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ORIGINAL RESEARCH

Study of plasma protein binding activity of isometamidium and its impact on anthelmintic activity using trypanosoma induced calf model

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Abstract

Aim: The objective of present study was to determine Plasma Protein Binding (PPB) activity and its effect on clinical efficacy of isometamidium after intramuscular administration in calves. The binding of drugs to plasma proteins is an important factor in controlling the availability and distribution of drugs. In general, PPB reduces the free fraction of drug available for therapeutic activity, since only the non-protein bound drug is pharmacologically active.

Materials and Methods: Six calves were used for PPB study and eighteen for clinical efficacy. Isometamidium was administered @ 0.5mg/kg intramuscularly as a single dose for PPB study. Equilibrium dialysis technique was used to determine the PPB activity. For clinical efficacy, infection with *Trypanosoma* was induced in calves of two groups, untreated control and experimental group. Infection was confirmed after 28 days by mice inoculation test. Isometamidium @ 0.5mg/kg was administered to experimental group. Haematoobiochemical and mice inoculation tests were performed after 7 days of drug administration (Day 35).

Result: The percentage of PPB activity of isometamidium was 86.71 ± 0.59 to 93.03 ± 0.63% against the concentration 9.76 ± 0.84 to 4.39 ± 0.20 g ml^{-1}. Higher percentage of PPB activity (>86%) suggests greater duration of safety by this drug. It was found that anthelmintic activity of isometamidium was substantially affected by higher PPB.

Conclusion: It was concluded that isometamidium has greater plasma protein binding capacity which did not hamper clinical efficacy of drug.

Keywords: anthelmintic, calf model, plasma protein binding activity, trypanosoma

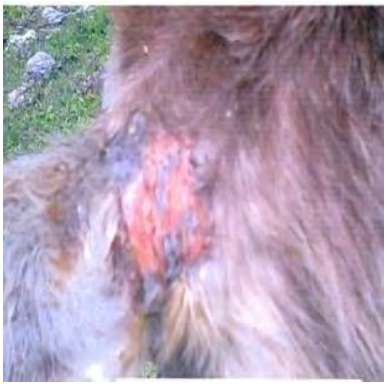
Introduction

"Surra" or Trypanosomiasis is caused by *Trypanosoma evansi* which is most widely distributed pathogenic mechanically transmitted vector borne haemoprotozoan disease. Animal Trypanosomiasis causes the death of 3 million head of cattle each year [1]. In cattle and buffalo the disease is manifested as peracute, acute, subacute or chronic form. The disease is underestimated in cattle and buffaloes mainly due to subclinical nature.

Chemotherapy and chemoprophylaxis is the bastion of control of surra in India. Currently drugs like diminazene aceturate, quinapyramine sulphate and chloride (Triquin, Antrycide Prosalt) are used against surra for treatment and prophylaxis in India. But major problems such as high price of drugs, less availability of drugs and the development of drug resistance are major tribulation faced in India. Isometamidium has been used as chemotherapy and chemoprophylaxis of disease in cattle, sheep and goats under conditions of natural tsetse challenge for more than 35 years [2] in countries other than India. Mode of action of isometamidium is not fully understood, but evidence is there that kinetoplastic topoisomerase type II of *trypanosoma* is selectively inhibited by the drug. The claimed duration of protection afforded by isometamidium is as long as 5 months [3].

Literature regarding disposition kinetics of isometamidium in various species like, sheep [4], goat [5], and pigs [6] are available but reports regarding its plasma protein binding are not available. To determine the dosage regimen plasma protein binding activity is required.

Plasma protein binding (PPB) has been shown to substantially affect tissue penetration, elimination half-life and the volume of distribution of antimicrobial agents [7]. Additionally, the impact of PPB on the microbial effect of antibiotics has been investigated by several *in vitro* studies [8]. In general, PPB reduces the free fraction of drug available for bacterial killing, since only the non-protein bound antibiotic is pharmacologically active [9]. Although, the effect of PPB on microbe killing is well documented for antibiotics especially β lactams, data on other classes of drugs like anthelmintics, are still rare [10]. Therefore, it is doubtful whether findings obtained from β-lactams can be extrapolated to other classes of drugs such as the anthelmintics.



