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Indocyanine Green (ICG) and Evans Blue:  
Comparative Study of Plasma Volume Measurement

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Running Head: Comparison of ICG and Evans Blue Dilution Techniques

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## Abstract

Plasma volumes (PV) were measured in two groups of rats by injection of ICG (n = 213) or Evans Blue (n = 100) via indwelling jugular cannulae. Both groups had similar ( $P > 0.05$ ) body weights, core temperatures, plasma protein concentrations, and corrected venous hematocrits. Serial blood samples were obtained over 10 min (8 samples, ICG) or 60 min (6 samples, Evans Blue). PV's were assessed directly by dye dilution, and blood (BV) and cell volumes (CV) were calculated. The coefficients of determination ( $r^2$ ,  $\bar{X} \pm SD$ ) of ICG and Evans Blue plasma dye disappearance curves were  $0.995 \pm 0.008$  ( $P < 0.01$ ) and  $0.677 \pm 0.215$  ( $P < 0.01$ ), respectively. The half-lives ( $t_{1/2}$ ) were  $3.1 \pm 0.5$  and  $256 \pm 139$  min ( $P < 0.01$ ) for the ICG and Evans Blue groups, respectively, and corresponding disappearance constants (K, fraction of dye disappearing/min) were  $0.23 \pm 0.04$  and  $0.0034 \pm 0.0017 \text{ min}^{-1}$  ( $P < 0.01$ ). The PV, BV, and CV values (ml,  $\bar{X} \pm SD$ ) for ICG were  $21.7 \pm 4.3$ ,  $36.0 \pm 5.2$ , and  $14.3 \pm 2.0$  and were not significantly different ( $P > 0.05$ ) from corresponding values for Evans Blue. In additional experiments (n = 12), PV was quantitated ( $21.9 \pm 3.2$  ml) by ICG followed by injection of known volumes of 0.9% saline ( $6.1 \pm 0.5$  ml). One h later, the predicted volume ( $28.0 \pm 3.6$  ml) was not significantly different ( $P > 0.05$ ) from the measured volume ( $28.2 \pm 4.1$  ml), and the correlation ( $r = 0.858$ ) was significant ( $P < 0.01$ ). The data indicate that ICG can be used to measure PV in rats and simultaneously assess changes in liver function (i.e. clearance rate). Because of its short  $t_{1/2}$ , PV can be reestimated within an h.

Key Words: Indocyanine green, Evans Blue, Plasma volume, Blood volume, Cell volume, Dye dilution, Rattus norvegicus.

### Introduction

Indocyanine green (ICG) has been used successfully to measure cardiac output (6,7,14,15), hepatic blood flow (1,3,13,18,19,20,23), and liver function (3,4,23). Bradley and Barr (2) reported a significant correlation between the ICG dilution and chromium<sup>51</sup> methods of blood volume measurement in human subjects. Ketterer et al. (13) found that volumes of distribution measured with ICG were not different ( $P > 0.05$ ) from plasma volumes measured with Evans Blue in dogs. Also, Caesar et al. (3) indicated that plasma volumes measured in human subjects with ICG were similar to those reported in other studies (4,8) using different methods.

ICG has a low toxicity (3,16,22), is rapidly distributed in the blood stream, (3,16,22), is essentially completely cleared via the liver (3,16,22), and has a short half-life (3,16,17,18,19,22,23). Therefore, in view of reported uses and properties, it seemed potentially useful for the rapid measurement and re-measurement of plasma volume in rats and, at the same time, survey any potential changes in hepatic clearance (function). This paper compares plasma, blood, and cell volumes in rats measured by ICG dilution and the classical Evans Blue method (5).

## Methods

### A. Preparation of Stock Dye.

Indocyanine green (ICG, 50 mg, Hynson, Westcott and Dunning, Inc.) was reconstituted by adding 12.5 ml of sterile, distilled water and 12.5 ml of 10% (W/V) bovine serum albumin (BSA, No. A-8022, Cohn Fraction V, Sigma Corp.) containing 280 mEq/L sodium and 10 mEq/L potassium.

Evans Blue (J.T. Baker Chemical Co.) was prepared by adding 33 mg of dye to 5 ml of sterile, distilled water and 5 ml of 10% BSA with electrolytes. Dye stocks were refrigerated at 5°C in foil wrapped flasks.

