Technical and physiological background of plasma volume measurement with indocyanine green: a clarification of misunderstandings

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Submitted 3 July 2006; accepted in final form 10 November 2006

Jacob M, Conzen P, Finsterer U, Krafft A, Becker BF, Rehm M. Technical and physiological background of plasma volume measurement with indocyanine green: a clarification of misunderstandings. J Appl Physiol 102: 1235–1242, 2007. First published November 16, 2006; doi:10.1152/japplphysiol.00740.2006.—The indocyanine green (ICG) dilution technique (DT) is frequently used for plasma volume (PV) measurement. However, because of inadequate knowledge about the properties of this dye, lack of accuracy has been attributed to the method. The aim of this report is to provide physiological background information about the ICG-DT to avoid some profound misunderstandings. When performing tracer dilution, one has to consider the tracer’s distribution space before interpreting the result. For ICG, the distribution space is the total PV, i.e., circulating + noncirculating PV, fixed within the endothelial glycocalyx. The distribution space of red blood cells and large molecules, in contrast, is only the circulating part of PV. Therefore, it is erroneous to compare directly PV derived from different tracer dilution methods. The transcapillary escape rate of ICG should not significantly influence measured PV if the method is performed properly, i.e., if a short time window of measurement is subjected to monoexponential extrapolation. A major problem of PV measurement in general is that the target of PV cannot reliably be determined simply from ICG dilution. After this fact was recognized, ICG was frequently used as a tracer for PV measurements (14, 15, 22, 39–42).

Tracer dilution techniques for measuring PV are generally based on intravascular injection of a bolus of the tracer followed by measurement of the concentration in the patient’s plasma after complete mixture (Fick’s principle). The distribution space can be calculated from this concentration and the amount of dye injected, and the investigator normally assumes that this space represents PV. However, several reports questioned the accuracy of the ICG-DT for several reasons (20, 23, 29). This report aims to summarize the knowledge about the ICG-DT, including the physiological background, to forestall profound misunderstandings of a well-established method for direct measurement of total PV.

TRACER

ICG, a water-soluble and relatively unstable 755-kDa tricarbocyanine dye, rapidly binds to plasma proteins, mainly albumin, after injection. Complete mixture with the plasma can normally be expected 2 min after venous application (14, 15, 22, 39–42, 47, 48). It is rapidly and exclusively eliminated via the liver, by secretion into the bile, and $t_{1/2}$ is only 2.5–3 min. ICG was found to be a safe, accurate, and precise tracer for PV measurements (14, 39, 47). Bound to plasma proteins, the peak extinction of ICG lies at 800 nm (Fig. 1). This wavelength also represents the “isobestic” point of hemoglobin, i.e., the wavelength at which the extinction of the oxygenated form equals that of the deoxygenated one. At 900 nm, ICG has no measurable extinction, in contrast to hemoglobin and other constituents of the plasma.

PRINCIPLE

Determining the concentration of any tracer before complete mixture with the plasma is pointless. However, the sought-after
concentration at injection time \((t_0)\), but after complete mixture of the dye, never really exists. It is a theoretical value due to the overlapping processes of elimination and mixing, i.e., the time window required to allow complete mixture of the dye with the patient’s plasma. To obtain the theoretical concentration at \(t_0\), one needs to determine the kinetics of early dye clearance. For this, it is necessary 1) to take several samples at reproducible times after complete mixture, 2) to measure the ICG concentrations in these samples, and 3) to extrapolate back to the theoretical concentration at \(t_0\).

EXTRAPOLATION TECHNIQUES AND TIME WINDOWS

At least during the first 5 min after recirculation, ICG elimination seems to consistently follow a monoeXponential kinetic \((48)\). However, beyond minute 5, the elimination kinetic inconstantly changes toward a biexponential, or even polyexponential, mode \((24, 45, 48)\). To avoid overestimation of the distribution space of ICG \((\text{DS}_{\text{ICG}})\), the samples used for monoeXponential extrapolation should only be taken up to minute 5. Otherwise, a “curve-fitting technique” is needed for computation of the theoretical concentration at \(t_0\) \((48)\) from a biexponential elimination kinetic. For clinical use, the monoeXponential extrapolation from values obtained during the first 5 min appears to be the better choice for several reasons: 1) it requires a shorter period of steady state of BV (important during surgery), 2) it provides PV almost “online” (see Whole blood method), 3) the investigator is not obliged to perform “black box” processing of the data, 4) immediate reinfusion prevents net blood loss (see Whole blood method), and 5) lower ICG doses \((0.25 \text{ vs. } 1 \text{ mg ICG per kilogram body wt})\) are required, accelerating recirculation.

