Trypanocidal effect of Diminazene aceturate by intranasal administration. Comparison among formulations

Alicia Graciela Fuchs*[1, 2]
María C. Soraires Santacruz*[3]
Octavio A. Fusco[4]
Alicia M. Hoffer[4]
Cong-Xi Zhang[5]
Fan Zhang[5]
Mónica I. Esteva[4]
Marcela S. Rial[4]
Laura E. Fichera[4]
Nicolás G. Cid[6]
Romina J. Glisoni[6]
De-Hua Lai[5]
Esteban J. Bontempi[4]

[1] Instituto Nacional de Parasitología, ANLIS "Carlos G. Malbrán". Buenos Aires, Argentina.

[2] Universidad Abierta Interamericana. Centro de Altos Estudios en Ciencias Humanas y de la Salud. Buenos Aires, Argentina.

[3] Cátedra de Química Medicinal, Departamento de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, - Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

[4] Instituto Nacional de Parasitología, ANLIS "Carlos G. Malbrán", Argentina.

[5] Center for Parasitic Organisms, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou, P.R. China.

[6] Instituto de Nanobiotecnología (NANOBIOTEC), Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

✉ Estados Unidos 929, C1101AAS / ejbon@yahoo.com

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ABSTRACT

Background: The standard treatment for nagana and surra, vector–born parasite diseases, is a single intramuscular (i.m.) dose of diminazene aceturate (DA), an aromatic diamidine. Due to discontinuity of public provision of veterinary services or to lack of access to remote areas, low income farmers inject livestock and other domestic animals by themselves. We tested a not explored administration route for drugs against parasitological infections: the intranasal one. DA dissolved in water did not reach an effective drug concentration. **Methods:** Mice were infected with *Trypanosoma brucei brucei* or *Trypanosoma evansi* and treated with different formulations of DA. Survival, parasitemia, body weight and behavior were recorded. **Results:** DA formulated with chitosan reached a lethal concentration for bloodstream parasites. Residual parasites were absent, as demonstrated by immunosuppression. **Conclusion**: The

* ALICIA GRACIELA FUCHS AND MARÍA CRISTINA SORAIRES SANTACRUZ CONTRIBUTED EQUALLY TO THIS WORK.

intranasal route is an effective, safer, and easier way to perform antiparasitic treatments in animals.

KEYWORDS

Berenil, Diminazene diaceturat, Intranasal, Tripanosomiasis, Aceite, Quitosan

Efecto tripanocida del aceturato de diminazeno por administración intranasal. Comparación entre formulaciones

RESUMEN

Antecedentes: El tratamiento standard para nagana y surra, enfermedades parasitarias transmitidas por vectores, es una única dosis intramuscular (i.m.) de diminazene diaceturato (Berenil), una diamidina aromática. Debido a la interrupción de la provisión pública de servicios veterinarios en varios países africanos, o a la falta de acceso a áreas remotas, granjeros de bajos recursos empezaron a inyectar al ganado por sí mismos. En este trabajo probamos una ruta de administración de drogas no explorada para infecciones parasitarias: la intranasal. Berenil disuelto en agua, sin aditivos, no alcanzó una concentración efectiva de droga. **Métodos**: Se infectó a ratones con Trypanosoma brucei brucei o Trypanosoma evansi y se los trató con diferentes formulaciones de Berenil. Se registró la supervivencia, parasitemia, peso corporal y comportamiento. **Resultados**: Berenil formulado con quitosan alcanzó una concentración letal para parásitos en el torrente sanguíneo. Por posterior inmunosupresión se demostró la ausencia de parásitos residuales. **Conclusión**: La ruta intranasal es una manera más efectiva, segura y fácil para efectuar el tratamiento antiparasitario en animales.

PALABRAS CLAVE

Berenil; Diminazene diaceturat; Intranasal; Tripanosomiasis; Aceite; Quitosan

INTRODUCTION

The etiological agent of African animal trypanosomiosis or nagana are the *Trypanosoma congolense*, *T. vivax* and *T. b. brucei*. It is present in Sub-Saharan countries and transmitted by tsetse flies. Surra is another veterinary parasitosis caused by trypanosomatids (*T. evansi*). It is found in South America, Southeast Asia and Africa, and it is transmitted by horse-flies.

