


Modeling the Evans Blue Dilution Method for the Measurement of Plasma Volume in Small Animals: A New Optimized Method

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Abstract—The measurement of plasma volume (V_p) in humans and animals is frequently performed by the Evans blue dye dilution method. However, after injection of Evans blue into the circulation, no steady state is observed because of delayed mixing and progressive leakage of dye out of vascular space. Various methods of calculation have been proposed, either with a single blood sampling 5–10 min after dye injection (Single point method), or with extrapolation at time zero of a logarithmic decay (Log linear method). We propose a method based on a two-compartment hypothesis taking into account the initial mixing and the leakage phase in the time course of dye concentration. Nineteen Sprague–Dawley rats were studied in various conditions and blood sampling was performed before and 2, 4 and 6 min after injection of 200 μg Evans blue. A mathematical model was designed to describe the two-compartment hypothesis and allowed the calculation of V_p and K_{out} (rate of disappearance of dye from vascular space). A Bland and Altman representation evidenced an overestimation of V_p with previous methods and the great dispersion of results with the single point method, especially when using the 6 min point. Calculation of K_{out} revealed more accurate with the model than the Log linear method, especially when the mixing rate is slow. We suggest using the two-compartment model to measure V_p with Evans blue technique in rats. This method also allows precise evaluation of the rate of dye leakage, which could be a good marker of vascular permeability to albumin.

Keywords—Plasma volume, Evans blue, Dye dilution technique, Mathematical model, Rat.

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INTRODUCTION

Evans blue dye dilution method has been widely used to measure plasma volume (V_p) in animals^{1–3,9,14,15} and in human beings.^{5,6,10,12,13} It is based on the dye dilution method, allowing the calculation of the volume of liquid (V) in which a dye of given mass (M_i) is injected and, after equilibration, a dye concentration (C) is measured.

$$V = M_i / C. \quad (1)$$

However, this equation supposes that the dye is uniformly distributed in the entire volume to be measured and that there is no escape or leak of the dye from this volume. In the present case, these conditions are not fulfilled since (a) Evans blue, injected in a vessel and sampled in another vessel, takes time to reach a stable blood concentration (mixing time), (b) Evans blue is linked to plasma proteins (mainly albumin) and may leak outside of the vascular bed for various physiological or pathological conditions.^{6,11}

Then, the “single point” method appears to be inaccurate since the concentration of the dye in the blood is not stable, due to the delay of homogenization and escape rate. Therefore, the method has been ameliorated by measuring the dye concentration at several time points after injection and extrapolating the $\log(\text{concentration})$ vs. time curve at time zero to obtain a theoretical dye concentration, to be used for plasma volume calculation according to Eq. (1).⁷ This method supposes that the dye concentration decreases with time following an exponential decay from a single compartment that receive instantly and uniformly at time zero the entire volume of dye. As this condition is not realistic, some

authors discard the first point(s) of concentration to skip the initial phase of diffusion of the dye in the dilution volume.⁵ However, it is not known how many points, therefore how much sampling time, have to be skipped to obtain a linear logarithmic decay.

Several studies have re-evaluated the various methods of V_p measurement with Evans blue in humans.^{5,7} However, the use of dye dilution techniques in small animals adds more constraints since the repeated blood withdrawals may impact total blood volume. Therefore, the number of withdrawals should be optimized and limited to the minimum required to obtain reliable V_p . In practice, most studies in rats have used a single point method with a blood withdrawal at 5–10 min after dye injection.^{1–3,9,14,15}

The present study demonstrates that this single point method gives inaccurate values for V_p and we propose a two-compartment model based method.

MATERIALS AND METHODS

Ethical Approval

Experimental protocols were approved by the Ethics Committee in Animal Experiment Charles Darwin, done in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EU) for animal care, and conducted in accordance with French laws for animal care (reference#11051 201708282253473v 2).