DETERMINING ICG CONCENTRATION

ICG concentration is determined by measuring the extinction specifically caused by ICG. Therefore, it is necessary to subtract the basal extinction at 900 nm (no ICG extinction) from the extinction at 800 nm (peak ICG extinction) in each sample \((\text{Fig. 1})\). In venous blood, the oxygenation of hemoglobin is not constant; therefore, for measurement of the specific extinction of ICG in whole blood samples, we need arterial, fully \(O_2\)-saturated blood to obtain a constant basal value at 900 nm. Before determination of unknown ICG concentrations, the system must be calibrated by measurement of two samples containing known ICG concentrations. This leads to the calibration equation \((\text{Fig. 2})\). Because ICG does not enter red blood cells but, rather, rapidly binds to plasma proteins after injection, we have to consider this only partial “dilution” of the dye in a whole blood sample (see Whole blood method) when calculating \(\text{DS}_{\text{ICG}}\).

**Plasma method.** For the plasma method \((14, 15, 20, 29)\), only one single venous cannula, inserted into a cubital vein, is needed. After calibration of the system (measurement of the specific extinction in two 5-ml samples of the patient’s plasma containing ICG at 2 artificially generated concentrations), the dye \((0.25 \text{ mg ICG/kg body wt})\) is injected into the patient’s circulation at \(t_0\). To prevent contamination of the venous catheter [mainly, the cone during connection and disconnection of the dye syringe \((\text{Fig. 3})\)] and, consequently, contamination of the future blood samples with dye, even though the catheter is flushed with saline after injection, we use a second three-way valve \((\text{Fig. 4})\). This valve is removed immediately after the dye is injected and the system is flushed, i.e., before the first timed sample is withdrawn.

Starting exactly 2 min after \(t_0\), blood samples \((2.5–3 \text{ ml each, taking } \sim 3 \text{ s})\) are withdrawn every \(20 \text{ s}\), up to the end of minute 5 \((10 \text{ samples in all})\), with each withdrawal starting \(\sim 1.5 \text{ s}\) before and ending \(\sim 1.5 \text{ s}\) after the intended point of time. The catheter is not flushed between the two samples; rather, a few drops of blood are allowed to drip out immediately before the next sample is withdrawn \((\text{Fig. 5})\).

After separation of the red blood cells from the plasma by centrifugation, the specific extinction is determined in every sample. Specific extinction at \(t_0\) is derived by monoeXponential extrapolation of the light absorption curve back to \(t_0\) \((\text{Fig. 6})\).
This absorption value is incorporated into the calibration equation and leads to the theoretical plasma concentration of the dye at $t_0$ ($CP_0$). $DS_{ICG}$ can then be calculated as follows: $DS_{ICG} = D \times CP_0^{-1}$, where $D$ is the amount of dye injected.

Menth-Meier et al. (28) demonstrated that it is appropriate to perform peripheral dye injection for PV measurement by ICG-DT. However, others advised induction of hyperemia before dye injection to accelerate recirculation (14, 28). Additionally, in preliminary, unpublished experiments ($n = 10$), we established that injection into one peripheral catheter and withdrawal of the timed samples from this and, simultaneously, an additional catheter on the other arm led to the same PV difference = $11 \pm 17$ (SD) ml ($P > 0.05$, Student’s paired t-test).

The “plasma method” is a scientific method that can be performed without causing discomfort to an awake volunteer. Because the samples must be processed as described above, it delivers $DS_{ICG}$ at $\sim 30$ min after dye injection. Because $\sim 40$ ml of blood loss occurs per measurement, repeated measurements should be avoided to prevent our target, $DS_{ICG}$, from being relevantly influenced by the method.

Whole blood method. In addition to a (central) venous cannula for tracer injection, an arterial line is needed for the whole blood method (22, 39 – 42). As explained above, at 800
nm, the oxygenation of hemoglobin is not relevant: the extinction is the same (isobestic point of hemoglobin). In contrast, extinction at 900 nm, leading to the basal value (see above), must be obtained with equally oxygenated hemoglobin in all samples.