For smallholder farmers the cost of nagana or surra is the treatment itself and the production impairment associated with the disease (decline in fertility, meat and milk production, draft power) [1]. The standard treatment for them consists in one i.m. or subcutaneous injection of diminazene aceturate (DA), at a concentration of 7 mg/kg body weight [2]. Commercial preparations halved this minimal concentration, contributing to the appearance of resistant strains in South-East Asia [3]. In fact, resistance up to 50 mg/kg has been reported in strains recovered from infected animals [4]. The treatment implies the visit of veterinary personnel, water dilution of the dry powder and i.m. injection into sick animals. With the privatization of this service in several African countries, low income and/or remote areas farmers began to inject animals by themselves [5], resulting in several added problems like erroneous route delivery or infection by the use of non-sterile water.

Non-traumatic routes delivery could solve some of these problems. The digestive route (per os) is not useful for DA, as it is sensitive to acidic pH. Nostril delivery has a permissible pH and does not require sterility. Moreover, it has the pharmacodynamic ad-

vantage of bypassing the first-pass effect (biotransformation in the liver), providing a rapid onset of action. Drugs are absorbed through the nasal epithelium to the venous capillary vessels and then distributed to the whole body. We worked with the acute model of these trypanosomiosis, when parasites thrive and reproduce in the bloodstream and other organs. In nagana, early elimination of parasites diminishes the possibility to reach the brain parenchyma and elicit neurological effects. We report herein the advances made to find a convenient formulation for DA to reach the bloodstream.

METHODS

Reagents and materials: The following products were used: Pluronic® F-127 (generous gift from BASF, Argentina), 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), chitosan and diminazene aceturate (Sigma, USA), fish oil (Garden House softgels), palm and coconut oil (generous gifts from Castoroil SACIAFEI, Argentina), peanut oil (Arrowana, Yihai Kerry, China), acetonitrile, triethylamine and acetic acid (Sintorgan, Buenos Aires, Argentina), cyclophosphamide (Microsules Argentina) and octanesulfonic acid sodium salt (Santa Cruz Biotechnology, USA).

PARASITES AND EXPERIMENTAL ANIMAL MODELS (INFECTION AND TREATMENT).

Mice infection *T. b. brucei* bloodstream strain 427 [6] was maintained in Creek's minimal medium [7]. *T. evansi* bloodstream parasites were obtained from infected animals. A) In *T. b. brucei* acute model of infection $3x10^4$ bloodstream form parasites injected intraperitoneally to female BALB/c mice (2 months old, 20-25 g, 3-4 animals per group) were lethal within 1 week. B) The same survival time was recorded when 10^5 *T. evansi* bloodstream parasites (STIB 805 or 806) infected Swiss Webster mice (2 months old, 40 g). STIB 805 is one of the least DA sensitive strains [8], while STIB 806 is DA-resistant [9].

DA treatment. The DA stock was freshly prepared in water at a 35 mg/ml concentration. From there, a working solution of 14 mg/ml was formulated, alone or with the following additives:

1) Pluronic® F-127 (20% w/w), a thermo-responsive linear poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer (PEO-PPO-PEO, MW: 12.6 kDa). It was liquid at 4°C, allowing instillation in the normal way, but gelified at body temperature.

2) Different cooking oils (10% v/v final concentration). Namely, fish, palm and coconut oils for *T. b. brucei* and peanut oil for *T. evansi*.

3) Chitosan (5 mg/ml), with the addition of 1% acetic acid to assist solubility.

The positive control group in all experiments was treated with one i.p. dose of 7 mg/kg DA. All of the other groups received the intranasal treatment. Mice were in supine position. Five µl of the DA working solution were administered to the opening of each nostril with a plastic tip, without physical contact. Infected mice were treated at the time of infection (*T. b. brucei*) or 24 h later (*T. evansi*).

DA TREATMENT EVALUATION

Experimental infection followed similar courses in mice infected with *T. b. brucei* or *T. evansi*. Parasitemia was assessed in Neubauer counting chamber (detection limit: 10^4 parasites/ml).