Material

Nineteen Sprague–Dawley rats, aged 11 weeks, were studied after various conditions of normoxia and hypoxia and training status (sedentary and endurance trained). This study was part of a protocol aiming to explore the effect of endurance training on physiological response to chronic (4 weeks) hypobaric hypoxia (barometric pressure = 435 mmHg; simulated altitude: 4460 m). Results of this study will be published elsewhere, the purpose of the present paper being strictly methodological.

Animals were anesthetized with intra-peritoneal pentobarbital injection (60 mg/kg). Food was not available for the animals at least 12 h before the experiment to avoid excessive lipemia and plasma turbidity.⁵ Hematocrit was first measured *via* blood sampling from the tail (70 μ L). Then, a catheter was inserted in the right jugular vein for injection of the dye and another one in the left carotid artery for blood sampling. Separate vessels for injection and sampling were used to avoid contamination of samples with remaining dye.

After initial blood sampling (time zero point), Evans blue (200 mg in 200 μ L of saline) was injected slowly and the catheter was flushed with a measured quantity of saline (usually 350 μ L) to ensure that the entire mass of dye was actually injected. Then at 2, 4 and 6 min after injection (time was carefully noted if different), blood sampling was performed and the volume withdrawn carefully noted (generally between 650 and 750 μ L).

Calibration and Dye Analysis

After centrifugation, dye absorbance was measured in plasma at 620 nm with spectrophotometry. Standard curve, performed in duplicate, was set up with 9 points of diluted Evans blue in saline solution from 1 to 20 mg/L. Dye concentration was obtained from an average of the pure and the twofold diluted plasma samples optical densities. Final concentration of Evans blue in plasma samples was calculated by subtracting absorbance of plasma before injection.

Method of Calculation

We made the hypothesis that the volume of distribution of the dye can be modeled by a double compartment with a transfer of dye from the injection compartment to the distribution compartment and a leak from the distribution compartment as shown in Fig. 1.

The system is then characterized by the following variables:

V_p : volume of distribution (plasma volume)

V_i : volume of liquid injected

M_i : mass of dye injected

C_p : dye concentration in the distribution compartment (plasma)

C_i : dye concentration in the volume of injection

K_{in} : inflow and outflow rate of dye between the injection compartment and the distribution compartment

K_{out} : outflow rate of dye from the distribution compartment (dye leak from plasma)

Our calculations were made following the principle of mass conservation: the total mass of dye injected is equal to the sum of mass in the two compartments plus the mass of dye leaking from the vascular compartment. The second principle used is that the net flux of dye from one compartment to another is proportional to the difference in concentration between the two compartments.

Then, the time course of dye concentration in the infusion and distribution compartments follows two differential equation taking into account the rates of input and output of dye to and from the plasma.

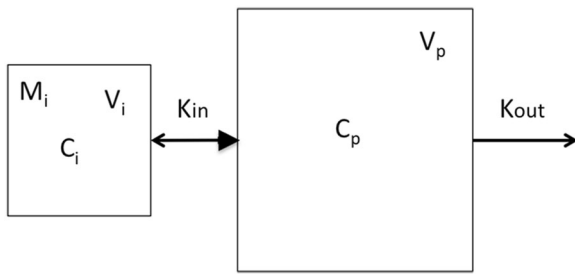


FIGURE 1. Two-compartment model for measurement of plasma volume with Evans Blue dye. Injection compartment: M_i mass of dye injected, V_i volume of liquid injected, C_i dye concentration in injection compartment, C_p dye concentration in the plasma compartment, V_p plasma volume.

$$V_p \cdot \frac{dC_p}{dt} = K_{in} \cdot C_i(t) - K_{in} \cdot C_p(t) - K_{out} \cdot C_p(t) \\ = K_{in} \cdot C_i(t) - (K_{in} + K_{out}) \cdot C_p(t) \quad (2)$$

$$V_i \cdot \frac{dC_i}{dt} = -K_{in} \cdot C_i(t) + K_{in} \cdot C_p(t) \quad (3)$$

