Optimum Conditions Of 5-Nucleotidase Activity In Sera Of KalaAzaric Patients.

Eaman A.S. AL-Rubaee *, ph. D.

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Summary:

Background: *This research focuses on the characterization studies of 5^{v}-Nucleotidase (5'-NT) of kala-azar patients' sera. 5V NT is one of the enzymes used as indicator of liver function test . In this study the optimum conditions for the activity of 5s - NT enzyme has been demonstrated for kala-azaric patients.*

Materials and methods: *Thirty five blood samples were obtained from Baghdad Hospitals from untreated infants and children with kala-azar. 5'-NT activity was measured by following the method of Wood and Williams.*

Results: *Optimum conditions of serum 5' - NT activity of kala- azaric patients obtained were 50 u L , 30 minutes ,0.8 mM,7.7 and 50'C) as (Volume of serum , incubation time , substrate concentration, pH and temperature) respectively*

Key words: *5 -Nuceotidase, serum and kala-azar.*

Introduction:

5'-Nucleotidase (E.C.3.1.3.5) is widely distributed in several mammalian tissues (1) as well as in microorganisms (2,3). The enzyme is phosphomonoesterase which catalyses the hydrolysis of all ribo nucleoside-5"-monophosphate (4,5). Commonly most tissues contain two isoenzymes of 5s-Nucleotidase (5v-NT), the intrinsic membrane one and a soluble cytosolic form (6-9). Clinical interest in this enzyme was a roused by the finding that its activity in human serum was frequently raised in liver disease and "seldom raised in bone disease (10,11).

Serum 5'-NT has a reference range for adults of (0) to (11) U/L at (37°) (12) and for normal children (2.5-11.9) U/L. While, for kala-azaric patients of (8.9) to (70.0) U/L at 37°C (13). The enzyme is stable at (4°C) for up to 4 days or at (-20°C) for several months (12).

Leishmaniases are among the most important protozon infections that affect humans in the world . The disease is wide spread in 88 endemic countries with 350 million people at risk , 12million people peranently affected, and an estimated annual incidence of 1.5-2 million cases.

Asurprisingly broad spectrum of clinical expressions is observed in humans ranging from asymptomatic to , cutaneous (CL), diffuce cutaneous, mucocutaneous and visceral (VL) diseases, and an intermediary form known as post-kala Azar dermal Leishmaniasis.(14,15). Visceral leishmaniasis (kala-azar) is caused by species of leishmania (Lu donovani complex, K changnsi, L. infantum) that disseminate hematogenously, infecting macrophages in the liver, spleen, bone marrow, and lymphnodes. The infection is zoonotic in most areas. Dogs and other carnivores are the most common reservoir (16). It has a vast geographic distribution involving millions of people, and although epidemiologic features may differ widely the important clinical features are generally similar in different geographic regions (16-18). There is no diagnostic enzyme for this disease and since 5v-NT is one of the enzymes used as indicator of liver function test (19,20), its activity in the sera of kala-azaric patients had been studied in a comparison with normal sera (12). In this study the optimum conditions for the activity of this enzyme has been demonstrated.

* *Basic Science Dept., Collage of Dentistry, University of Baghdad.*

Material and methods:

Blood samples were obtained by vein puncture and allow to clot at room temperature, then the samples were separated by centrifugation at 3000 r.p.m. for 15 minutes. Thirty five pathological samples were obtained from Baghdad Hospitals from untreated infants and children with kala-azar. The diagnosis was based on bone marrow aspiration and clinical examination.

5v-NT activity was measured by following the method of Wood and Williams (1981) (21). Serum and barbiturate / HCl buffer are mixed and incubated at 37°C for 5 minutes, for temperature equilibration. Substrate is added to start the reaction, which is allowed to proceed for exactly 30 minutes. Nickel chloride is then added, followed rapidly by SDS, stannous chloride/hydrazine sulfate and acid molybdate.

This stops the enzyme reaction and develops the color. The contents of the test tube are well mixed and left for 15 minutes before measuring the absorbance (A) at 618 nm vs the reagent blank. One enzyme activity unit is defined as the amount of the enzyme that produces one (i mole of phosphate per minute at 37°C by hydrolysis of ester bond present in (5v-AMP).

Protein concentration was carried out following lowry method (22) where bovine serum albumin was used as standard.

The effect of enzyme concentration was carried out by incubating (0.8 mM) 5v-AMP with different protein concentration range from (0.42) to (5.88) mg/ml (equal to (5-70) uL of serum)which may represent enzyme concentration in the bach sample of cases . The effect of substrate concentrations ranged between (0.01-1.0) mM in (0.5 mL) of incubation mixture which consist of (0.4 mL) of (0.04 M) barbiturate /HC1 buffer, (0.05 mL) of substrate and (0.05 mL) of serum. The effect of incubation time on the reaction velocity of the enzyme 5v-NT was determined after incubation for different time intervals (5-50) minutes using (0.8 mM) of the substrate as a final concentration at 37°C.