### B. Experimental Animals.

Male Sprague-Dawley rats (Charles River CD strain) were caged individually in an environmental chamber (13 x 11 x 6 ft) maintained at 26°C and 49 ± 17% relative humidity. The air was replaced at a rate equivalent to 1.4 room vol./h. Fluorescent lighting was controlled automatically for a 0600-1800 h light cycle. All animals (ICG group, n = 213; Evans Blue group, n = 100) were fed a diet of Charles River chow and water ad libitum. They were fasted 24 h before use to minimize and subtle effects of hormonal or nutritional status on plasma volume. Experiments were begun each day at 0800 h.

### C. Temperature Measurement.

Core temperatures (rectal probe inserted 6.5 cm) were measured using interchangeable thermister probes (YS 1700 Series, C-8415-21, Cole-Parmer Instrument Co.) and a digital thermometer (581 C Digital Thermometer, Digitec, United Systems Corp.).

### D. Surgical Procedure.

All animals were cannulated via the right external jugular vein according to a method developed in this laboratory (12).

### E. Blood Sampling.

All sampling syringes were prerinsed in 5% BSA to minimize dye adsorption. Stock dye (1.0 ml) was injected at room temperature, and  $T = 0$  was the midpoint of infusion (2 sec). The cannula was immediately rinsed with 0.1 ml of 0.9% saline. ICG blood samples were drawn at 1, 2, 3, 5, 6, 7, 8 and 10 min and those for Evans Blue at 10 min intervals for 1 h. Hematocrits (HCT) were corrected (0.96) for "plasma trapping" (9), and heparinized plasma was prepared by centrifugation at  $8,000 \times g$  for 10 min. Plasma protein concentrations were read from a refractometer.

### F. Calculations and Statistics.

Plasma dye concentrations ( $\mu\text{g/ml}$ ) were read against the plasma blank using a Cary 15 spectrophotometer at a  $\lambda_{\text{max}} = 805 \text{ m}\mu$  for ICG and  $618 \text{ m}\mu$  for Evans Blue. Optical density units were converted to plasma concentration by reference to standard curves of dye in plasma constructed with each dye lot and found to be linear in the concentration range used in this study (ICG =  $0-4 \mu\text{g/ml}$ ; Evans Blue =  $0-50 \mu\text{g/ml}$ ). The formulas used in the calculations were:

$$\text{Plasma volume (ml)} = \text{Dye injected } (\mu\text{g}) - \text{Conc. at } T = 0 (\mu\text{g/ml}) \quad (1,19,22)$$

$$\text{Blood volume (ml)} = \text{Plasma volume (ml)} - 1 - (0.96 \text{ HCT} - 100) \quad (1,9)$$

$$\text{Cell volume (ml)} = \text{Blood volume (ml)} \times (0.96 \text{ HCT} - 100) \quad (9)$$

$$\text{Half-life (min)} = (\log_{10} \text{ y-intercept}) - 2 - (\log_{10} \text{ y-intercept}) - \text{slope} \quad (19,22)$$

$$\text{Disappearance constant (min}^{-1}\text{)} = \ln 2 / t_{1/2} \quad (3)$$

$$\text{Rate of Removal (\%/min)} = (2.303) \times (-100) \times (\text{slope})$$

The two-sample t-test was used to determine statistical significance, and differences between means resulting in  $p < 0.05$  were considered significant (21).

## Results

For the ICG group, the mean body weight was  $501 \pm 27$  g ( $\bar{X} \pm SD$ ), the corrected venous hematocrit  $40.1 \pm 4.8\%$ , core temperature  $38.6 \pm 0.6^{\circ}\text{C}$ , and plasma protein concentration  $7.5 \pm 0.5$  g/100 ml, and these values were not significantly different ( $P > 0.05$ ) from those of the Evans Blue group. Both groups also had similar ( $P > 0.05$ ) plasma, blood, and cell volumes (Table 1). However, the half-lives ( $t_{1/2}$ ), disappearance constants (K), and rates of removal between groups were significantly different ( $P < 0.05$ ).

