Evaluation of Evans Blue extravasation as a measure of peripheral inflammation

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Method Article

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Abstract

Plasma extravasation is one of the characteristic symptoms of an inflammatory response and of neurogenic inflammation, a phenomenon which occurs following nerve damage, and can lead to significant changes in the control of the cutaneous circulation[1]. Evans Blue binds with high affinity to serum albumin, and the complex has been often used to quantitatively assess the extent of extravasation that accompanies peripheral and neurogenic inflammation[2,3]. Here we present a protocol for evaluating Evans Blue extravasation in rodents with the aid of local application of capsaicin to initiate a neurogenic response exciting the local nociceptors, causing pain, axon reflex vasodilatation and plasma extravasation due to local release of inflammatory mediators. The method is inexpensive, precise and efficient, and can be adapted to assess extravasation in a range of experimental pathological conditions such as parotitis[4] or craniofacial muscle inflammation[5], and their dependence on different proteins, receptors or pharmacological treatments[6-9], in addition to those eliciting neurogenic inflammation.

Reagents

- Sodium pentobarbital \(0.34 mg/ml dilution in saline; Dolethal, Vétoquinol S.A. France)
- Hair removal cream \(Dermo-tolerance anti-irritant; Vichy laboratories, France)
- Sterile distilled water
- Sodium chloride \(NaCl, M = 58.44, Panreac, cat. no. 131659)
- Evans Blue \($C_{34}H_{24}N_6O_{14}S_4Na_4$, MW = 960.81, Sigma, cat. no. E2129)
- Capsaicin powder \($C_{18}H_{27}NO_3$, Sigma, cat. no. M2028)
- Base L-200 cream base \(Cofares, Spain)
- Propylene glycol \($C_3H_8O_2$, M = 76.10, Panreac, cat. no. 141545)
- Formamide \($HCONH_2$, M = 45.04, Sigma, cat. no. F7503)

Equipment

- Adult male Sprague-Dawley rats \(other strains and mice can be used as well) - Gauze - Flat spatula - Insulin syringes \(1ml; BD Plastipak, cat. no. 300013) - Needle \(0.5mm; BD Microlance, cat. no. 300600 \(25G)) - Surgical instrument tools: one pair of forceps, spittle and scalpel - Pipette and pipette tips \(Brand, cat. no. 702521 and 702516) - Plastic tubes \(Brand, cat. no. 114835) - 1,5 ml Eppendorf tube \(Brand, cat. no. 780500) - Multiwell plate P96 \(Sarstedt) - Aluminium foil sheet - Heater \(JP Selecta SA) - Weighing scales - Perfusion pump \(Masterflex, model 7521-00) - Spectrophotometer \(Molecular Devices)

Procedure

1.Prepare Evans Blue

Prepare the EB solution for injection in 0.9% saline at a concentration of 50 mg/ml. Sonicate. Once

prepared, the solution can be stored at 4ºC. **TIP**: *It is advisable to prepare in advance.*

2.Prepare capsaicin cream

Mix 22.6% of Base L-200, 3.7% of Propylene glycol and 73.7% of distilled water and then add 1.6 % of capsaicin powder. Store at room temperature.

3. Preparation and collection of the tissue

a. Cut aluminium foil into 7-8 cm square pieces and label them with sample numbers.
b. Prepare Pentobarbital working solution for anesthetizing the rats.
c. Inject the anaesthesia intraperitoneally \(50 mg/Kg) depending on the weight of the rat and wait until it is deeply anesthetized.
d. Cut carefully the vibrissae on both sides of the snout with curved scissors.
e. Apply the depilatory cream to the whisker pad area and leave for 7 or 8 minutes.
f. Remove the cream with a flat spatula, taking care not to injure or irritate the area.
g. Rinse the area with a gauze wet in distilled water
h. Prior to the injection of EB, vasodilatation should be induced in the tail vein for easier cannulation. Immerse the rat tail in warm water to induce vein vasodilatation and improve their visualization.
TIP: *Be careful not to overheat the water to prevent flaking of the skin. Temperature should not exceed 43ºC. Infrared lamps can be used, as well as vasodilating agents such as Xylazine and Acepromazine.*
i. Inject the prepared EB solution \(50 mg/kg of 50 mg/ml solution) intravenously in the lateral vein of the tail with an insulin needle at a rate of 0.01 ml/g of animal weight **\(Figure 1A)**. The lateral veins are located on both sides of the centerline of the tail and are very shallow so that the injection should be almost parallel to the surface. Once injected, remove the needle and press the injection site with cotton to stop bleeding. Time should be measured from the moment the skin turns blue.
TIP: *Make sure that the vein is not broken during the injection and all the EB is injected properly. It is best to begin the injection into the distal part of the tail. If a lump is formed at the injection site, it means that the vein is broken, and a subsequent injection should be performed more proximally. In this case the syringe should be refilled to replace any lost volume of EB.*
j. Five minutes after the injection, apply capsaicin or vehicle cream \(according the group it belongs to) in the whisker pad area on both sides of the snout.
k. Wait 10 minutes after application of the cream and remove the cream with the aid of a gauze **\(Figure 1B)**.
l. Perfuse the animal through the ascending aorta with saline.
m. Quickly remove the whisker pads. Dissect the area between the nose and the inferior lip. Remove the top layers \(about 5 mm in depth) making sure not to include the intraoral epithelium.
n. Wrap each pad separately in a piece of aluminium foil, leaving the upper side of the package open to allow better drying.