Optimum pH for 5"-NT activity was determined by measuring the enzyme activity according to Wood and Williams (1981) using (0.04 M) barbiturate/HC 1 buffer with different pH value (7.0-8.5) at optimum concentration of 5v-AMP as a substrate. Then, the optimum temperature for enzyme activity was determined by incubating the enzyme with (0.8 mM) of substrate at different temperature (10-100)°C for 30 minutes.

Results:

In routine clinical chemistry practice, 5v-NT has been measured in serum. The levels of 5sNT activity are increased in hepatobiliary diseases and malignancy. Although 5v-NT is specific •for ribonucleotide-5"-phosphates, it cannot simply be assayed by the addition of serum to substrate and the monitoring of product formation because nonspecific alkaline and acid phosphatases also hydrolyse this class of phosphate esters. This problem has stimulated the imagination of investigators and has led to a large number of methods with different approaches to assess 5s-NT activity in the presence of nonspecific phosphatases and to monitor the 5"-NT reaction. Wood and Williams (21) present a sensitive colorimetric determination of 5"-NT based on the measurement of liberated phosphate by reaction with stannous chloride/molybdenum blue. No protein precipitation is required. The method is simple, sensitive, economical, and easy to use, and involves relatively stable reagents. By using this method a series of experiments arranged so the concentration of the enzyme is always the same, but the substrate (reactant) concentration is increased from one experiment to the next [Fig. (1)]. We find that in the low substrate concentration experiments the rate increases as we increase substrate concentration, but as the experiments involve higher and higher substrate concentrations, we find that we reach a maximum reaction rate (at [S]=0.8 mM). More substrate does not increase the rate any more. At this point, we say that the enzyme is saturated (it cannot handle any more) to increase the rate again.

Increasing [E] will increase the reaction rate. Results in Fig. (2) indicate that (4.2 mg/ml) protein of serum gives maximum enzyme activity.

Fig. (3) shows the relationship between the incubation time of the reaction and the reaction rate (V). It is obvious that the velocity increased with increasing time and (30 minutes) of incubation has been found the time for maximum enzyme activity. As shown in Fig. (4), the rate of enzyme catalyzed reactions will increase with increasing temperature until an optimum temperature is reached (50°C). Above the optimum temperature rate falls off as the enzyme conformation is disrupted. The effect of pH on 5v-NT activity in sera of kalaazaric patients was examined using (0.04 M) barbiturate/HC 1 buffer with pH range from 7.0 to 8.5 [Fig. (5)] and pH=(7.7) was found to be the pH optimum. In conclusion, the optimum conditions of serum 5'-NT activity of kala-azaric patients obtained were (50 uL, 30 minutes, 0.8 mM, 7.7 and 50°C) as (volume of serum, incubation time substrate concentration, pH and temperature) respectively.

Fig.(1) : Effect of substrate (5`- AMP) concentration on the 5`-NT activity in sera of kala- azaric patients.

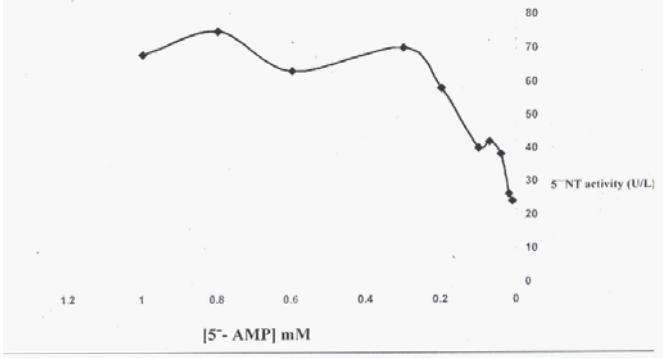


Fig.(2) : Effect of (5`- NT) concentration on the anzyme activity in sera of kala- azaric patients.

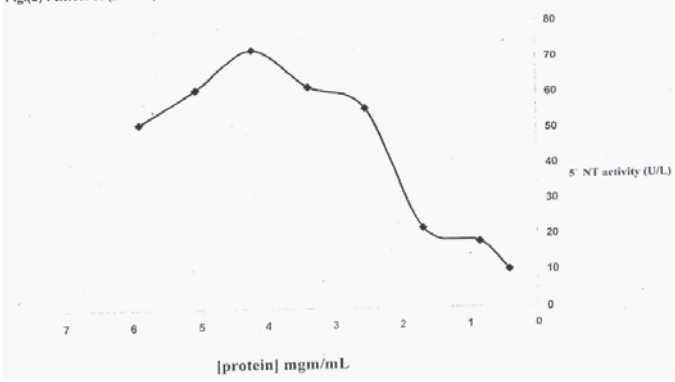


Fig.(3) : Effect of incubation time on serum 5"-NT activity of kala- azaric patients.