To test the ICG dilution technique, follow-up experiments were conducted in which plasma volume was measured by ICG dilution before (B) and after (A) intravenous injection of a known volume of 0.9% saline (Table 2). The time between the first and second plasma volume measurement was 1 h, and 2 min were allowed for saline mixing after injection. As shown in Table 2, the beginning plasma volume (ICG  $PV_B$ )  $21.9 \pm 3.2$  ml was expanded to (ICG  $PV_A$ , measured)  $28.2 \pm 4.1$  ml by injection of  $6.1 \pm 0.5$  ml of saline. The "predicted" value ( $28.0 \pm 3.6$  ml) was not significantly different ( $P > 0.05$ ) from the "measured" value ( $28.2 \pm 4.1$  ml), and the correlation ( $r = 0.858$ ) was significant ( $P < 0.01$ ). The average percentage difference ( $\% \Delta$ ) between "predicted" and "measured"  $PV_A$  was  $-0.7 \pm 2.1\%$  ( $\bar{X} \pm SEM$ ).

ICG half-lives ( $t_{1/2}$ , min) before ( $3.0 \pm 0.1$ ) and after ( $2.9 \pm 0.1$ ) saline injection were significantly different ( $P < 0.05$ ), and the average percentage difference ( $\% \Delta$ ) was  $-3.5 \pm 1.2\%$  ( $\bar{X} \pm SEM$ ).

### Discussion

ICG has been used successfully to measure cardiac output (6,7,14,15), hepatic blood flow (1,3,13,18,19,20,23), and liver function (3,4,23). Also, plasma volumes estimated by ICG dilution (3,4,13,23) check well with values obtained by other methods in human subjects (3,4,8,23) and dogs (13). Coupled with the properties of low toxicity and rapid distribution into the circulatory system (3,16,22), and short  $t_{1/2}$  (3,16,17,18,19,22,23), ICG seemed suited to remeasure plasma volumes and simultaneously evaluate changes in liver function in rats following experimental intervention.

In this study, identical plasma volumes/kg body weight ( $43.4 \pm 6.9$  ml/kg,  $\bar{X} \pm SD$ ) were obtained for both techniques. These values agree well with  $40.3 \pm 5.8$  ml/kg and  $41.1 \pm 5.6$  ml/kg in human subjects reported by Caesar et al. (3) and Gray and Frank (8), respectively. Half-lives, disappearance constants, and rates of removal of the two groups (Table 1) were significantly different ( $P < 0.05$ ). The  $t_{1/2}$  of ICG ( $3.1 \pm 0.5$  min) agrees with reported values in human subjects (17,18,19,23), as does the Evans Blue  $T_{1/2}$  (18,19). Also, the ICG disappearance rate ( $0.23 \pm 0.04$   $\text{min}^{-1}$ ) and rate of removal ( $22.2 \pm 5.2$  %/min) check well with values previously reported for human subjects (3,18).

To test further the ICG dilution method, additional experiments were conducted. The data indicate that ICG can be used to reestimate changes in plasma volume in rats following experimental intervention after a short period of time. The "predicted" plasma volume (Table 2) was similar ( $P > 0.05$ ) to plasma volumes measured after saline injection, and the correlation ( $r = 0.858$ ) was significant ( $P < 0.01$ ). The ICG  $t_{1/2}$ 's before and after saline injection were different ( $P < 0.05$ ). However, the significance of this is questionable since the

coefficient of variability of each mean was approximately 3%. Also, the average percentage difference between half-lives was  $-3.5 \pm 1.2\%$ . Certainly, a 3% change does not represent a precipitous alteration in hepatic function, especially when compared to the approximately 240% reported by Wiegand et al. (23), 200-600% (18) or 300-400% reported by Rowell et al. (19) or 170% reported by Ritz et al. (17).

It has been concluded that ICG can be used to measure plasma, blood, and cell volumes reliably in the rat model and simultaneously assess changes in liver function (clearance rate). Because of ICG's short  $t_{1/2}$ , PV can be reestimated within an h.

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The views, opinions, and findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other official documentation.

In conducting the research described in this report, the investigators adhered to the 'Guide for Laboratory Animal Facilities and Care,' as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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Table 1

Comparison of plasma, blood, and cell volumes and half-lives, disappearance constants, and rates of removal of ICG and Evans Blue groups.

|   | <u>ICG (n = 213)<sup>1</sup></u> | <u>Evans Blue (n = 100)<sup>1</sup></u> |
|---|----------------------------------|---|
| Plasma volume (ml)                          | 21.7 ± 4.3                       | 21.3 ± 3.5                              |
| Blood volume (ml)                           | 36.0 ± 5.2                       | 36.3 ± 5.9                              |
| Cell volume (ml)                            | 14.3 ± 2.0                       | 14.9 ± 3.0                              |
| Half-life (min)                             | 3.1 ± 0.5 <sup>+</sup>           | 256 ± 139                               |
| Disappearance constant (min <sup>-1</sup> ) | 0.23 ± 0.04 <sup>+</sup>         | 0.0034 ± 0.0017                         |
| Rate of removal (%/min)                     | 22.2 ± 5.2 <sup>+</sup>          | 0.3 ± 0.2                               |

<sup>1</sup> Values are  $\bar{x} \pm$  SD.