A typical time course of dye concentration obtained with this model is shown on Fig. 2 with a normal (a) and a logarithmic (b) scale in ordinates. It clearly shows the limits of the previous methods using either a single point (the dye concentration varies with time) or a linear logarithmic regression (the linearity of the

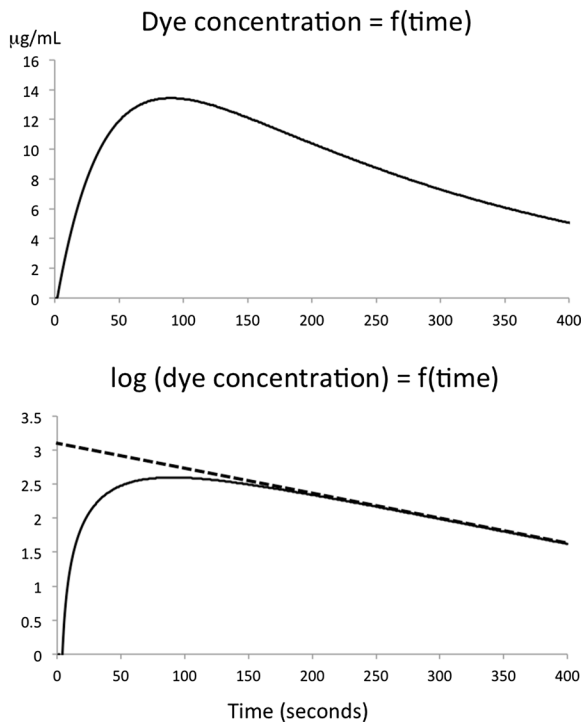


FIGURE 2. Typical time pattern of dye concentration with the two-compartment model (above) and the same time pattern with a log scale for dye concentration (below), with a straight line to extrapolate for dye concentration at time zero.

regression is unrealistic during a certain period of time until mixing is homogeneous).

Using this mathematical model, we used an optimization method to calculate V_p , K_{in} and K_{out} , knowing M_i and the values of C_p from blood sampling at 4 time points (0, 2, 4 and 6 min after injection). A least squares method of optimization was used to find the best values of K_{in} , K_{out} and V_p that would minimize the sum of the differences between square values of C_p measured by blood sampling and values of C_p predicted by the model. Some examples of measured C_p values and corresponding best theoretical Cd curves are shown on Fig. 3.

The efficacy of the calculation (ERR) was evaluated as the sum of the 3 differences (squared values) between model and observed values of C_p at 2, 4 and 6 min, as a percentage of the sum of the 3 observed C_p squared values. The calculation takes into account the volume of blood withdrawn at each time point, the volume of dye solution injected and the volume of saline used to flush the dye after injection.

A Scilab version of the calculation is available in supplemental data.

Comparison with previous methods

We applied to the same data points two previous methods to calculate plasma volume and we compared them to the values obtained with our model method.

“Single point” method: we applied the simple calculation shown in Eq. (1) by using either the 2 min, 4 min or 6 min value of dye concentration.

“Log Linear” method: we plotted the \log_{10} of dye concentration at 2, 4 and 6 min and linearly extrapolated the curve to time zero to obtain a theoretical time zero dye concentration and we then applied Eq. (1) (Fig. 2, lower panel).

Statistics

Methods were compared between each other using a paired student t test. Statistical significance was considered when p value < 0.05 . A Bland and Altman representation was used to compare the previous methods to the model method.

RESULTS

In most cases the adequacy of the model to the observation was very good (with an error ERR of less than 1%), as shown in Fig. 3.

