Potency Test: Effective Intraperitoneal Dose 50 of ophidian antivenom

1. TARGET

Ensure that the sample to be analysed complies with the defined power specifications.

1. BASIS

An amount of venom (to ensure death of the animals in less than 48 hours) is mixed with varying volumes of antivenom, the mixtures being adjusted to a constant volume with sterile saline solution. After an incubation period, aliquots of the mixtures are injected intraperitoneally into groups of at least 5 mice. The results provide information on the ability of the antivenom to neutralise the lethal effect of a given venom. Neutralisation lethality is expressed as mg of venom neutralised by one mL of antivenom. Use the Probit statistical method to calculate the 50% Effective Dose, defined as the ratio of mg of venom/mL of antivenom at which 50% of the injected mouse population is protected.

1. MATERIALS AND REAGENTS

- Reference poisons.
- 3 mL glass or plastic syringes.
- 26/½ or 27/½ gauge needles.
- Phosphate buffered saline, pH 7.2 (PBS).
- CD-1 strain mice weighing 16-18 grams.
- Bain Marie Precision Scientific 185.
- Ophidic antivenoms to be tested.

1. PROCEDURE

- Determine the DL_{50} of the venoms with which you are going to challenge the snake antivenoms.
- Choose five levels to test, which will depend on the type of antivenom tested. These levels should preferably have the same logarithmic interval between them.
- To prepare the venom/antivenom mixtures, it is suggested to follow the steps given in the example below, adjusting them to the particular conditions of each determination. Calculations for potency tests against other poisons are included at the end of this method.
- Follow the instructions below for the different types of antivenom. The following is an example of the procedure: suppose you have a polyvalent antivenom and you need to know its potency against the venom of the snake *Bothrops asper*. It has been determined that the 50% Lethal Dose of *Bothrops asper* venom from Costa Rica, intraperitoneally, is approximately 62.5 µg, when using white mice weighing 16-18 grams. Based on this

information, a dose of venom corresponding to 4 LD_{50} is established in order to guarantee the death of the mice.

62.5 µg X 4= 250 µg of venom to be inoculated per mouse.

- Since at least 5 mice per level will be inoculated, each receiving a volume of 0.5 mL, a minimum final volume of 2.5 mL is required. In this case, it is preferred to have an excess, so a final volume of 3.5 mL per level is chosen.
- Arbitrarily, a volume of the venom solution to be made available per level (1 mL) is defined, such that a total amount of venom of 250 µg X (3.5/0.5)= 1.75 mg per mouse is required to be dissolved in 1 mL per level. In other words, the working solution of venom will be a concentration of 1.75 mg/mL. Since 1 mL of this poison solution is added to each level, we will have 1.75 mg of poison in each tube.
- After determining the concentration of the poison solution to be used, calculate the amount of poison that needs to be weighed, taking into account the number of levels you want in the test. For example, if 5 levels are prepared:

  1 mL x 5 levels = 5 mL of poison solution
- Calculate an excess of the poison solution:

  6.0 mL of poison solution x 1.75 mg/mL = 10.5 mg of poison
- 10.5 mg of venom is required for all test levels. This amount of venom is dissolved in PBS to obtain the calculated venom solution concentration (1.75 mg/mL). For this case, 6 mL of PBS should be used.

- To find the volume of antivenom to be added at each level, proceed as follows: For the level of 2 mg of venom/mL of antivenom, (1.75 mg/2.0 mg) X 1 mL = 0.875 mL of antivenom.

  A similar calculation applies for the 3 mg/mL and 4.5 mg/mL levels:

  (1.75 mg/3 mg) X 1 mL = 0.583 mL of antivenom.

  (1.75 mg/4.5 mg) X 1 mL = 0.438 mL of antivenom.
- No poison control is included to avoid animal suffering.
- Finally, the tube volumes are adjusted to a final volume of 3.5 mL with PBS. The following table summarises the above:

LEVEL (mg/mL)	ANTIVENOMINE (mL)	PBS (mL)	POISON (mL)*	MICE PER DOSE
2.0	0.875	1.625	1.0	5
3.0	0.583	1.917	1.0	5
4.5	0.438	2.062	1.0	5

*1.75 mg/mL working solution of venom.

