 35 to 40 kg; age 1.5 to 2 years). Group 1 received micellar diminazene at a dose of 1 mL per 20 kg (2.5 mg/kg body weight). Group 2 received a 7% solution of diminazene aceturate at a dose of 1 mL per 20 kg (3.5 mg/kg body weight).

Blood samples were collected from the jugular vein at 0.5, 2, 4, 6, 8, 24, and 48 h after drug administration. The blood was separated into plasma and erythrocytes by centrifugation ($1,000 \times g$ for 15 min). The concentration of diminazene was measured in both fractions. The diminazene concentration in blood plasma was determined by spectrophotometer (Cary 100; Varian, USA) using the method described by Klatt and Hajdu [8].

Therapeutic efficiency

The animal experiments were conducted on two farms and in two veterinary clinics located in three different regions of Stavropol Territory, Russia, where babesiosis is endemic.

The effectiveness of the preparation for treating sheep babesiosis caused by *Babesia bigemiuna* was assessed during the spring infection outbreaks of 2003 and 2005.

Altogether, we evaluated 1,281 animals in our study including 246 that were treated for therapeutic purposes and 1,035 that were treated prophylactically. The sheep belonged to different age-sex groups. Babesiosis in the sheep included in our study was diagnosed according to epizootological and clinical data, xenodiagnostic results, and the findings from a laboratory study of peripheral blood smears. For diagnosing babesiosis, blood was collected from the ear vein, and the smears were made with the first drops. The smears were air-dried, fixed in 96% alcohol, and stained by the Romanovsky-Gimza method [12].

The piroplasmicidal effectiveness of neozidin M was tested by using a 5% variant. The novel (micellar) form of diminazene was administered to all animals at a concentration of 1 mL per 20 kg of body weight or 2.5 mg/kg diminazene (the active substance).

Intracellular penetration of the water-soluble and micellar forms of diminazene

Rat peritoneal macrophages were isolated by a conventional method [4]. We used Tween 80 as a nonionic SAS, which was conjugated to fluorescein isothiocyanate (FITC) as described by Storz [20]. According to the quality certificate supplied by the manufacturer, the CCMF1 value for this SAS was 1.5×10^{-4} M.

Diminazene in rat peritoneal macrophages was identified by using an indirect immunochemical method with phage (primary, 1×10^{13} phage particles/mL), rabbit anti-phage

(secondary, 1 : 500), and goat anti-rabbit FITC-labeled (tertiary; Fluka, Japan) antibodies.

Mini-antibody clones specific for diminazene were selected by using a sheep combinatorial phage library kindly provided by Professor W. J. Harris, University of Aberdeen, Aberdeen, UK. Fluorescence analysis with a DM LB fluorescence microscope (Leica, Germany) was used to visualize green FITC fluorescence, which indicated the presence of diminazene at the site being examined. This test was also used to detect Tween 80 (which had also been conjugated with FITC) on the surface of and inside the rat peritoneal cells.

The peritoneal cells were prepared for microscopy by the following method. Smears were made from 50 μ L of a cell suspension. The smears were washed with cold acetone, air-dried, and then washed with phosphate buffered saline (PBS; pH 7.2). After that, the smears were incubated for 1 h in a suspension of specific phage anti-diminazene antibodies diluted in PBS to 1×10^{13} phage particles/mL. The smears then were washed with PBS, incubated for 1 h with a solution of rabbit antiphage antibodies that were diluted 1 : 500 in PBS, and washed with PBS. Finally, the smears were incubated for 1 h with a solution of FITC-labeled anti-rabbit antibodies (diluted 1 : 100 in PBS) and were washed with PBS. After drying, the smears were analyzed with a DM LB fluorescence microscope.

Results

Intracellular penetration of the water-soluble and micellar forms of diminazene

It is known that SASs promote micelle formation and drug stability. Therefore, it was important to conduct experiments to evaluate the interaction of Tween 80 with peritoneal cells. Fig. 1 shows peritoneal macrophage cells cultured in the presence of FITC-labeled Tween 80. The green fluorescence observed on and inside the cells

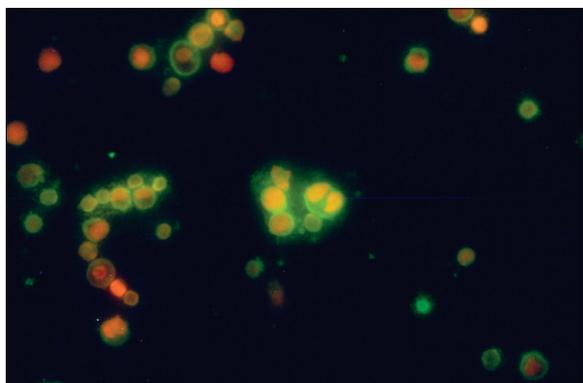


Fig. 1. Rat peritoneal cells cultured with FITC-labeled SAS micelles (green). $\times 400$.