4. Tissue processing

a. Place the tissue in aluminium foil in an oven at 56ºC for two days to dry.
b. After two days, take the tissue out of the oven and immerse each one of the samples in a plastic tube

with formamide to extract the EB out of the tissue. Add 8 ml of formamide per 1 g of dry tissue.
TIP: *Cover the tubes with aluminum foil.*
c. Leave the plastic tubes containing the sample embedded in formamide in an oven at 56ºC over two days.
d. After two days in the oven, extract the formamide with a pipette \(it should be blue) to a covered, labelled eppendorf tube and discard the tissue.
e. Prepare the dilutions for the standard curve: dilute EB in the formamide solution in exponential concentrations. The concentrations used in our study, adjusted to the level required for the absorbance of the samples obtained, were as follows:
0 ; 1 ; 2 ; 4 ; 8 ; 16 ; 32 ; 64 ; 128 µg/ml
TIP: *Prepare just before measuring the absorbance of the samples. A standard curve is necessary for each experimental session*
f. Take 200 µl of the formamide extracted from each sample and put each one in a different well of a P96 multiwell plate. In the same plate put 200 µl of the corresponding dilutions for the standard curve.
h. Measure the absorbance with the help of a spectrophotometer at a wavelenght of 620 nm. We use the program Shortcut to Spf.

5. Data fit

a. Obtain absorbance values from the solutions forming the standard curve. These values should grow exponentially \(**Table 1**).
b. Adjust the standard curve to a theoretical sigmoidal curve. The regression parameters \(a, b, x_0, y_0) can be estimated from the experimental absorbance data using any statistical program \(such as Sigma Plot 8.0). Then, the non-linear regression formula is applied:
$y=y_0+a/\(1+exp\(-\(x-x_0)/b))$
where the independent variable x indicates the concentration, the dependent variable y refers to the absorbance and a theoretical standard curve is obtained \(**Figure 2**). This allows us to calculate any point with respect to the formula, thus obtaining a set value.
c. Extrapolate the concentration of extravasated EB obtained in the study from the known concentrations of EB and formamide measured in the standard curve obtained in step **5b**. The values can be calculated by isolating the variable x from the previous equation:
$x=x_0-ln\(\(-y+y_0+a)/\(y-y_0))$ ** b
d. TIP:_this step is necessary if the tissue collection and spectrophotometric analysis was performed on various days. If all spectrophotometry measurements were obtained in one session, this step can be skipped._
After obtaining the x value, all data should be normalized for comparison with each of those obtained in different experimental sessions. The data are normalized with respect to the cut off point x_c that is given by assuming that the derivative of the sigmoid curve at $x=x_c$ is equal to 0.0005,
$dy/dx \(x=x_c) = 0.0005$
This very low arbitrary value establishes that at the cut off point, the absorbance curve is saturated \(practically unchanged, as indicated by a derivative close to zero).

Finally, x concentrations are converted into dimensionless magnitudes by transforming them into percentages with respect to x_c

% = x ** 100/x_c

This procedure allows for normalizing the data from different experimental sessions on separate days, which otherwise would lead to non-comparable data.

Timing

Whole protocol duration: 4 days including incubation times Steps 3a-b, 5 min Steps 3c-g, 20 min Steps 3h-l, 20 min Steps 3m-n, 10 min Step 4b, 2 days Step 4c, 15 min Step 4d, 2 days Steps 4e-h, 1 hour These times are approximate as they depend of the number of samples processed.

