ANALYTICAL CHARACTERIZATION OF ANTIVENOM PHARMACEUTICAL POTENTIAL BY ASSAY OF L-AMINO ACID OXIDASE ACTIVITY

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abstract: The antivenom capacity of an immunoglobulins preparate of ovine origin is analised. Antivenom is "in vitro" analised from the point of view of its inhibiting potential on L-amino acid oxidase from Vipera ammodytes venom. The activity of L-amino acid oxidase, (LAO), is assayed using a spectrophotometric method, at 507 nm, with peroxidase and chromogenic system – phenol, 4-aminoantipyrine. Regarding the antivenom efficiency, the results provided by "in vitro" method are different from the results provided by "in vivo" method, on testing animals. The method is very useful for monitoring purification and concentration of antivenom antibodies.

1. Introduction

The antivenoms are pharmaceutical preparations containing specific antibodies for the venom of interest [1,2]. They are obtained from serum/plasma of hyperimmunised animals with venom provided by one or many species [3,4].

Actually, the first antivenoms are the serums obtained after coagulation of blood from hyperimmunised animals. Subsequently, methods for concentration and purification of antibodies have been issued in order to decrease clinical risks, [5÷10].

The antivenom activity is assayed following the reaction generated by injection of well-defined mixes of venom and antivenom in testing animals (mice, rats) [11÷14]. As all venoms (in particular those provided by snakes) contain several enzymes, the antivenom potential can be expressed as antienzymatic activity by "in vitro" determination of activity of certain enzymes from mixes of venom and antivenom.

Even if "in vivo" techniques involve several inconveniences (long analysis times, testing animals, personnel), they are accepted to express the antivenoms potency, mainly because they are close to the medical problems raised by the treatment of snakes bites [15÷19].

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In order to obtain antivenoms of great therapeutic efficiency, the assay methods for antivenom activity by determination of antienzymatic activity are extremely useful in the process of immunoglobulins purification and concentration.

This paper presents an assay method for an ovine antivenom potential by determination of L-aminoacid oxidase activity from mixes of venom and antivenom for Vipera ammodytes.

2. Experimental

2.1. Principle

The enzymatic activity of L-amino acid oxidase for mixes of crude serum or immunoglobulins concentrate (antivenom) and venom, in different ratio, specified in the corresponding tables, was recorded.

The immunoglobulins concentrate was obtained by a method which combines precipitation with caprylic acid and dialise [19, 20], from serum of animals (*Ovis aries*, 2 years old males), previously hyperimmunised with Vipera ammodytes venom.

The enzymatic activity of L-amino acid oxidase was determined by a UV-VIS molecular absorbtion spectrometric method based on coupling of two reaction catalised by L-amino acid oxidase and peroxidase. The method allows determination of H_2O_2 produced into the system, in presence of phenol and 4-aminoantipyrine, in controlled conditions of *p*H, temperature and time, [21].

The antivenom potential (proficiency or efficacy) of crude serum or of antibodies concentrate are considered by the inhibition of the L-amino acid oxidase from venom.

2.2. Reagents and apparatus

- Spectrometer of molecular absorbtion, 1 cm cells, micropippetes;
- 1 mg/ mL venom solution in buffer 0.2 M TrisHCl; *p*H=7.5;
- 1mg/ml (approx. 100 U.I./ml) peroxidase in buffer 0.2 M TrisHCl; *p*H=7.5;
- Chromogenic solution (0.2M 4-aminoantipyrine, 0.2M phenol);
- Crude serum or antibodies concentrate;
- Buffer solution 0.2 M TrisHCl; *p*H=7.5;
- Aminoacids standard solution, L-tirosine or L-phenilalanine, 0.1-2.5 mM, 30 mM.

2.3. Procedure

The reagents are tempered at 25^o C for 45 minutes.

The volumes of antigene (0.05 mL venom solution) and antibody (crude serum or immunoglobulins concentrate) are tempered at 37^o C for 30 minutes. If the volumes are too small and do not reacts risk occurs, 0.1 mL of Tris buffer solution is added. The solution obtained is mixed.

0.5 mL Tris buffer solution, 0.25 mL chromogenic solution, 0.1 mL peroxidase solution and 0.25 mL 30 mM L-phenilalanine (or L-tyrosine) solution are added, then the mixture is rapidly brought to the volume of 2.5 mL using Tris buffer solution. The variation of sample absorbance versus blank at λ = 507 nm is measured and the result is interpoled from the calibration curve, previously obtained using standard solutions of amino acids and long reaction time (approx. 3–4 hours) or standard solutions of H_2O_2, peroxidase, 4-aminoantipyrine and phenol.