As in the plasma method, immediately before the injection of the tracer, a two-point calibration, consisting of measurement of the specific extinction of two arterial blood samples containing two known, but distinct, ICG concentrations (Fig. 2), must be performed. Then the tracer (0.25 mg ICG/kg body wt) is injected into the (central) venous cannula at \( t_0 \). For measurement of ICG concentration, blood is continuously drawn via the arterial catheter through a cuvette by means of a calibrated pump (20 ml/min). The blood is slightly heparinized and immediately reinfused. The cuvette is attached to the spectrophotometer, and as many timed measurements as possible are taken. We prefer taking measurements every 10 s between minutes 2 and 5 (22, 40–42).

The specific extinction at \( t_0 \) is derived by monoexponential extrapolation of the light absorption curve resulting from the respective measuring time points (Fig. 6). If this absorption value is introduced into the calibration equation, the theoretical whole blood concentration of the dye at \( t_0 \) (\( \text{CB}_0 \)) is obtained. \( \text{CP}_0 \) is calculated as follows: \( \text{CP}_0 = \text{CB}_0 \times (1 - \text{Hct}_{LV})^{-1} \), where \( \text{Hct}_{LV} \) represents the large vessel hematocrit determined in blood samples withdrawn from the arterial line. \( \text{DS}_{\text{ICG}} \) is then calculated as follows: \( \text{DS}_{\text{ICG}} = D \times \text{CP}_0^{-1} \).

The whole blood method is much more convenient for the investigator than the plasma method and provides the plasma volume online, which can be important in the perioperative setting. Additionally, because of reinfusion of the samples, this method saves blood and, therefore, is ideal for intraoperative repeated measurements without impact on the target.

FROM \( \text{DS}_{\text{ICG}} \) TO PV: PHYSIOLOGICAL BACKGROUND OF A TECHNICAL METHOD

As explained above, when the ICG-DT is correctly performed, the result is \( \text{DS}_{\text{ICG}} \), nothing else. The point is to correctly interpret this value to establish the “real” PV, and this is obviously a serious problem for some investigators (20, 23, 29): PV derived via ICG-DT was, e.g., “checked for correctness” by calculating RCV from PV and \( \text{Hct}_{LV} \) and expecting this RCV derived from ICG (RCV\(_{\text{ICG}}\)) to be constant in the absence of any bleeding or transfusion if the ICG-DT was consistent. In other cases, PV derived from ICG-DT (PV\(_{\text{ICG}}\)) was expected, as a proof for consistency of the method, to be constant before and after an intervention (e.g., induction of general anesthesia or volume loading). Such an assumption is invalid, however (see below). The transcapillary escape rate of albumin was also blamed to relevantly influence the results. However, central physiological properties of the microvasculature must be taken into consideration.

The \( f_{\text{cell}} \) phenomenon. There is an important, but inconstant, difference between \( \text{Hct}_{LV} \) and whole body hematocrit (\( \text{Hct}_{WB} \)), derived from measuring PV with the ICG-DT and RCV with fluorescein-labeled red blood cells. The quotient \( \text{Hct}_{WB} \times \text{Hct}_{LV}^{-1} \), occasionally termed the “f\(_{\text{cell}}\) ratio” in the literature, ranges from 0.73 to 1.10 (44). In some clinical trials, the \( f_{\text{cell}} \) ratio was assumed to be 0.89–0.91 and was used as a correction factor to derive PV from BV or RCV and \( \text{Hct}_{LV} \) (5). However, the \( f_{\text{cell}} \) ratio is not constant during many clinical interventions, e.g., volume loading (22, 40). Therefore, use of no factor or the same correction factor before and after such an intervention can lead to erroneous calculations of BV. This is illustrated in Table 1 by the “RCV\(_{\text{ICG}}\),” calculated without correction for \( f_{\text{cell}} \) before and after volume loading. The calculation is based on \( \text{Hct}_{LV} \) and PV obtained using the ICG-DT (PV\(_{\text{ICG}}\)). As readily apparent, the reason for a seeming “decrease” in RCV\(_{\text{ICG}}\) without any actual loss of red blood cells is an impressive increase in the \( f_{\text{cell}} \) ratio. Consequently, it would be erroneous to equate this apparent, not directly measured, RCV\(_{\text{ICG}}\) value with the real RCV derived from dilution of fluorescein-labeled red blood cells (RCV\(_{\text{FI}}\)) (33). As a result, the premise that RCV\(_{\text{ICG}}\) must remain constant if red blood cells are not removed or lost (29) is not justified. Whether an intervention such as induction of general anesthesia, which is connected with vasodilatation and other hemodynamic responses, can also lead to a change in \( f_{\text{cell}} \) ratio is not known. The \( f_{\text{cell}} \) ratio does not seem to be systematically affected by sex, age, or systemic diseases. Corresponding work on animals (1, 27) and healthy (30, 46) and diseased humans (11, 52)
Report