Survival after treatment was studied for 2-3 months. To confirm absence of viable parasite, animals were immunosuppressed by three i.p. doses of 200 mg cyclophosphamide/kg, 4 days apart between injections, and kept under observation for an additional month.

EVALUATION OF SHORTER IN VITRO DA EXPOSITION TIMES

T. b. brucei parasites (around 1 x 10^6/ml) were incubated with 2 µM DA (1 µg/ml) for variable periods of time and then centrifuged to get rid of the drug. The zero-time control was not exposed to the drug. Each time point was then adjusted to an initial parasite concentration of 35 x 10^3/ml drug-free medium and loaded into sterile 96-well plates (200 µl/well). After 3 days, cells were counted using a Z2 Coulter Counter (Beckman) and the percentage of growth inhibition was calculated.

PLASMA DA DETERMINATION BY HPLC

Chromatographic conditions and preparation of standard solutions.

A modified Gummow´s method was used [10]. Analyses were carried out in a HPLC Shimadzu with

a Shodex C18-4D column. The isocratic elution was achieved with a mobile phase consisting of 30/70 acetonitrile /0.005M octanesulfonic acid sodium salt in water containing 0.1% triethylamine, adjusted to pH 3.2 with acetic acid. The mobile phase was filtered through 0.22 µm Magna Nylon hydrophilic membrane filters (Thomas Scientific, USA) and degassed by sonication prior to use. The flow rate was 1.0 ml/min. The volume of injection was 30 µl and the total run time was 7 min. The PDA detector was set at a wavelength of 368 nm.

To obtain the calibration curve, eight different dilutions of the DA stock solution (1 mg/ ml methanol) were freshly prepared. Tubes containing 50 µl of blank mouse plasma were spiked with a fixed amount of the internal standard DAPI (final concentration: 5 µg/ml) [11] and with different amounts of the freshly prepared DA stock solution (1 mg/ ml methanol), to reach final concentrations from 10 to 5000 ng/ml. All tubes were completed with methanol to a final volume of 100 µl, vortexed and centrifuged. Clear supernatants were dried under a stream of nitrogen gas, dissolved in mobile phase and then injected into the HPLC. Three DA quality controls were also prepared (50, 500 and 2500 ng/ ml) in order to assess measurements reproducibility during sample analysis.

SAMPLE PREPARATION

Naïf mice received the i.n. treatment (20 mg/kg DA/ 10% fish oil) and at different post-instillation times 200 µl blood were collected from the tail vein in heparin containing eppendorf® tubes. After centrifugation (5000 rpm, 5 min) plasma was separated and the volume measured. Then DAPI was added to attain a 5 µg/ml concentration. After the methanol extraction, drying and resuspension, the supernatant was immediately injected into the HPLC.

RESULTS AND DISCUSSION

An instillation volume of 5 µl was selected, because larger volumes could reach the stomach [12] where DA could be degraded [13] (Fig. 1). In the initial experiment DA was formulated with water and administered by the i.n route. Even an excess of 320 mg/kg was inefficient to diminish lethality (100% of the Balb/c mice died within 15 days post *T. b. brucei* infection).

DA was then formulated with Pluronic® F-127. We had again to increase the doses to obtain protection: administration of 320 mg/kg (8 days treatment) showed 100 % protection, and the treated animals were alive, parasite free and without development of any signs of toxicity for 90 days post infection.

Then, we assayed a new formulation with fatty acids with documented performance. One of the parameters considered was the presence in the composition of Docosahexaenoic acid (DHA), an omega-3 fatty acid with an essential role in neuron´s plasma membrane [15]. It interacts with membranes, modifying its permeability to different substances [16]. Fish oil is a convenient source. It is composed of variable amounts of 23 different omega fatty acids [www.nordicnaturals.com/ie/faq_ie.php], being eicosapentaenoic acid and DHA the main two. The cheapest and less purified product (not pharmacological grade) is composed of 18 % eicosapentaenoic acid and 12% DHA, for a total content of 30% omega-3. The emulsion of DA and 10 % fish oil was well accepted by the animals. DA concentration, decreased up to 20 mg/ kg, conferred 100 % protection to the infected animals until the end of the experiment (3 months) (Fig. 2). Fish oil alone, used to treat another experimental control group, did not increase animal survival.

