

Estimation of Hirudin antigenicity by radial immunodiffusion assay in Mammalian blood after leeching

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Abstract:

Hirudin is a strong thrombin inhibitor that does not need a cofactor for its activity. Present study is to determine the antigenic nature of hirudin through radial immunodiffusion assay. Venous blood from the human volunteers was collected after phlebotomy and the recombinant hirudin was used for determining the antigenic nature of hirudin. The result showed formation of antibodies in experimental male and female cases, due to insertion of natural hirudin by leeching.

Key words : *Poecilobdella viridis*, antigen antibody, immunodiffusion assay.

Introduction:

The use of leeches reached a peak in the nineteenth century for various indications: laryngitis, ophthalmic problems, cerebral apoplexy, obesity, and mental disorders. In 1884, Haycraft discovered hirudin which is the main anticoagulative substance in leeches' saliva Whitaker *et al*, (2004). In 1955, Markwardt was the first to isolate hirudin from the pharyngeal glands of leeches. Hirudin is a protein consists of 65-amino-acid, that is a direct inhibitor of thrombin. Hirudin is an anticoagulant present in the leech saliva. Hirudin prevents or dissolves the formation of clots and thrombi (i.e., it has a thrombolytic activity), and has therapeutic value in blood coagulation disorders. Today hirudins are produced as recombinant proteins based on the leech anticoagulant protein sequence. Recombinant hirudin has been particularly useful as an alternative to heparin in patients with heparin-induced thrombocytopenia who require parenteral antithrombotic treatment. Rcombinant forms may also be used in myocardial infarction (MI) and unstable angina. Reiss *et al*. (1995),

Presently Hirudin is used as an anticoagulant in various medical treatments . Potzsch B *et al*,(1996). study estimates the antigenic nature of hirudin.

In this set of experiment r- Hirudin procured from Dr. Jurgen Hofirger NAMOS (Nanotechnology of Biomimeties on surfaces), Germany. It was used as antigen to test its immune reaction in vitro. For this 5 volunteers were applied leeches (one each) for different duration's and antibodies against the leech saliva were allowed to form in volunteers for 96 hr. and then venous blood from the volunteers was removed after 96 hrs of leeching. Serum was separated, which contain antibodies against natural hirudin and other ingradients in leech saliva. The serum was used for immunoelectrophoresis, by Radial Immuno Diffusion Assay.

Radial Immunodiffusion Assay

Single radial immunodiffusion (RID) is used extensively for the quantitative estimation of antigens. The antigen antibody precipitation is made more sensitive by the incorporation of antiserum in the agarose. Antigen (Ag) is then allowed to diffuse from wells cut in the gel in which the antiserum is uniformly distributed. Initially, as the antigen diffuses out of the well, its concentration is relatively high and soluble antigen antibody adducts are formed. However as ag diffuses farther from the well, the Ag -Ab complex reacts with more amount of antibody resulting in a lattice that precipitate to form a precipitin ring. Thus, by running a range a known antigen concentration on the gel and by measuring the diameters of their precipitin rings, a calibration graph is to be plotted. Antigen concentrations of unknown samples, run on the same gel can be found by measuring the diameter of precipitin ring and extra plotting this value on calibration graph.

Materials and Method

Leeching is done to five healthy individuals, having the normal blood parameters. The techniques uses for the present study are - Agarose, 10x Assay buffer, Antigen, Antiserum, Gel punch with syringe, Glass plate, Template.

Procedure

1. 10 ml of 1.0 % agarose (0.1 g /10 ml) was prepared in a buffer by heating slowly till agarose dissolves completely. Care was taken to avoid scroch or froth the solution.
2. The molten agarose was allowed to cool to 55 °C.
3. 120 µl of antiserum was added to 6 ml of agarose solution mixed thoroughly for uniform distribution of antibody.
4. Agarose solution containing the antiserum was poured on to a grease free glass plate set on a horizontal surface. Left it undisturbed to form a gel.
5. Wells was formed with the help of a gel puncher using the template.
6. 20 µl f the antigen was added to the wells.

7. The gel plate was kept in a moist chamber (box containing wet cotton) and incubated overnight at room temperature.
8. The edges of the circle were marked and measured the diameters of the rings.
9. Graph of diameter of ring (on y axis) versus concentration of antigen (on x axis) on a semi -log graph sheet was plotted.
10. Determined concentration of unknown by reading the concentration against the ring diameter from the graph.

Antigen antibody reaction :

Five volunteers were applied leeches (one each) for 1 hr and antibodies against the leech saliva were allowed to form in the volunteers for 96 hours. and then venous blood from the volunteers was taken after 96 hr of leeching, serum was separated, which contained antibodies against natural hirudin and other ingredients in leech saliva. This serum was used for immunoelectrophoresis, by radial immunodiffusion assay.