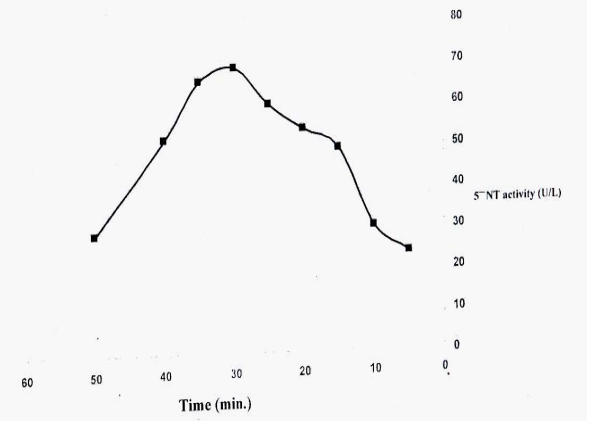


Fig.(4) : Temperature – activity profile of serum 5"-NT in sera of kala- azaric patients.

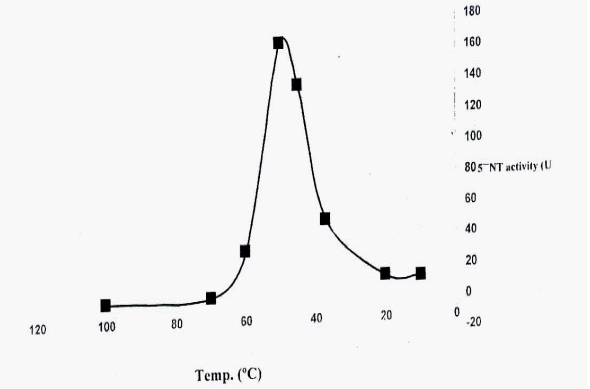
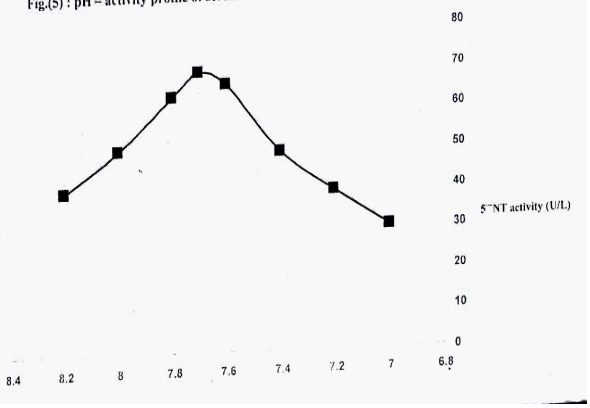


Fig.(5) : pH – activity profile of serum 5"-NT in sera of kala- azaric patients.



Discussion:

When substrate levels are at saturation and the enzyme has reached maximum reaction rate (Vmax.), increasing [E] will increase the reaction rate. Results in Fig. (2) indicate that (4.2 mg/ml) protein of serum gives maximum enzyme activity. The figure also shows there was a loss of enzyme activity upon using more than (4.2 mg/ml) of protein and this may be due to product inhibition (23,24) and polymerization (enzyme convert from active monomeric form to inactive polymeric form) with the increasing of protein concentration (25).

At the start of the reaction the initial velocity (V°) is high due to the large concentration of substrate present. As the reaction proceeds the concentration of products falls and reaction rate slows. As the products of the reaction accumulate they may actually inhibit the enzyme (this is an important control mechanism in metabolic pathways) (5).

Increasing temperature will increase the rate of chemical reactions generally (kinetic energy of reactants is raised which increases the chances of favorable collisions). Enzyme catalyzed reactions will also increase with temperature due to the same reasons. However, catalytic activity depends on the enzyme having the correct conformation (molecular shape). As temperature increases the conformation of the enz me is disrupted due to the increased kinetic energy of the enzyme molecule itself (5). As shown in figure (4). Above the optimum temperature rate falls off as the enzyme conformation is disrupted.

The rate of enzyme catalyzed reactions is sensitive to pH. The conformation of an enzyme can be disrupted when the ionization state of its amino acid side chains is altered by changes in pH (affects ionic and hydrogen bonds that are partly responsible for holding a protein in its biologically active conformation). Previous work reported pH ranged between (7.5-7.9) as optimum pH for 5"-NT activity from different sources (10,26).

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