Values of V_p calculated with the 5 distinct methods are shown in Fig. 4. Bland and Altman representations are shown in Fig. 5. There is a systematic significant

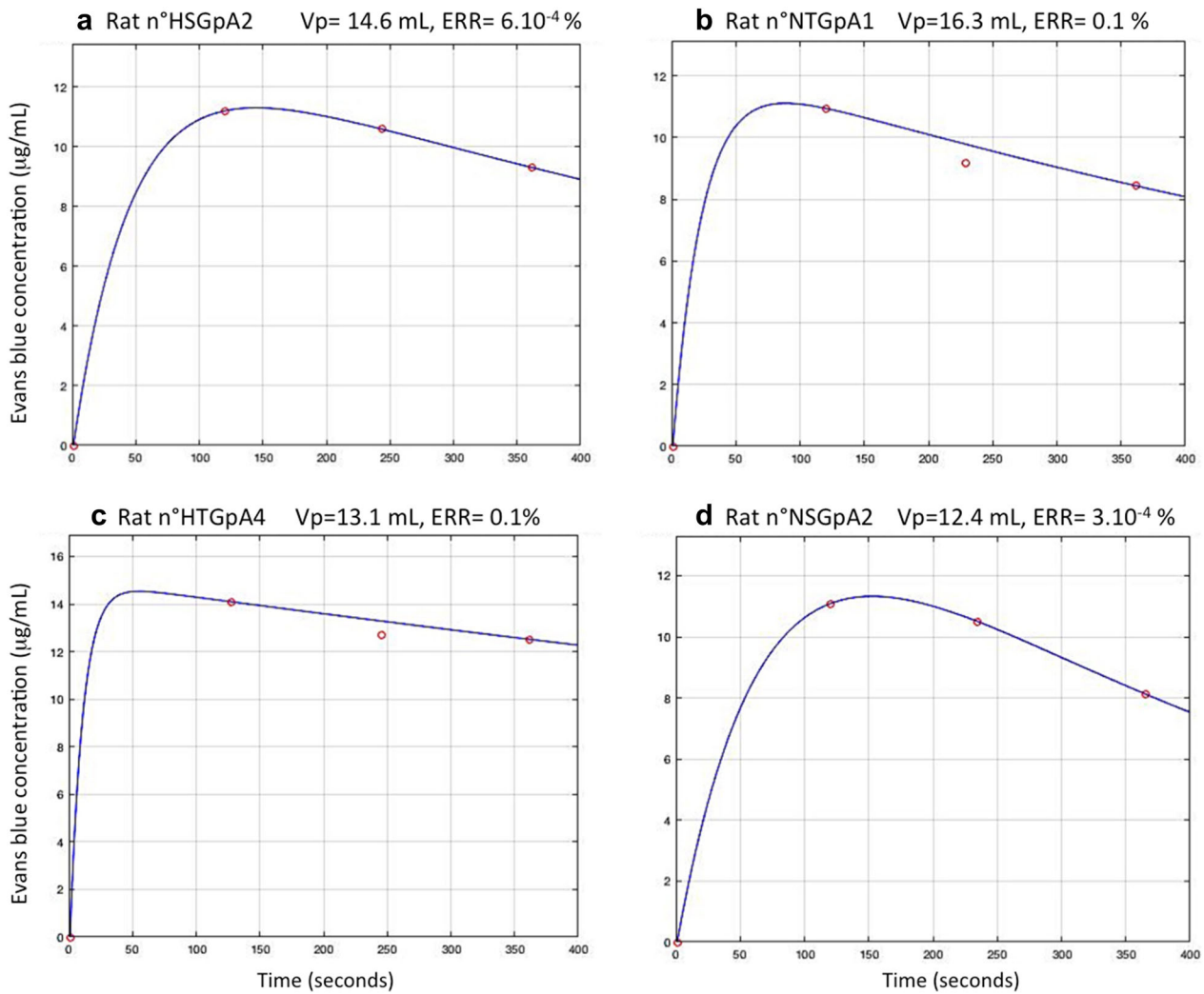


FIGURE 3. Examples of plasma volume (V_p) measurements in four animals, with estimated error (ERR), in various experimental conditions: (a) hypoxia, sedentary; (b) normoxia, trained; (c) hypoxia, trained; (d) normoxia, sedentary.