Antibodies produced after leeching were present in serum of all the volunteers. They reacted with the r- hirudin to form a lattice that precipitate to form a precipitin ring. The serum samples containing antibodies were mixed with the gel and the 4 wells in the gel were filled up with different concentrations of r – Hirudin (5 μ l, 10 μ l , 1 μ l 5 μ l and 20 μ l). The diameters of precipitin rings obtained after incubation of 48 hours at 37 ⁰C were measured and a standard graph (diameter of precipitin rings against r-hirudin concentration) was prepared (Table. 1). (Fig. 1,2,3).

Then another group of 5 healthy volunteers were applied with leech (one each) for 1 hour and immediately after leeching the venous blood was removed from the nearby site of leech bite. Plasma was separated which contained the injected leech saliva (hirudin + other biochemical secretions). This hirudin is treated as the natural antigen. 20 μ l of plasma was poured into a well of freshly prepared immunoelectrophoresis gel mixed with antibodies as above. The plate was then incubated at 37 ⁰C for 48 hours and then the diameter of precipitin ring was measured. The experiment was repeated there. The average diameter was plotted on the standard graph to know the quantity of hirudin injected (Fig.1).

It is found that a leech of weight about 3 g usually injects 4.25 μ g of hirudin during one bite of 1 hr.

Results and Discussion:

As the leech bite continues, it reduces congestion due to the anticoagulant effect of leech saliva, which contains thrombin inhibitor hirudin, apyrase, collagenase, hyaluronidase, factor Xa inhibitor and fibrinase I and II Knobloch et al., (2007). A leech consumes 5mL to 15mL of blood and induces oozing on the site of attachment of between 50mL and 100mL of blood during the 24-hour to 48-hour period after the leech is detached.

Presently, Hirudin is used in various medical treatment modalities after US FDA has cleared it for use in medicine. Various studies have demonstrated its efficacy as

anticoagulant. Huhle et al., (2000) and Nowak (2001). As a polypeptide natural recombinant hirudin may elicit an immunological response in humans, this may also be due to the long phylogenetic distance from invertebrates (Song et al., 1999). In the present study, generation of antihirudin antibodies was observed using immunodiffusion test. The r - hirudin radial immunodiffusion demonstrated binding of the human anti-hirudin, antibody to r - hirudin (Fig . 4.52, and 4.53) . The generation and disappearance of anti hirudin antibodies were investigated in patients with heparin induced thrombocytopenia who were treated with recombinant hirudin for about 5 days by Song et al., (1999).

The production of anti hirudin antibodies raise the question as to whether the generation of anti-hirudin antibodies interferes with the anticoagulant activities of r hirudin. The radial immunodiffusion assay in the present investigation demonstrated *in vitro* binding of the human anti hirudin antibody to r hirudin. However, the hematological investigation indicated that the antibodies against hirudin have no influence on the thrombin inhibition property of hirudin as the clotting time was found to be increased in all the volunteers tested. However, the experimental results of prothrombin time demonstrated that prothrombin time was not affected indicating that there are no binding sites of hirudin on prothrombin. To confirm this, help from various Bioinformatics sites is taken.

Table 1: Radial immunodiffusion assay of antihirudin raised against hirudin.

S.No.	Quantity of r-hirudin (μg)	Diameter of precipitin ring (mm)
1	0.844	6.0 \pm 0.26
2	1.688	8.0 \pm 0.15
3	2.532	11.4 \pm 0.12
4	3.376	12.8 \pm 0.18
5	4.250*	134.0 \pm 0.20

Values are \pm SE of 5 observations.

* Quantity of Hirudin calculated from graph. It indicates quantity of Hirudin injected in one bite during full meal.