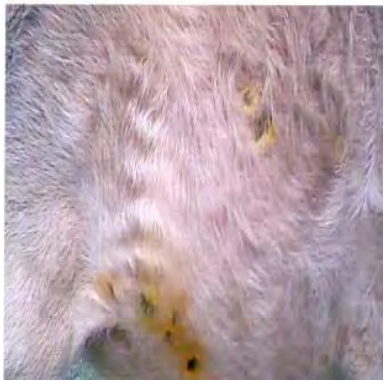


Plate-1. Urticarial patches at the base of ear and on belly of group II calves after 28 days of induction of *Trypanosoma evansi.*

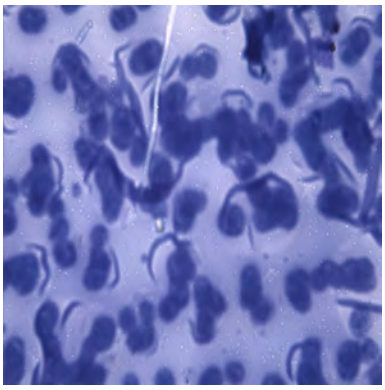


Plate-2. Slide of blood film from group II mice showing *Trypanosoma evansi.*

The present study was carried out with an attempt to determine the plasma protein binding activity of isometamidium in calves and to determine the effect of plasma protein binding on drug efficacy.

Materials and Methods

Chemicals: Isometamidium hydrochloride (ISMM), technical grade, a synthetic trypanocide was provided as gift by Alembic Limited, Veterinary Division, Mumbai, India. The purity of compound was >90%. All chemicals used in the experiment was obtained from E.Merck (India), Rankem and Sigma Chemicals (USA).

Calves: Twenty four healthy calves of about 6 months weighing 40-50 kg were used for this experiment. Six animals were used for plasma protein binding study and other eighteen for drug efficacy. The composition of feed given was 3 parts of paddy straw, 1 part of mustard cake and 1 part of wheat husk. Water was provided ad-libtum. These animals were dewormed. After 21 days of last administration of anthelmintic, the animals were acclimatized in experimental condition for 7 days and were stall fed only.

Ethical approval: This study was performed after the approval from Institutional Ethical Committee and all procedures were carried out in accordance with the Guidelines laid down by the International Animal Ethics Committee. At the end of study all animals were rehabilitated.

Isometamidium was administered at 0.5 mg/kg bw (1% solution in normal saline) intramuscularly to six animals. 0.5 mg/kg bw was used for study of plasma protein binding and clinical efficacy as this is the minimum therapeutic dose of isometamidium used in field conditions for most of the species. Reports regarding its use in large animals are scanty and no reports regarding its PPB study and clinical efficacy are available. So, we have taken the dose level of 0.5mg/kg bw of PPB for clinical efficacy study. Ten ml of blood was also collected from jugular vein at 40, 60, 90 and 120 min post drug administration, for plasma protein binding study. Plasma was then separated by centrifugation at 3000 rpm for 30 min and stored at 4^0C in refrigerator till further use.

Determination of plasma protein binding activity: Equilibrium dialysis technique as described by Mandal *et. al.* [11] was used in this experiment to determine plasma protein binding activity of isometamidium. Appropriate size of dialysis bag (size 20/32", M/S Sigma chemical Co., USA) were cut into suitable pieces, washed thoroughly with distilled water and kept immersed overnight in phosphate buffer (pH-7.4) at 37^0C. A small part of each piece of dialysis bag was inserted around in one end of a glass tube (2-3") having both the ends open and then secured tightly with thread. A tight knot was put on the other end of the extended dialysis bag so as to make a closed bag sufficiently voluminous enough to hold 5 ml of plasma. The dialysis tube containing 5 ml of plasma was then suspended in a test tube of large size containing 5 ml of phosphate buffer solution (pH-7.4) and then placed into the incubator in standing position for 24 hr at 37^0C. Test tubes were taken out for subsequent drug analysis in plasma of tube and phosphate buffer outside the tube. The protein content of plasma of each sample was also estimated by Biuret method [12]. The protein binding activity was expressed as percentage. The binding capacity, dissociation and association constants were calculated using the method of least square regression technique described by Pilloud [13].

Determination of efficacy of isometamidium: *Trypanosoma evansi* was procured from Dept. of Parasitology, West Bengal University of Animal and Fishery Sciences, where they were maintained by serial passaging in mice. All eighteen animals were divided in three groups, each group containing six animals. Group I was kept as control and group II and III were induced with trypanosomiasis. About 1 ml of blood was collected intracardially from mice and mixed with 1 ml of Alsever's solution and injected subcutaneously in calves of group II and III. Group II was kept as untreated control and group III was administered Isometamidium.