- Incubate the venom-antivenom mixtures at 37 °C for 30 min, sealing the mouths of the tubes with parafilm.
- Inoculate, intraperitoneally, 0.5 mL per mouse with each of the prepared levels. Inoculate 5 mice per level, making sure to properly identify the mice.
- Record the number of mice killed at 24 and 48 hours.
- Calculate the Effective Dose 50% using the Probit method.

SPECIFICATION

Accepted values for antivenom (final product) at the Clodomiro Picado Institute:

- Each mL of **PoliVal-ICP** ophidic antivenom should neutralise at least 3 mg of *Bothrops asper* venom, 2 mg of *Crotalus simus* venom, 3 mg of *Lachesis stenophrys venom*.
- Each mL of **PoliVet-ICP** ophidic antivenom should neutralise at least 2.5 mg of *Bothrops asper* venom and 2 mg of *Crotalus simus* venom.
- Each mL of **Anticoral-ICP** mL ophidian antivenom should neutralise at least 300 µg of *Micrurus nigrocinctus* venom.
- Each mL of Pan-African serum (**EchiTab-Plus-ICP**) should neutralise at least 3 mg of *Echis ocellatus* venom, 2 mg of *Bitis arietans* venom and 0.2 mg of *Naja nigricollis* venom.
- Each mL of lyophilised **EchiTab-ICP** Pan-African ophidian ophidian antivenom should neutralise at least 3 mg of *Echis ocellatus* venom, 2 mg of *Bitis arietans* venom, 0.4 mg of *Naja nigricollis* venom and 0.1 mg of *Dendroaspis polylepis* venom.

1. REFERENCES

- Solano, G. et al. (2010) Study of the design and analytical properties of the lethality neutralization assay used to estimate antivenom potency against *Bothrops asper* snake venom. Biologicals 38, 577-585.
- World Health Organization (WHO), 2016. Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins.
- Gené, J.A. and Robles, A. (1987) Determination of the 50% lethal dose by the Spearman-Karber method. Revista Médica del Hospital Nacional de Niños (Costa Rica) 22, 35-40.
- Pan American Health Organization (1977) Manual of procedures. Production and control tests in the preparation of diphtheria, tetanus, botulinum, antivenom and gas gangrene antisera.
- World Health Organization (1981) Progress in the characterization of venoms and standardization of antivenoms. WHO, Geneva.

Determination of Effective Intravenous Dose 50 of ophidian antivenom

1. OBJECTIVES

Ensure that the sample to be analysed complies with the defined power specifications.

1. BASIS

An amount of venom (to ensure death of the animals in less than 48 hours) is mixed with varying volumes of antivenom, the mixtures being adjusted to a constant volume with saline. After an incubation period, aliquots of the mixtures are inoculated into mice intraperitoneally. The results provide insight into the ability of the antivenom to neutralise the lethal effect of a given venom. Lethality neutralisation is expressed as mg of venom neutralised by one mL of antivenom. With the application of statistical methods such as the Probit method, the 50% Effective Dose is determined, defined as the ratio of mg of venom/mL of antivenom at which 50% of the inoculated mouse population is protected.

1. MATERIALS AND REAGENTS

- Bain Marie Precision Scientific 185.
- Automatic pipettes
- Reference poisons; generally used in the immunisation of horses to produce antivenom.
- Antivenom to be tested.
- 1 mL plastic syringes.
- 26 gauge needles.
- Physiological saline solution (PSS).
- White mice weighing 20-22 grams.

1. PROCEDURE

- Determine the DL_{∞} the poisons with which you are going to challenge the ophidian antivenoms
- Choose three or four levels to test, which will depend on the type of antivenom tested. These levels should have the same logarithmic interval to each other.
- To prepare the mixtures of poison and antivenom, it is suggested to follow the steps given in the following example, adjusting them to the particular conditions of your determination. Calculations for potency testing against other poisons are included at the end of these instructions.