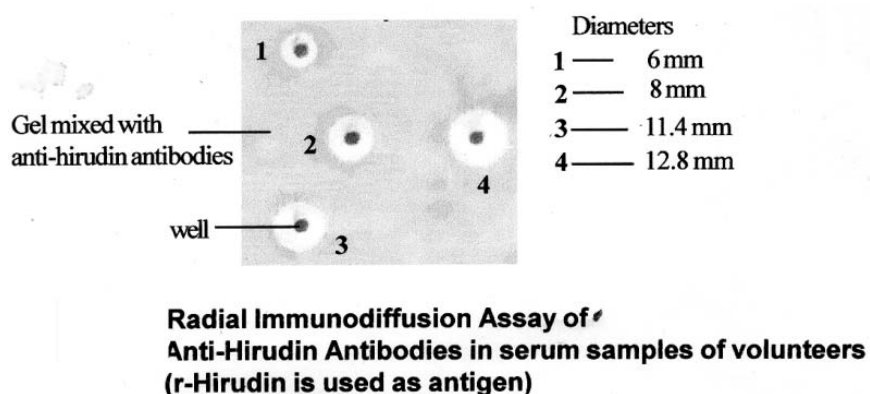


Figure - 1

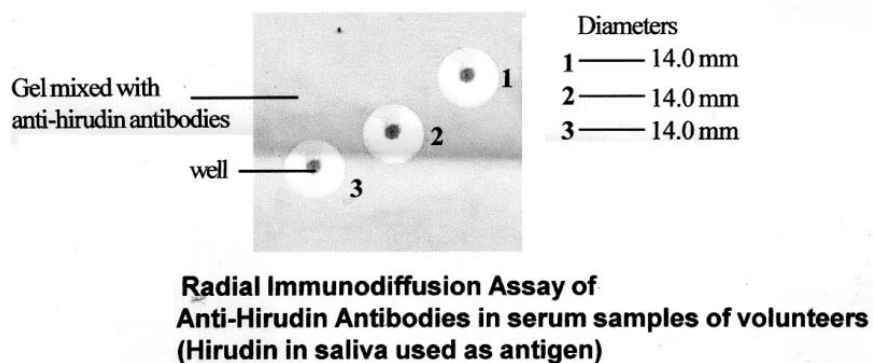


Figure - 2

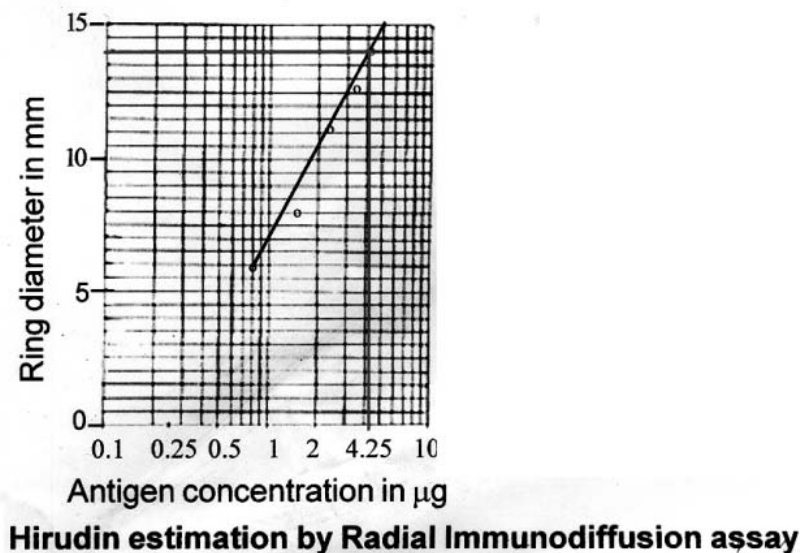


Figure - 3

Reference:

- [1] Huhle G, Hoffmann U, Hoffmann I, et al. A new therapeutic option by subcutaneous recombinant hirudin in patients with heparin-induced thrombocytopenia type II: A pilot study. *Thromb Res* 2000; 99: 325–334.
- [2] Knobloch K, Gohritz A, Busch K, Spies M, Vogt PM. [Hirudo medicinalis-leech applications in plastic and reconstructive microsurgery—a literature review]. *Handchir Mikrochir Plast chir*. 2007 Apr; 39 (2):103-7.
- [3] Nowak G. Clinical monitoring of hirudin and direct thrombin inhibitors. *Semin Thromb Haemost* 2001; 27:537–541.

- [4] Potzsch B, Greinacher A, Reiss F-C, et al. Recombinant hirudin as anticoagulant in cardiac surgery: experiences with eleven patients [abstract]. *Ann Hematol.* 1996;72:A4.
- [5] Reiss F-C, Lower C, Seelig C, et al. Recombinant hirudin as a new anticoagulant during cardiac operations instead of heparin: successful for aortic valve replacement in man. *J Thorac Cardiovasc Surg.* 1995;110:265-267.
- [6] Song, H.F.;W.Liux; Z.M.Tang (1999): Pharmacokinetic and peripheral platelet count after single dose of recombinant human thrombopoietin in rhesusmonkey, *Acta Pharmaco) Sin.*20 (3):224-248.
- [7] Whitaker IS, Rao J,Izadi D,Butler PE: Historical Article : *Hirudo medicinalis*: ancient origins of ,and trends in the use of medicinal leeches throughout histroghut history. *Br J Oral Maxillofac surg* 2004,42(2):133-137.