If the concentration interpoled from the curve has the value:

$$c(\text{mM})=0.2\text{x}Abs(\text{units}) \tag{1}$$

for a reaction time, t(min.), then the enzymatic activity can be calculated using the following formula [21]:

$$\text{LAO activity}=\frac{0.2\cdot 2.5\cdot Abs}{0.05\cdot t(\text{min})}\,\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}\ \text{venom} \tag{2}$$

or

$$\text{LAO activity}=\frac{10\cdot Abs}{t(\text{min})}\,\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}\ \text{venom} \tag{3}$$

3. Results and Discussions

Based on the experimental results, we decided to express the antivenom efficiency as the volume (quantity) of antivenom needed to neutralize 50% of the L-aminoacid oxidase activity of a certain quantity of venom, EED_{50} – 50% enzymatic effective dose or 50% enzymatic equivalent dose. This approach employs the theory of general mechanisms of enzymatic inhibition and allows a more precise analytical characterization of the pharmaceutical potential of venom.

Variation of absorbance versus time for mixes of crude serum with venom in different ratio (given in Table 1) is presented in Fig. 1.

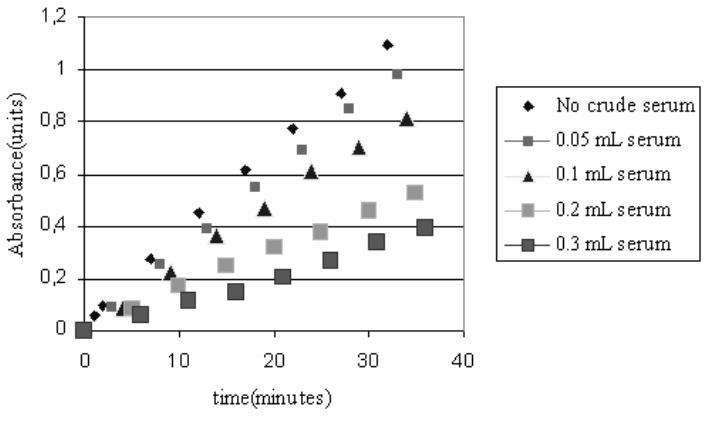

Fig. 1 *Variation of absorbance at λ=507 nm using crude serum*

Table 1. Determination of L-amino acid oxidase activity with crude serum

Parameter (measurement unit)	0.050 mg venom incubated with: crude serum (mL) 0	crude serum (mL) 0.050	crude serum (mL) 0.100	crude serum (mL) 0.200	crude serum (mL) 0.300
LAO activity (µmol/min/mg)	0.342	0.297	0.239	0.150	0.109
(Activity $_{Max.}$- Activity) / Activity $_{Max.}$	0	0.132	0.301	0.561	0.681

Variation of the enzymatic activity as ratio between the difference between maximum activity and activity and the activity corresponding to a certain serum volume is presented in Fig. 2.