<sup>+</sup> P < 0.05 between corresponding  $\bar{x}$ 's ± SD's.

Table 2

Test of ICG dilution method by intravenous injection  
of known volume of 0.9% saline.

| ICG PV <sub>B</sub> (ml) <sup>1</sup> | PV <sub>A</sub><br>(Predicted,ml) | PV <sub>A</sub><br>(Measured,ml) <sup>2</sup> | %Δ<br>(Average percentage<br>difference) |
|---------------------------------------|-----------------------------------|---|--|
| 20.7                                  | 26.4                              | 26.3  | 0.4                                      |
| 23.8                                  | 30.4                              | 32.4  | -6.6                                     |
| 25.1                                  | 31.9                              | 34.2  | -7.2                                     |
| 18.1                                  | 23.4                              | 25.2  | -7.7                                     |
| 24.5                                  | 31.2                              | 27.8  | 10.9                                     |
| 22.3                                  | 28.6                              | 26.8  | 6.3                                      |
| 22.7                                  | 28.8                              | 27.1  | 5.9                                      |
| 21.4                                  | 27.5                              | 30.7  | -11.6                                    |
| 20.6                                  | 26.8                              | 28.9  | -7.8                                     |
| 27.9                                  | 34.0                              | 33.8  | 0.6                                      |
| 19.3                                  | 25.5                              | 25.5  | 0.0                                      |
| <u>16.3</u>                           | <u>21.6</u>                       | <u>19.9</u>                                   | <u>7.9</u>                               |
| 21.9 ± 3.2 <sup>3</sup>               | 28.0 ± 3.6 <sup>3*</sup>          | 28.2 ± 4.1 <sup>3</sup>                       | -0.7 ± 2.1 <sup>+</sup>                  |

<sup>1</sup> Measured PV before (B) experimental treatment.

<sup>2</sup> Measured PV after (A) experimental treatment.

<sup>3</sup>  $\bar{x} \pm SD$ .

\* Not significant (Predicted vs. Measured).

<sup>+</sup>  $\bar{x} \pm SEM$ .

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( $r^2$ ,  $\bar{x} \pm SD$ ) of ICG and Evans Blue plasma dye disappearance curves were  $0.995 \pm 0.008$  ( $p < 0.01$ ) and  $0.677 \pm 0.215$  ( $p < 0.01$ ), respectively. The half-lives ( $t_{1/2}$ ) were  $3.1 \pm 0.5$  and  $256 \pm 139$  min ( $p < 0.01$ ) for the ICG and Evans Blue groups, respectively, and corresponding disappearance constants ( $K_1$ , fraction of dye disappearing/min) were  $0.23 \pm 0.04$  and  $0.0034 \pm 0.0017 \text{ min}^{-1}$  ( $p < 0.01$ ). The PV, BV, and CV values (ml,  $\bar{x} \pm SD$ ) for ICG were  $21.7 \pm 4.3$ ,  $36.0 \pm 5.2$ , and  $14.3 \pm 2.0$  and were not significantly different ( $p > 0.05$ ) from corresponding values for Evans Blue. In additional experiments ( $n = 12$ ), PV was quantitated ( $21.9 \pm 3.2$  ml) by ICG followed by injection of known volumes of 0.9% saline ( $6.1 \pm 0.5$  ml). One h later, the predicted volume ( $28.0 \pm 3.6$  ml) was not significantly different ( $p > 0.05$ ) from the measured volume ( $28.2 \pm 4.1$  ml), and the correlation ( $r = 0.858$ ) was significant ( $p < 0.01$ ). The data indicate that ICG can be used to measure PV in rats and simultaneously assess changes in liver function (i.e. clearance rate). Because of its short  $t_{1/2}$ , PV can be reestimated within an h.

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