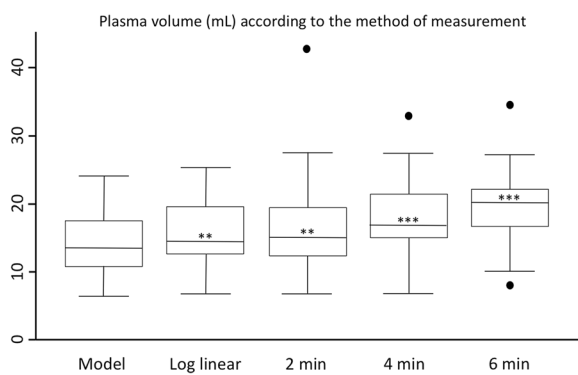


FIGURE 4. Values of plasma volume as a function of method of calculation. Model: two-compartment model; Log linear: extrapolation to time zero of log(dye) concentration as a function of time; 2 min, 4 min, 6 min: « single point method » using the Fick equation for one single time point. Box-and-whisker plot showing the median, upper and lower quartiles and most extreme measurements. ** $p < 0.01$, *** $p < 0.001$ vs. Model.

difference between the previous methods and the model method, with higher values of V_p when calculated with previous methods. The Bland and Altman representation evidences the overestimation of V_p with previous methods and highlights the great dispersion of results with the single point method, especially when using the 6 min point, with an overestimation of around 5 mL for plasma volume. If we consider that a variation of less than 10% is acceptable in the measured value of V_p , we can accept a difference in V_p between our method and the other methods not higher than 2 mL. In all cases, the dispersion seen in the four methods in Fig. 5 shows values of differences higher than 2 mL.

The escape rate of dye from plasma is given by K_{out} and, as calculated by the model method, its value is $6.7 \pm 3.9\%/min$. With the Log linear method, the slope of the log(concentration) vs. time curve is