Since fish oil is unavailable or expensive in some places, we tried plant oils, like palm and coconut oil. Palm oil is used for cooking in several African countries. Instead of DHA they contain another polyunsaturated fatty acids (palm oil contains 10% w/w of omega-6 linoleic acid, while coconut oil has 2% w/w of omega-3 linolenic acid). We observed that mice receiving one dose of DA 20 mg/kg + plant oil was 100% protected. We cannot rule out a contribution of the oils, as certain protection against trypanosomatids, in terms of longer survival and lower parasitemia, was reported using vegetable oils alone [17, see review 18].

Another plant oil tested was the one extracted from peanut, rich in linoleic and arachidonic acids. Mice (Swiss Webster) infected with *T. evansi* STIB805 and treated 24 h later needed a concentration of 280 mg/ kg to clear parasitemia and completely suppress mortality until the end of the experiment (60 days). These higher doses displayed several phenotypic effects: animals were apathetic, lose weight (Fig 3), showed

reduced mobility and a sunken abdomen. Since after treatment completion the group regained weight and motility, the adverse effects were reversible in this model.

These protection results are valid within the limits of the respective models. For example, we tried a more resistant strain: STIB 806. It could endure a 3 days i.p. treatment with 28 mg/kg [9] and *in vitro* showed an EC_{50} higher than the one from STIB 805 (12.5 compared to 10.7 ng/ml) [8]. In accordance with those results, the i.n. treatment did not extend the lifetime of the infected animals.

To complete the characterization of the effects of the drug we evaluated the presence of relapsing parasites hidden in different regions of the body [19]. An immunosuppressive treatment implemented on surviving animals showed that none of the fish oil treated animals suffered a relapse. In contrast, some of the plant oil treated animals died.

At this point we wanted to determine the *in vivo* circulating amount of DA by HPLC. When setting-up the method, we took into consideration that DA is unstable at acidic pH [19] and that degradation products have been reported [20]. Nasal pH is close to 6 [21], so degradation in that environment is probably of little significance (half-life > 1 week). After the samples were collected and processed, they were stored in the mobile phase (pH 3.2) in waiting for the HPLC run. In control samples, we did not detect degradation products. However, we observed compound destruction (lower peaks) in samples stored for more than 2 weeks.

For the HPLC experiment we used naïf animals, because previous studies showed no significant difference in the AUC (area under the concentration-time curve) between *T. congolense* infected and noninfected animals [22 and references therein]. We adopted a liquid chromatography method with a sensitivity of 30 ng/ml, showing linearity over the expected range. DA retention time was 3.1 min (Figure 4). Since samples had to be processed, an internal control (DAPI) with a different retention time (4.6 min) was added to every plasma sample of treated animals before the methanol extraction. Comparing the added amount to the one detected by HPLC made possible to establish the percentage of recovery in every sample. Normally it was around 70%. This factor was applied to the AUC of the DA peak to calculate the initial amount. Blood was extracted at different time points and analysed. A peak (160 ng/ml) was obtained 15 minutes post-instillation, and 15 ng/ml were still present at 120 minutes.

This result was useful to calculate the efficiency of nose delivery. Each mouse (20 g body weight) was treated with 20 mg/kg DA. Estimating 100% absorption and 1.5 ml of circulating blood volume, it should have reached 270 µg/ml. But we found 160 ng/ml, which is about 1,000 times less.

We also wanted to compare this *in vivo* result with *in vitro* determinations. For *T. b. brucei*, the reported EC50 for DA [23 and our own results] were in the order of 0.5 µM (257 ng/ml). But this kind of determination implies survival upon continuous exposure to the drug. To assess the minimal DA-parasite contact time, an *in vitro* assay was set-up. *T. b. brucei* bloodstream parasites were subjected to 2 µM DA (1 µg/ml) (4 times higher than the EC50) for variable periods of time. Exposition times as short as 30 minutes diminished growth by 34%. This result is in accordance with the irreversible damage suffered by the drug-sensitive CP 2137 clone 1 after incubation for the same time and with the same DA concentration [24].