Confirmation of *Trypanosomiasis*: Animals started showing symptoms like dullness, staring gaze, corneal opacity, emaciation, anorexia, pyrexia (Temp 104 ^{0}F-105^{0}F), posterior paralysis etc. on 28 days after post infection of *Trypanosoma evansi*. (Plate-1).

Table-1. Plasma protein binding activity of isometamidium in calves after I/M administration (mean values of 6 replicates with S.E.)

Time (hr)	Concentration in plasma (g/ml)	Concentration after Incubation (g/ml)		Bound protein %
		Plasma	Buffer	
0.66	4.39 ± 0.20	3.88 ± 0.05	0.32 ± 0.02	93.03 ± 0.63
1	4.03 ± 0.03	3.72 ± 0.03	0.31 ± 0.004	93.16 ± 0.03
1.5	9.76 ± 0.84	8.44 ± 0.77	1.32 ± 0.06	86.71 ± 0.59
2	6.07 ± 0.60	5.3 ± 0.57	0.77 ± 0.03	87.68 ± 0.9

Table-2. Plasma protein binding constant ($_i$), dissociation rate constant (K) and association rate constant (K_a) of Isometamidium

Time (hrs)	?$_i$ (mol g^{-1})	$K_?$ (Mol L^{-1})	K_a (L mol^{-1})
0.66	0.96×10^{-8}	1.22×10^{-6}	0.82×10^{6}
1	1.28×10^{-8}	2.06×10^{-6}	0.49×10^{6}
2	0.92×10^{-8}	2.07×10^{-6}	0.46×10^{6}
Mean±S.E.	$1.03 \times 10^{-8} \pm 0.12 \times 10^{-8}$	$2.04 \times 10^{-6} \pm 0.02 \times 10^{-8}$	$0.56 \times 10^{6} \pm 0.01 \times 10^{-8}$

The confirmation of infection was done by inoculation test in mice. About 2ml of blood was collected from jugular vein of calf and injected intraperitoneally in mice. Blood smear was prepared from tail vein after cutting tail of mice and was examined under microscope for *trypanosoma* at every 24 hr interval. The smear was stained by Leishman's stain. *Trypanosoma* was confirmed in mice after 48 hrs of injection (Plate-2).

Isometamidium hydrochloride solution (1%) was prepared in pyrogen free distilled water and was administered intramuscularly to animals of group III at 0.5 mg/kg bw to each animals after 28 days of infection with *Trypanosoma* i.e. after confirmation of infection. In order to determine the clinical efficacy. haematobiochemical parameters like Alanine Amino Transferase (ALT) [14], blood glucose [15], Aspartate Amino Transferase (AST) [14] and haemoglobin [16] were assessed. Blood was collected from jugular vein of animals of groups I, II and III for haematobiochemical tests on day 0 (day of inducing *trypanosoma*), day 28 (confirmation of infection) and on day 35 (7 days after administration of isometamidium). The clinical symptoms of each animal were monitored regularly till 35 days. On 35th day blood samples were collected and mice inoculation test was also performed.

Statistical analysis: Mean values, standard error and analysis of variance of the tabulated data, were calculated using SPS 10.0 version of statistical software.

Results

Results obtained in relation to plasma protein binding of isometamidium are presented in Table 1. The binding capacity (β_i), association constant (K_a) and dissociation constant (K_β) of isometamidium with plasma protein in calves have been summarized in Table 2. The percentage of protein binding activity of isometamidium was 86.71 ± 0.59 to 93.03 ± 0.63% against the concentration 9.76 ± 0.84 to 4.39 ± 0.20 g ml^{-1}. The equilibrium association constant (K_a) was $0.56 \times 10^{6} + 0.01 \times 10^{-8}$ litre mole^{-1} and dissociation constant (K_β) was $2.04 \times 10^{-6} \pm 0.02 \times 10^{-8}$ Mole litre^{-1}.

Results obtained pertaining haemobiochemical tests are shown in Figures 1, 2, 3 and 4. ALT and AST activity increased significantly (*P<0.01*) in groups II and III calves on day 28 compared to day 0 but decreased significantly (*P<0.01*) on day 35 in group III. Blood glucose level and haemoglobin level decreased significantly in group II and III on day 28 compared to day 0 but these values were found to increase significantly on day 35 in group III calves in comparison to calves not treated with isometamidium i.e. untreated control group II. Mice inoculation tests was found to be negative in group III calves in comparison to group II calves when blood collected on day 35 was tested. Group II calves showed significantly higher concentration of *Trypanosoma evansi* in blood film on mice inoculation test.