VP methods of measurement (mL) – Bland & Altman

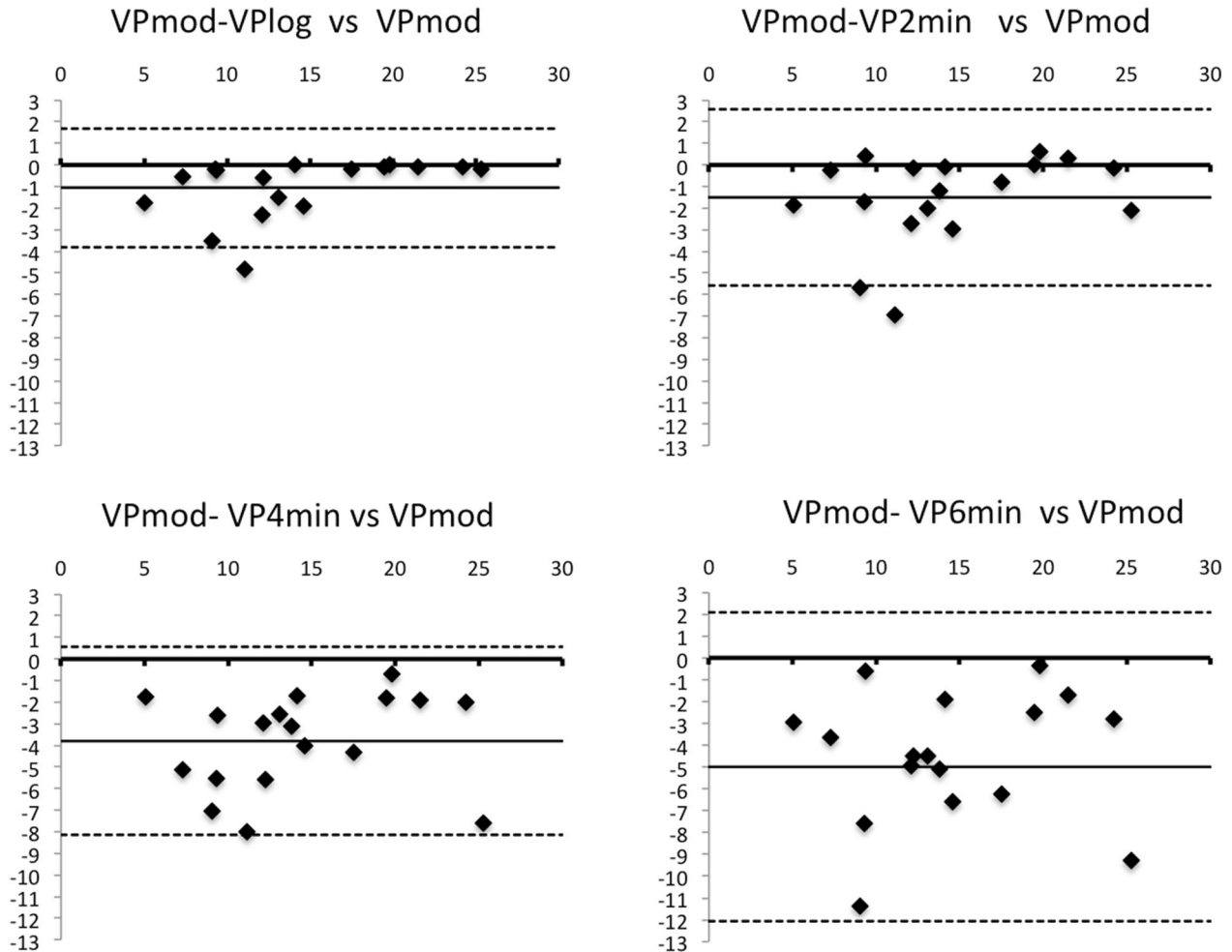


FIGURE 5. Bland and Altman representation for comparison of Log linear (VP_{log}) and single point (VP_{2min} , VP_{4min} , VP_{6min}) methods with the model method (VP_{mod}) for the calculation of plasma volume. All values in mL. Solid line: mean difference between each method and the model method. Dotted lines: mean difference $\pm 1.96 \times SD$.

$5.0 \pm 2.7\%/min$. The difference between methods is significant ($p = 0.004$). A Bland and Altman representation shows a significant discrepancy between the two methods (Fig. 6a). When expressed as a function of K_{in} (as given by the model), the difference of value for K_{out} between the two methods appears high for low values of K_{in} (Fig. 6b), i.e. when the rate of mixing is low so that the maximal dye concentration is reached lately.

DISCUSSION

Although the dilution technique using 125 -iodine labeled human serum albumin has been considered as the reference method to measure plasma volume,⁴ the method using Evans Blue (T-1824) is widely used to

avoid the manipulation of radionuclides. Moreover, it is less expensive and safer for environment and patient's health. The purpose of the present study is therefore, not to compare a new method to the 125 -iodine albumin method but to ameliorate the most commonly used Evans Blue method. We propose a more accurate method of measuring plasma volume using Evans blue dye dilution technique in rats. This method takes into account the initial mixing period of the dye and the escape rate of dye out of the vascular space. Moreover, this method allows the evaluation of the escape rate of the dye out of the vascular space.

The single point method seems particularly inaccurate since the time of sampling will determine the value of concentration used to calculate V_p and this concentration varies with time due to mixing and leak phenomena. This method underestimates the value of