- The following is an example of the procedure: suppose you have a polyvalent antivenom and you need to know its potency against the venom of the snake *Bothrops asper*. It has been determined that the 50% Lethal Dose of *Bothrops asper* venom from Costa Rica, by the intravenous route, is 16 µg, when using white mice weighing 20-22 grams. Based on this information, a dose of venom corresponding to 5 Lethal Dose 50% is established in order to guarantee the death of the mice.

  16 µg X 5 = 80 µg of venom to be inoculated per mouse.
- Since at least 5 mice will be inoculated per mixture, each receiving a volume of 0.2 mL, a minimum final volume of 1.0 mL is required. In this case, it is preferred to have an excess, so a final volume of 2 mL is chosen.
- Arbitrarily define a volume of physiological saline solution or PBS in which the venom is to be dissolved (1.0 mL), such that a total amount of venom of 80 µg X (2.0/0.2)= 0.8 mg is required, which will be dissolved in 1.0 mL. In other words, the working solution of venom will be 0.8 mg/mL. Since 1.0 mL of this poison solution is added to each tube, we will have 0.8 mg of poison in each tube.
- To find the volume of antivenom to be added to each tube, proceed as follows: For a level of 2 mg of venom/mL of antivenom, (0.8 mg/2.0 mg) X 1 mL = 0.400 mL of antivenom.
- A similar calculation applies for the 3 mg/mL and 4.5 mg/mL levels:

  (0.8 mg/3 mg) X 1 mL = 0.266 mL of antivenom.

  (0.8 mg/4.5 mg) X 1 mL = 0.178 mL of antivenom.
- Finally, the tube volumes are adjusted to a final volume of 2.0 mL with physiological saline or PBS. The following table summarises the above:

LEVEL (mg/mL)	ANTIVENOMINE (mL)	PBS (mL)	POISON (mL)*	MICE PER DOSE
2.0	0.400	0.600	1.0	5
3.0	0.266	0.734	1.0	5
4.5	0.178	0.822	1.0	5

*Working solution of venom 0.8 mg/mL.

- Incubate the venom-antivenom mixtures at 37°C for 30 min, sealing the mouths of the tubes with parafilm.
- Inject, intravenously, 0.2 mL per mouse with each of the prepared mixtures. Inject at least 5 mice per level making sure to properly identify the mice.
- Record the number of mice killed at 24 and 48 hours.
- Calculate the Effective Dose 50% using the Probit method.

SPECIFICATION

Accepted values for antivenom (final product) at the Clodomiro Picado Institute:

- Each mL of Pan-African serum (**EchiTab-Plus-ICP**) should neutralise at least 3 mg of *Echis ocellatus* venom, 2 mg of *Bitis arietans* venom and 0.2 mg of *Naja nigricollis* venom.

- Each mL of lyophilised **EchiTab-ICP** Pan-African ophidian ophidian antivenom should neutralise at least 3 mg of *Echis ocellatus* venom, 2 mg of *Bitis arietans* venom, 0.4 mg of *Naja nigricollis* venom and 0.1 mg of *Dendroaspis polylepis* venom.

1. REFERENCES

- Solano, G. et al. (2010) Study of the design and analytical properties of the lethality neutralization assay used to estimate antivenom potency against *Bothrops asper* snake venom. Biologicals 38, 577-585
- World Health Organization (WHO), 2016. Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins.
- Characterization of venoms and standardization of antivenoms, R.D.G Theakston.
- Gené, J.A. and Robles, A. (1987) Determination of the 50% lethal dose by the Spearman-Karber method. Revista Médica del Hospital Nacional de Niños (Costa Rica) 22, 35-40.
- Pan American Health Organization (1977) Manual of procedures. Production and control tests in the preparation of diphtheria, tetanus, botulinum, antivenom and gas gangrene antisera.
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