This experiment showed that the drug is rapidly absorbed. The aminopurine transporter P2/TbAT1 seems to be the main responsible for its uptake [review 25]. This experiment also showed that DA exerts its action even after disappearance from the external medium, suggesting a strong interaction with its target. In the parasite, the more accepted one is kinetoplast DNA [26, 27], although another drug targets like beta-trypsin [28], diamine oxidase and S-adenosylmethionine decarboxylase [29] have been described.

As a final point, it should be considered that in the *in vivo* protection experiment DA concentration does not have to achieve 100% lethality by itself. The animals have immunocompetence and can thus cope with the remnant parasites. In fact, in our acute model not all infected animals died when injected with a lower number of parasites (10,000), implying BALB/c mice mounted a strong protective response upon infection.

Finally, we tested another additive, more accepted by the pharmaceutical industry. Chitosan is a polysaccharide extensively studied for nasal delivery [30]. A formulation of chitosan 5 mg/ml and DA 14 mg/ml (7 mg/kg) was effective when tested in animals (n=

7). Moreover, there was no parasite activation after immunosuppression.

In summary, we demonstrated that *in vitro* the parasite was sensitive to short DA exposition times, suggesting that the metabolic damage was not easily circumvented. In the *in vivo* experiments complete parasite destruction ensued, as evidenced by the zeroing of parasitemia, the long survival time of the treated animals, and the absence of relapse after immunosuppression. This could be a consequence of both, the drug treatment and the adjusted immunological response of the host. It should be considered that some indirect DA effects could also contribute to its action. DA activates Angiotensin-Converting Enzyme 2 [31], and the resulting vasodilatation influences drug absorption. For example, treatment with a vasoconstrictor diminished 20% fentanyl i.n. absorption [32, 33], and also increased Tmax and decreased Cmax for butorphanol [34]. Besides, DA attenuates the anti- *T. congolense* inflammatory response ligated to pathology, contributing to its curative effect [35].

These results demonstrate that DA, formulated with chitosan and applied by the i.n. route, is as effective as the applied by the i.p./ i.m. routes. It could facilitate the way smallholder farmers treat nagana and surra, providing a safe intranasal treatment. The lack of previous diagnosis performed by experienced personnel is not an impediment. At the rural level the diagnosis is normally retrospective, based on response of sick animals to treatment.

As a bonus, the control of animal nagana could contribute to the eradication of human parasitosis. It has been described that livestock practices in villages are related to human sleeping sickness cases [36]. Several farm animals (cattle, pigs, goats and sheep) have been reported as reservoirs of the sleeping sickness agent *T. b. rhodesiense* [37-39]. Added to this, recombination between animal and human strains could pose novel health risks [40].

Paramount to increase treatment effectivity and to avoid resistance emergence are several important measures, like the use of weigh bands for correct dose calculation [41], the training of farmers and community animal health workers [42], the access to good quality drugs [43], and the abstention from using DA as prophylactic treatment.

CONCLUSIONS

The benefits of the i.n. route could improve food production by individual farms. This delivery pathway could also be attractive for human treatment, either for sleeping sickness with new less toxic diamidines currently in development [44, 45], or for other prospective uses of DA [46].

ABBREVIATIONS

DA: diminazene aceturate; DAPI: 4′,6-Diamidino-2-phenylindole dihydrochloride; DHA: docosahexaenoic acid; i.m.: intramuscular; i.n.: intranasal; i.p.: intraperitoneal; EC_{50}: half maximal effective concentration

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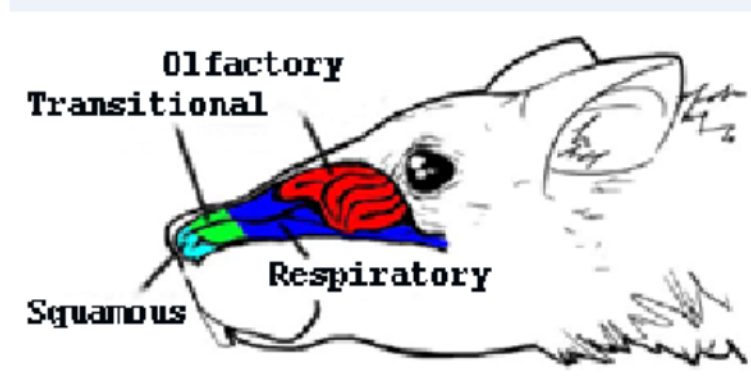

FIG. 1. ANATOMICAL VIEW OF MOUSE NASAL CAVITY [14]. DRUG WAS DEPOSITED INTO THE RESPIRATORY AREA OF THE NOSTRIL, WHOSE SURFACE IS SPECIES SPECIFIC.