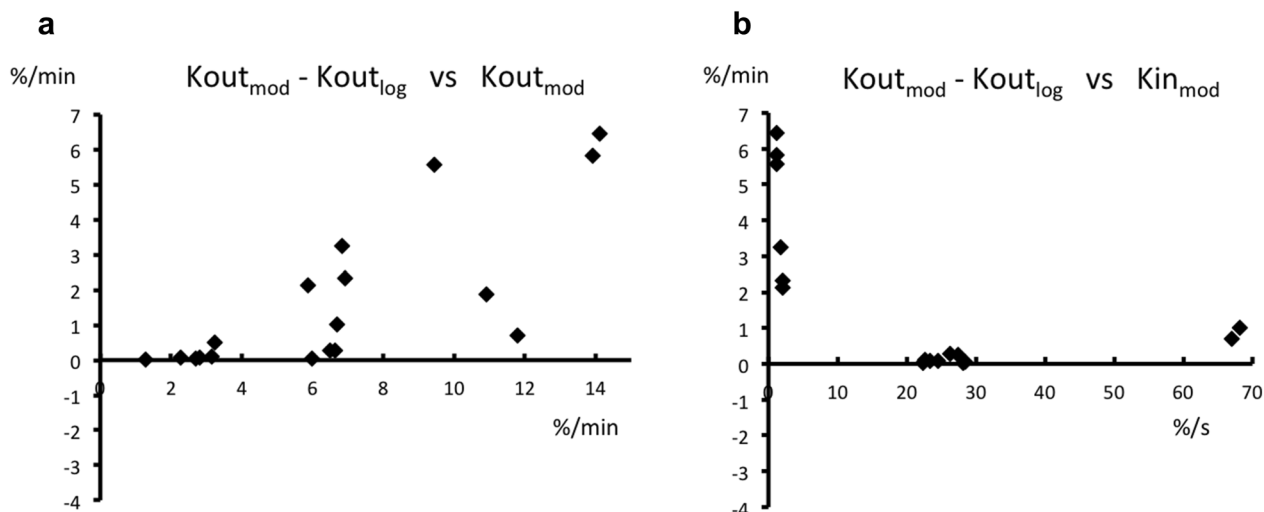


FIGURE 6. (a) Bland and Altman representation for comparison of Log Linear ($K_{out_{log}}$) with model ($K_{out_{mod}}$) methods of calculation for escape rate of dye from plasma (in %/min). (b) Difference between Log linear and model methods for the calculation of K_{out} , as a function of K_{in} . K_{out} = escape rate of dye from plasma compartment (%/min). K_{in} = rate of dye injection (%/s).

dye concentration and therefore overestimates the value of V_p (up to an average of 5 mL with the 6 min point).

The Log linear method gives results closer to our model method. However, since there is an overlap between the initial mixing period and the time window used for the linear extrapolation, there is also a small underestimation of dye concentration and overestimation of V_p (average of 1 mL).

Most authors, including Gibson & Evans in their initial publication,⁶ agree that the method with extrapolation to time zero is more accurate than the single point method.^{5,7} However, values obtained by extrapolation and by a single 10-min point were found similar in humans.⁸

Our method allows the calculation of the escape rate of dye from plasma (K_{out}). As the dye is mainly linked to plasma proteins, this parameter K_{out} may be a good estimation of protein leakage rate from plasma. Evans blue extravasation correlates with vascular leakage of serum albumin, and its leakage into the brain parenchyma indicates blood–brain barrier (BBB) disruption.¹⁶

The present method used to calculate K_{out} is more accurate than the use of the slope of the Log linear relationship, especially when the initial rate of mixing is low, for unknown physiological or pathological reasons such as low local blood flow at the point of injection or low injection rate. Since this method has a general approach for dye dilution techniques, it can be used for other animals or in humans, provided that injected dye mass and times of sampling are adjusted to the material studied. This new method does not add

any complexity to the classical methods and induces no significant extra cost when compared to the single point methods since dye concentration is measured by spectrophotometry. The small Scilab program annexed to the present paper is free for use and very simple to handle.

CONCLUSION

We suggest using a two-compartment model to measure plasma volume with Evans blue dye technique in rats. Four blood withdrawals are necessary (before and 2, 4 and 6 min after dye injection). Precise monitoring of sampling times and volume of liquid injected or withdrawn is necessary. This method also allows the precise evaluation of the rate of dye leakage, which could be a good marker of vascular permeability to albumin. “Single point” methods should not be used since they give inaccurate results.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (<https://doi.org/10.1007/s10439-018-02114-y>) contains supplementary material, which is available to authorized users.

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