Discussion

The percentage of protein binding activity (Table- 1) was above 86% which has been reflected by higher binding capacity (β_i: $1.03 \times 10^{-8} \pm 0.12 \times 10^{-8}$ Mol gram^{-1}) and lower dissociation constant (K_β: $2.04 \times 10^{-6} \pm 0.02 \times 10^{-8}$ Mol litre^{-1}) of isometamidium (Table-2) with the plasma protein. The higher plasma protein binding activity suggests greater half life value of drug and so frequency of administration is low. High plasma protein-binding capacity of isometamidium serves as a circulating reservoir and influences the therapeutic efficacy of the drug and provides protection for a variable period. The drug therefore is suggested to provide greater duration of protection. Similarly, Bacchi [17] reported that suramin (isometamidium) has an extremely long half-life in humans, 44–54 days, the result of avid binding to serum proteins indicating protection for a longer period and greater therapeutic efficacy of the drug.

The results of ALT and AST activity suggested that *Trypanosoma evansi* may cause hepatic damage leading in increase in ALT and AST activity. But ALT and AST activity in isometamidium treated animals of group III started to decrease when measured on day 35 compared to the activity on 28 day suggesting ameliorating effect of drug against hepatotoxicity produced by

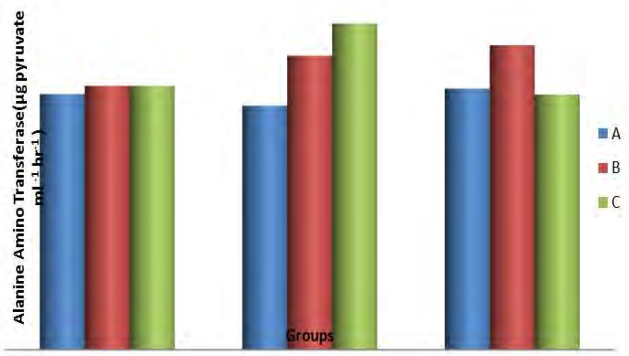


Figure-1. Effect of Isometamidium on Serum ALT activity (µg pyruvate ml $^{-1}$ hr^{-1}) in calves after single dose I/M administration. A , 0 day (induction of trypanosomiasis); B, after confirmation of infection (28 day); C, 7 days post-administration of isometamidium (35 day). Group I : Control ; Group II : Experimental control / untreated control; Group III : at the dose rate of 0.5 mg/kg

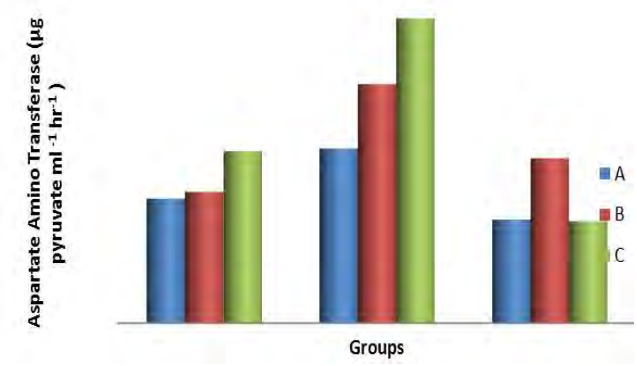


Figure-2. Effect of Isometamidium on Serum AST activity (µg pyruvate ml $^{-1}$ hr^{-1}) in calves after single dose I/M administration at different dose levels. A , 0 day (induction of trypanosomiasis); B, after confirmation of infection (28 day); C, 7 days post-administration of isometamidium (35 day). Group I : Control ; Group II : Experimental control / untreated control; Group III : at the dose rate of 0.5 mg/kg