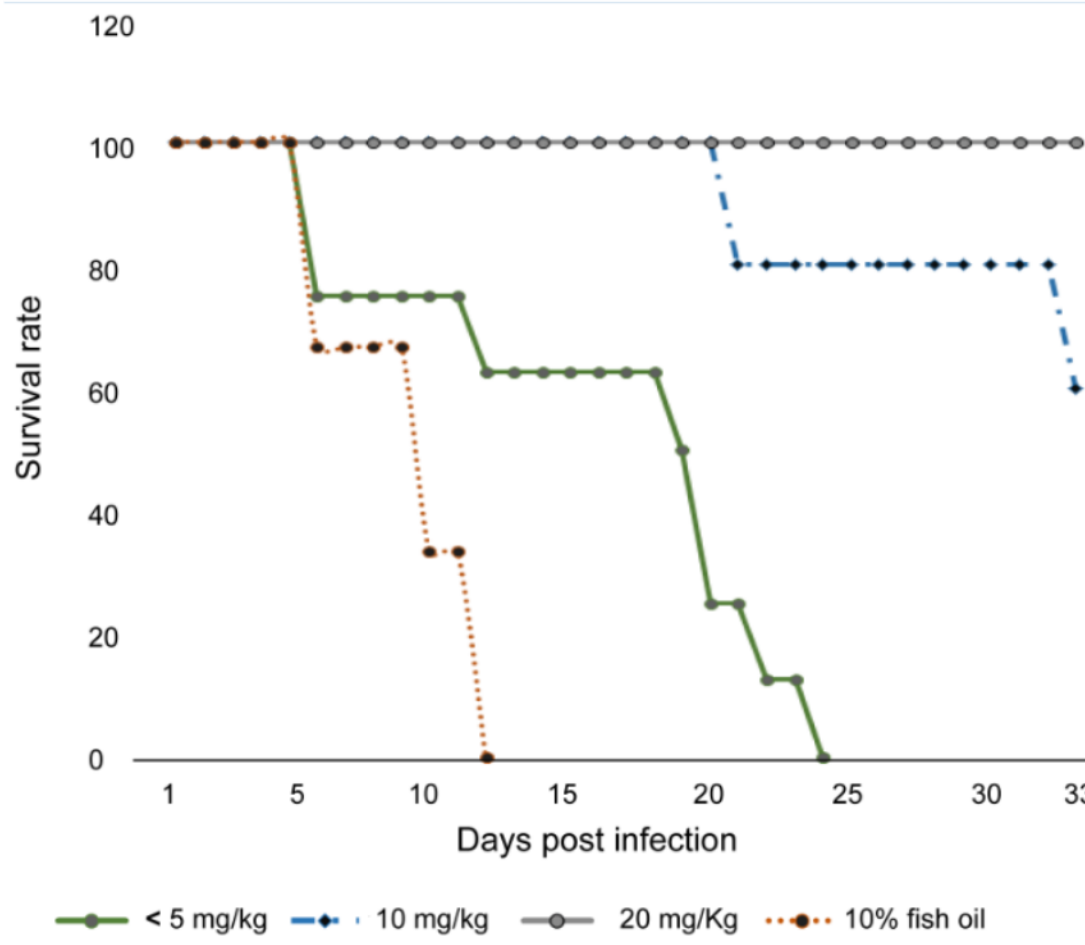

FIG. 2. SURVIVAL OF *T. B. BRUCEI* INFECTED BALB/C MICE TREATED WITH A SINGLE DOSE OF DIFFERENT CONCENTRATIONS OF DA/ FISH OIL OR FISH OIL ALONE BY I.N. ADMINISTRATION AT THE TIME OF INFECTION. EVERY LINE REPRESENTS RESULTS FROM 2 INDEPENDENT EXPERIMENTS.