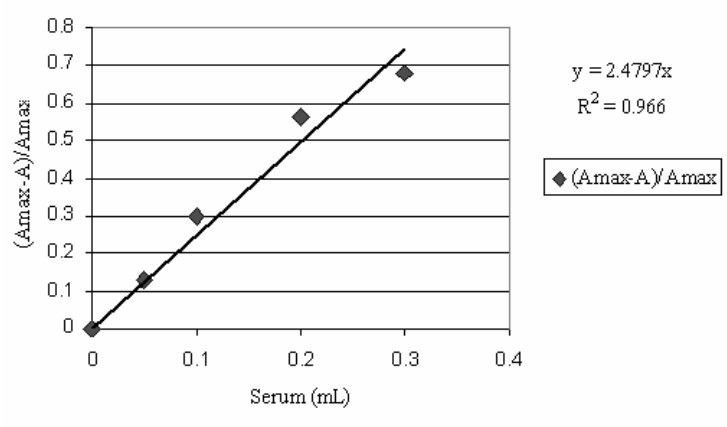

Fig. 2 *Variation of LAO activity versus volume of crude serum*

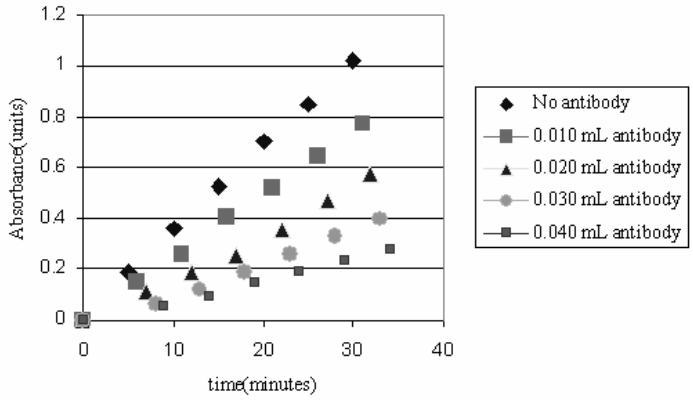

Fig. 3 *Variation of absorbance versus time, using antibody preparate, at λ=507 nm*

The efficacy (equivalency) of crude serum can be calculated with good precision for total inactivation of venom by interpolation from the graph presented in Fig. 2. However, we consider more appropriate to express the equivalence for 50% inactivation of the enzymatic activity of LAO in venom.

For 0.050 mg 50% inactivated venom, the equivalence will be:

$$EED_{50} = 0.5/(0.05 \cdot 2.480) = 4.03 \text{ mL crude serum/mg venom}$$

Variation of the absorbance versus time for mixes of immunoglobulins concentrate with venom in ration included in Table 2 is presented in Fig. 3.

Table 2 – Assay of anti-L-amino acid oxidase activity with immunoglobulins concentrate

Parameter (measurement unit)	0.05 mg venom incubated with: Ig conc. (mL) 0	Ig conc. (mL) 0.010	Ig conc. (mL) 0.020	Ig conc. (mL) 0.030	Ig conc. (mL) 0.040
LAO activity (μmol/min/mg)	0.340	0.251	0.181	0.122	0.081
(Activity Max.- Activity) / Activity Max.	0	0.262	0.468	0.641	0.762

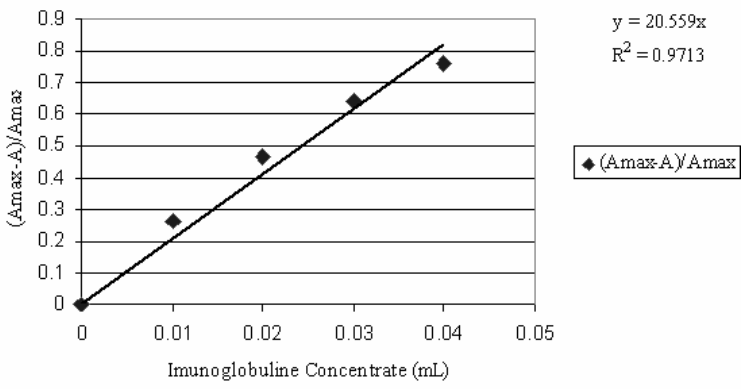

Fig. 4 *Variation of LAO activity versus volume of immunoglobulins concentrate.*

By interpolation from previous graph, for 0.050 mg venom, the equivalency of the immunoglobulins concentrate can be calculated as follows:

$$EED_{50} = 0.5/(0.05 \text{x} 20.559) = 0.486 \text{ mL Ig concentrate / mg venom}$$

By extrapolation for total inactivation of venom, the equivance can be calculated as follows: $2 \text{x} EED_{50} = 0.972$ mL Ig concentrate / mg venom

One could consider that the obtained immunoglobulins preparate is 8-10 times more concentrated in antibodies for L-amino acid oxidase from Vipera ammodytes venom than the crude serum.

The results obtained using this method are different from the results obtained using "in vivo" methods. Therefore, using the same immunoglobulins concentrate, by intraperitoneal injection in mice, we obtained the equivalence of 0.595 mL Ig concentrate/ mg venom. This difference can be explained taking into account that the immunoglobulins concentrate consists of a mix of antibodies for each venom protein, with different imunogene capacity. For this reason, the results obtained using other "in vitro" methods it is expected to be different, considering the enzymatic activity used to express the venom efficacy.

4. Conclusions

An enzymatic method used to determine the ovine antivenom potential by determination of anti-L-amino acid oxidase activity was tested.

The method proved to be useful mainly during purification and concentration of antivenom antibodies from crude serum in order to obtain the antivenom with great clinical efficiency.

Regarding the antivenom efficacy, the results provided by this method are different from the results provided by "in vivo" method mainly because the biological methods provide results that include and accumulate the all venom effects on the body of testing animals.

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