Troubleshooting

Careful choice of controls is required. Plenty of evidence supports the existence of significant vasodilatation and extravasation on the contralateral side following the induction of unilateral inflammation[11,12] or neuropathy[10]. It is therefore crucial that the nerve injury data is compared to data from sham operated animals. **CRITICAL STEPS** **3e** It is important to use a mild depilatory cream \(for sensitive skin) and not to exceed the time recommended by the manufacturer. **3f** Be careful not to injure or scratch the area, it is preferable to use a wet gauze. **3h** When using mice, albino strains or light-coloured animals are recommended \(if possible) due to easier visualization of the tail vein. **4b** Formamide is light-sensitive **5a** If the samples were collected on different days, it is necessary to perform the standard curve calculations for each day of experiment. The experimental results obtained can be normalized against the standard curve of each experimental session. See **5d**.

Anticipated Results

Table 1 shows a sample of the absorbance values obtained in a real experimental session, and **Figure 2** shows a graph of the standard curve and the experimental curve calculated from the standard curve. We have used the measurement of EB in studies of the effects of removal of dietary polyunsaturated fatty acids in a neuropathic pain model in rats[10]. We have also adapted this method in mice, to show that COT/TPL2 deficient mice show an impaired inflammatory process[9].

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Figures

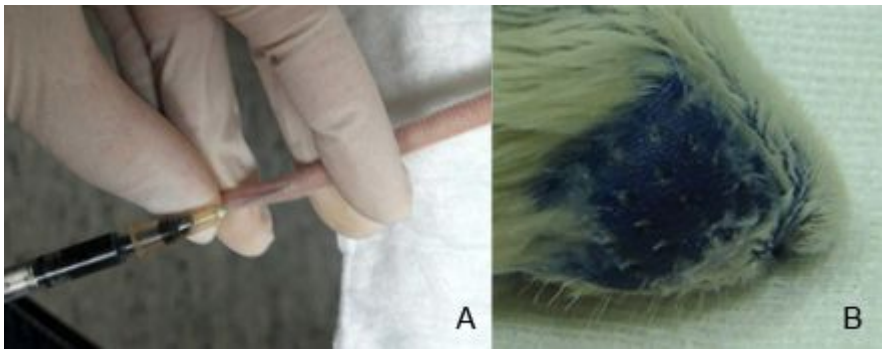

Figure 1

Evans Blue administration (A) EB injection in the lateral vein of the rat. The blue colour of the vein can be observed, giving an indication of a correct injection. (B) EB extravasation after capsaicin application. A strong blue colouring can be observed in the area treated with capsaicin cream whilst the rest of the skin has a light blue shade.

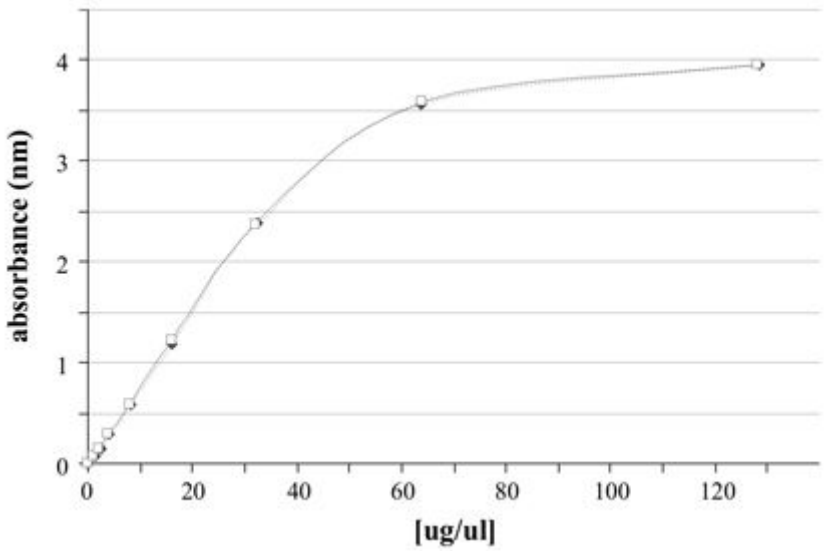

Figure 2

Example of results obtained from the Spectrophotometer Example of an original (grey diamonds) and theoretical curve (white squares) used for calculating the experimental absorbance values fitted to the theoretical standard curve.

Standard Curve	
[µg/µl]	Absorbance (nm)
0	0
1	0.075
2	0.144
4	0.294
8	0.589
16	1.189
32	2.389
64	3.571
128	3.958

Figure 3

Table 1 Example of standard curve values Example of actual absorbance values obtained from solutions of known concentration prepared for the standard curve at a wavelenght of 620 nm. The left column

shows the concentrations of EB used for the generation of the standard curve. The column on the right shows the values of absorbance obtained in the spectrophotometer for each of these concentrations.