suggests that the SAS accumulated in the cytoplasm after passing through the cell membrane (the nuclei appear yellow). The presence of diminazene in the peritoneal cells was observed by using mini-antibodies. Figs. 2 and 3 show the immunochemical detection of diminazene in the cells, in which the compound appears as green fluorescence owing to the formation of a complex between diminazene and the mini-antibodies.

Pharmacokinetic parameters of specific forms of diminazene

The pharmacokinetic parameters of specific forms of diminazene depend on its chemical properties and the mode of administration. Intramuscular injection of water-soluble diminazene results in a sufficiently rapid increase

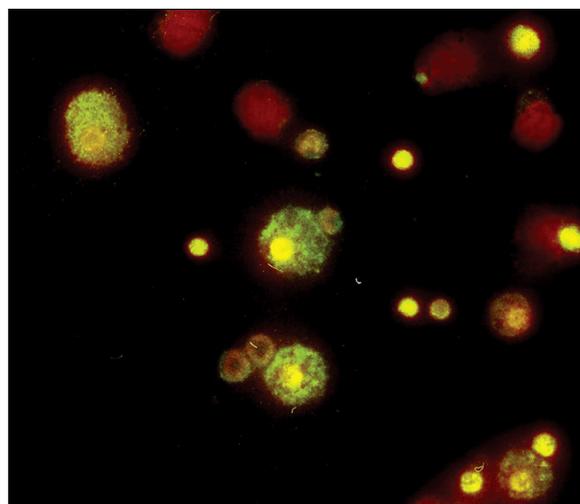


Fig. 2. Rat peritoneal cells cultured in the presence of micellar diminazene. $\times 400$.

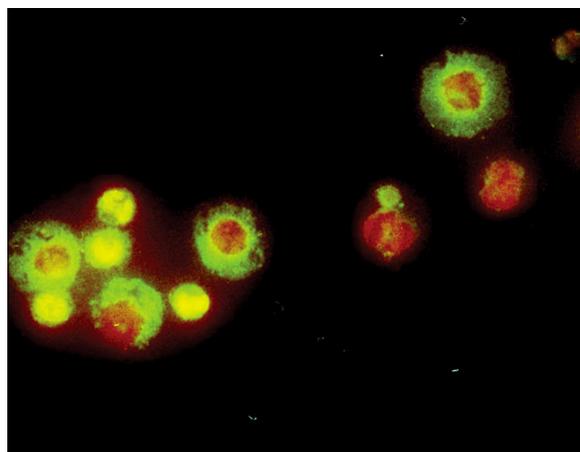


Fig. 3. Rat peritoneal cells cultured in the presence of aqueous diminazene. $\times 400$.

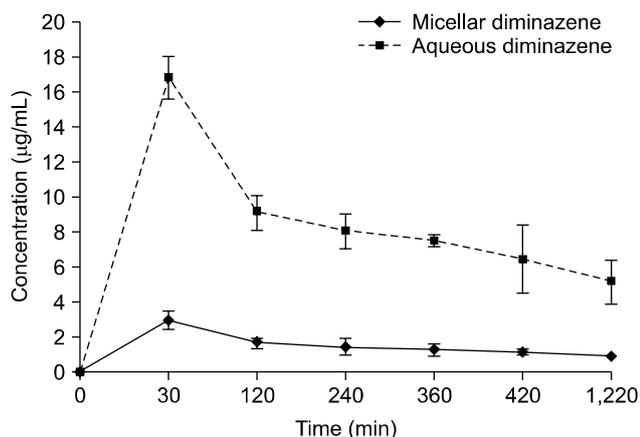


Fig. 4. Kinetic parameters of intramuscularly injected aqueous and micellar diminazene in sheep plasma.

of the drug concentration in blood. The pharmacokinetic curve is described by a two-chamber model in which the drug concentration often falls below the therapeutic level in the second phase.

In this study, the maximal concentration of aqueous diminazene in sheep plasma was $16.86 \mu\text{g/mL}$ (30 min). The half-life of diminazene was 10 ± 1.5 h. The concentration of micellar diminazene also peaked at 30 min after administration, but the maximal value was much lower ($2.97 \mu\text{g/mL}$). The half-life of the drug was 4 ± 1.5 h. With both forms of the drug, 97% of the administered dose was eliminated from the animals within 96 h (Fig. 4).