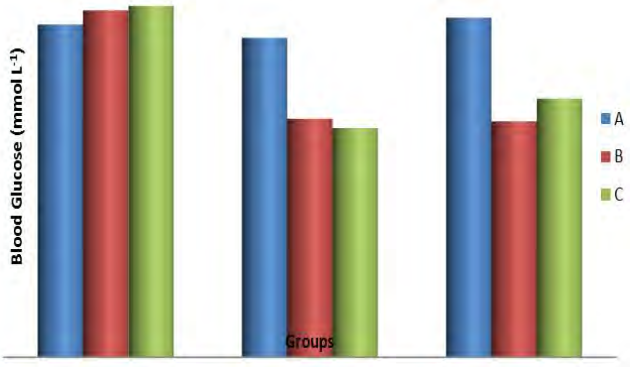


Figure-3. Effect of Isometamidium in Blood Glucose (m mol L^{-1}) in calves after single dose I/M administration at different dose level. A , 0 day (induction of trypanosomiasis); B, after confirmation of infection (28 day); C, 7 days post-administration of isometamidium (35 day). Group I : Control ; Group II : Experimental control / untreated control; Group III : at the dose rate of 0.5 mg/kg

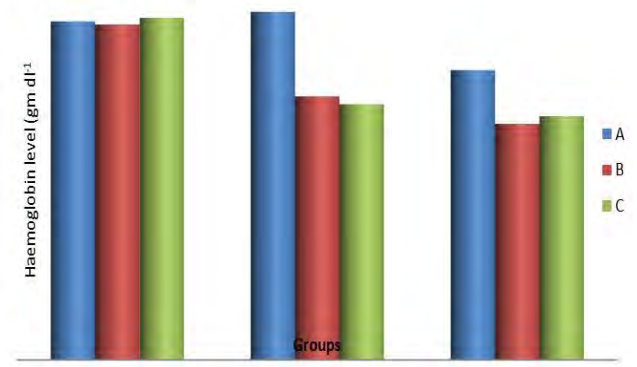


Figure-4. Effect of Isometamidium on Haemoglobin level (gm dl^{-1}) in calves after single dose I/M administration at different dose levels. A , 0 day (induction of trypanosomiasis); B, after confirmation of infection (28 day); C, 7 days post-administration of isometamidium (35 day). Group I : Control ; Group II : Experimental control / untreated control; Group III : at the dose rate of 0.5 mg/kg

Typanosoma. Multiplication of trypanosome in cells of reticuloendothelial system and striated muscle results cellular damage. Further *trypanosome* causes direct traumatic effect on striated muscle leading to increase in AST activity. Hilali *et. al*. [18] also reported significant alteration in the levels of serum AST and LDH enzymes in buffalo calves infected with *Trypanosoma evansi*. Similar results were also shown by Peni *et. al*. [19], where liver toxicity was reported in *trypanosoma* infection which they explained due to mononuclear infiltration of interstitial tissues of liver with minor cellular damage leading to increase in serum ALT activity.

Decrease in blood glucose level and haemoglobin group II and III on day 28 in comparison to group I suggested toxic effect of *Trypanosoma evansi* infection. Excessive utilization of the blood glucose by *trypanosomes* for their metabolism has been thought to account for the hypoglycaemia observed during trypanosomosis [20]. Ahmed and Malik [21], reported severe reduction in haemoglobin level and total erythrocyte count in Nubian goats and proposed that *trypanosome* leads to increased red blood cell destruction and extravascular and intravascular haemolysis by immune (trypanosome Ag-Ab complex and anti erythrocyte Ab) reaction. Hilali *et. al*. [18] and Youssif *et. al.* [22] also reported similar decrease in haemoglobin after trypanosome infection in buffalo calves and goat respectively. The significant increase in haemoglobin and blood glucose level in calves of group III on day 35 in comparison to group II suggested ameliorating potential of isometamidium against toxicities produced by *Trypanosoma evansi*. This effectiveness of isometamidium was confirmed with mice inoculation test which showed significant decrease in *Trypanosoma evansi* in blood picture of calves treated with isometamidium (group III). Furthermore, in group II animals infected but not treated exhibited progressive parasitaemia which is in agreement with Peni *et.al*., [23].

These results suggested higher clinical efficacy of drug isometamidium against *Typanosoma evansi*. These results suggest that higher plasma protein binding does not impair parasiticidal activity of isometamidium.

Conclusion

The study therefore concludes that isometamidium

has greater plasma protein binding capacity than the dissociation constant and so remains as depot in the body for longer duration. The drug isometamidium showed higher clinical efficacy against *Typanosoma evansi* on single dose I/M administration. Also it was found that higher plasma protein binding activity of isometamidium did not hamper the anthelmintic activity of this drug. However, the significance of plasma protein binding for anthelmintics and clinical outcome remains subject to further investigations.

Authors' contribution

All authors contributed equally. All authors read and approved the manuscript.

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Competing interests

Authors declare that they have no competing interest.

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