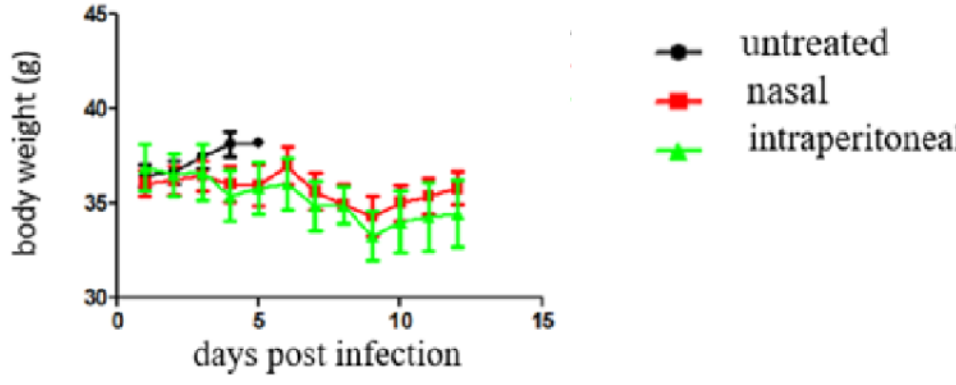

FIG. 3. EFFECT OF INFECTION (STIB805 *T. EVANSI*) AND TREATMENT ON SWISS WEBSTER MICE WEIGHT. AT DAY 5 THE PARASITIC LOAD WAS IN THE ORDER OF 108 / ML (UNTREATED GROUP), WHILE TREATED GROUPS HAD AN UNDETECTABLE LEVEL ALL ALONG THE EXPERIMENT. NO SIGNIFICANT DIFFERENCE IN WEIGHT LOSS WAS OBSERVED BETWEEN BOTH TREATMENT DELIVERY ROUTES.

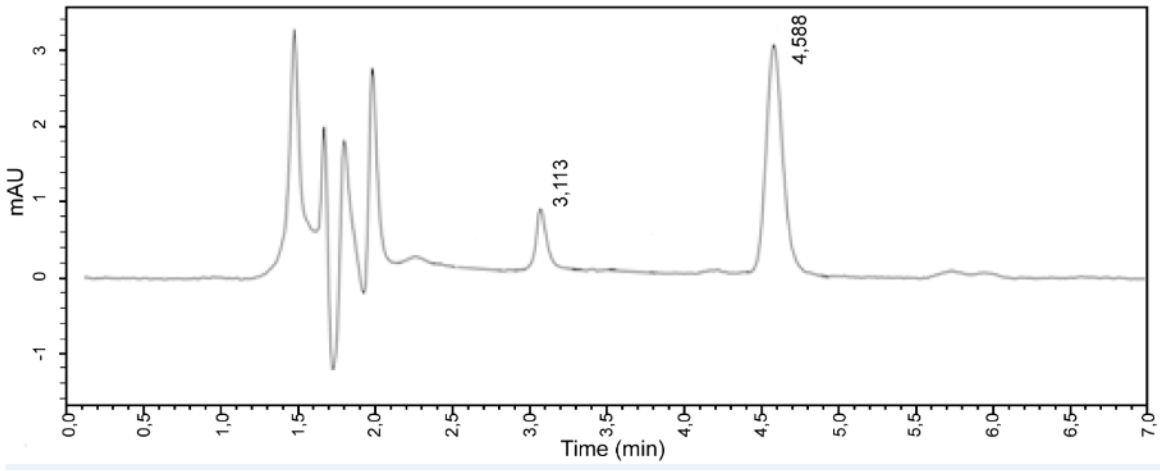

FIG. 4. TYPICAL HPLC CHROMATOGRAM. SEPARATION OF METHANOL-EXTRACTED PRODUCTS FROM A PLASMA SAMPLE CONTAINING ORIGINALLY 310.81 NG/ML DA AND 5 □G/ML DAPI.