A substantially different pattern of diminazene accumulation and elimination was observed when the drug concentration was measured in erythrocytes (Fig. 5). For micellar diminazene, two concentration peaks were recorded: $2.1 \mu\text{g/mL}$ at 2 h after administration and $2.4 \mu\text{g/mL}$ after 22 h. The kinetics of changes in aqueous diminazene concentrations in sheep erythrocytes were the same as those for plasma with a peak concentration of $1.87 \mu\text{g/mL}$ recorded at 4 h after drug administration.

Therapeutic efficiency

The therapeutic effects were observed after 10 to 14 h. After 24 h, the animal body temperature decreased to normal and appetite was restored. No blood protozoa were detected when the blood smears were examined at 1 to 2 days after treatment. No side effects or complications were recorded.

For comparison, we used the preparation veriben (CEVA Santé Animale, France), which was administered as a 7% solution at a concentration of 3.5 mg/kg of body weight. We did not observe any statistically significant differences in therapeutic effectiveness between the preparations. However, the dosage of the micellar form was significantly lower (by 30%), and apparently decreased the adverse side

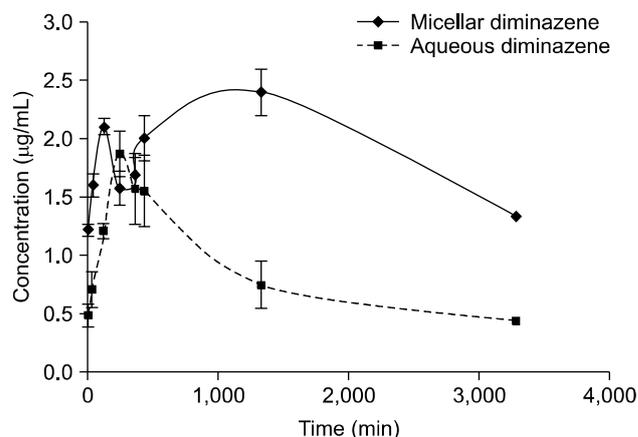


Fig. 5. Kinetic parameters of intramuscularly injected aqueous and micellar diminazene in sheep blood erythrocytes.

effects of the preparation.

Discussion

Despite the favorable prospects for using micelles as an active component for the delivery of injectable drugs containing hydrophobic substances [13,21], the biological properties of micelles are poorly understood. This is especially true for the biodynamics of micellar systems and their ability to interact with cellular structures. Therefore, we examined the *in vitro* interaction of micelles formed with diminazene and Tween 80 with cells. We subsequently performed experiments to detect diminazene and Tween 80 in the intracellular space. In the rat peritoneal cells, diminazene delivered via both micellar and water-soluble systems can be observed (green-yellow fluorescence of the cells). In the control cells (rat peritoneal cells cultured without diminazene), no fluorescence was observed (data not shown). Considering that Tween 80 can accumulate on the cell surface and penetrate into the cells, we presume that this reagent also facilitates diminazene delivery into the cells by changing membrane permeability.

We next determined the distribution of micellar and aqueous diminazene in blood plasma and erythrocytes according to pharmacokinetic parameters. We assumed that the penetration of SASs through the cell membrane might permit more active accumulation of the drug in the erythrocytes. Our results show that micellar diminazene accumulated in the erythrocytes more actively than did the aqueous form of the drug. These findings indicate that the micellar form is likely to promote higher therapeutic effectiveness of the drug. To assess the effectiveness, we performed a number of trials.

When analyzing the epizootological data, we accounted for the distribution area of the specific ticks (transmitters of the disease), the season, and the disease prevalence in the

region. The xenodiagnostic results showed that large numbers of pasture ticks (transmitters of *Babesia*) belonging to the genera *Boophilus*, *Ixodes*, and *Hyalomma* were found on the animals. The clinical symptoms included anorexia, depression, brachycardia, proventricular atony, a body temperature increase of 1.5 to 2°C, and hemoglobinuria (in severe cases). A final diagnosis of a particular blood protozoan disease was made after observing the characteristic paired pear-shaped forms of endoglobular parasites in the peripheral blood smears.

In summary, the findings from the present study demonstrate that micellar diminazene has pharmacokinetic characteristics that indicate the ability of this form of the drug to effectively penetrate into erythrocytes. This results in the subsequent drug accumulation at the sites of infectious agents. Further research on this drug form is recommended to explore its wider application in veterinary parasitological practice.

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