PLASMA VOLUME MEASUREMENT WITH INDOCYANINE GREEN

Table 1. Measured and calculated PV and RCV before and after volume loading with hetastarch

<table>
<thead>
<tr>
<th></th>
<th>Before Volume Loading</th>
<th>After Volume Loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVICG, ml</td>
<td>2.984±685</td>
<td>3.581±706</td>
</tr>
<tr>
<td>RCVFl, ml</td>
<td>1.231±171</td>
<td>1.238±155</td>
</tr>
<tr>
<td>HctWB</td>
<td>29.7±5.1</td>
<td>26.1±4.6</td>
</tr>
<tr>
<td>HctLV</td>
<td>35.3±4.5</td>
<td>27.3±5.7</td>
</tr>
<tr>
<td>fcell ratio</td>
<td>0.840±0.078</td>
<td>0.953±0.067</td>
</tr>
<tr>
<td>RCVICG, ml</td>
<td>1.628</td>
<td>1.345</td>
</tr>
<tr>
<td>PVcire, ml</td>
<td>2.256</td>
<td>3.297</td>
</tr>
<tr>
<td>ESL, ml</td>
<td>728</td>
<td>284</td>
</tr>
</tbody>
</table>

Measured values [plasma volume (PV) measured by indocyanine green dilution technique (PVICG), red cell volume (RCV) measured by fluorescein labeled red blood cells (RCVFl), whole body and large vessel hematocrit (HctWB and HctLV), and f cell ratio (HctWB/HctLV)] are means ± SD (n = 10); data are from Ref. 40. RCVICG represents RCV calculated from PVICG and HctLV; PVcirc is circulating part of PV derived from RCVFl and HctLV, and ESL is calculated volume of endothelial surface layer (ESL = PVICG − PVcire).

confirmed only the well-known high variability mentioned above.

What can be the reason for this variability in fcell, especially during interventions? A careful look at the individual distribution space of the different tracers used to determine PV offers a likely explanation. Recent intravital microscopic images revealed that there are actually three compartments of BV: 1) RCV, 2) a circulating part of PV (PVcire), and 3) a noncirculating part of PV, which is bound by the endothelial glycocalyx (EG) (54). The first images of the EG showed only a thin (20–30 nm) layer, because conventional fixation techniques almost completely destroy the endothelial surface layer (ESL) before electron microscopy. In the past 15 years, however, indirect and direct approaches demonstrated that, at least in the small vessels, there is a 0.4- to 1.2-μm-thick ESL containing noncirculating plasma, including plasma proteins (Fig. 7). This exclusion zone for flowing red blood cells is in dynamic equilibrium with the flowing plasma (37). Investigations using fluorescence videomicroscopy of skin capillaries showed that ICG enters the ESL within 1 min (2, 4).

Using the red blood cells as a tracer to quantify the circulating part of the plasma, we proceeded to compare PVICG with PVcire, as derived from RCVFl and HctLV. Because PVICG represents the whole body PV, the difference was presumed to represent the volume of the whole ESL of the circulatory system. The total ESL determined in such a manner was 732, 717, and 431 ml in three separate experiments (22, 40). These findings were occasionally criticized as being unrealistic, since only 10% of whole body blood is found in systemic capillaries (23, 29). However, the ESL should not be restricted to capillaries. Although it was demonstrated in small vessels that this layer leads to a considerable decrease in hematocrit with respect to HctLV (37, 54), larger blood vessels are also lined by an ESL, possibly with an even larger dimension. If it is assumed that ~350 m² is the total area of the endothelial interface with blood (37) and ~1 μm is the average thickness of the ESL (17, 54), a total ESL volume of ~350 ml can be expected. Because 1 μm is the assumed average height of the ESL in small vessels and it is likely that the whole body ESL is considerably thicker, our estimation introduced above seems quite reasonable. However, as demonstrated in Table 1, volume loading caused a dramatic decrease in the calculated noncirculating PV, presumably because of a decrease in ESL thickness. This, rather than a lack of consistency of the ICG-DT, is the reason for the apparent decrease in “RCVICG” in these patients.

If PV is the sought-after measure, one has to consider which part, the circulating or the total PV, is being stained (Fig. 7). Therefore, the properties of the ESL must be considered. It is an exclusion zone for large neutral and anionic molecules such as ≥70-kDa dextrans, but it is highly permeable for and, consequently, part of the distribution space of small neutral free dyes (e.g., 0.4-kDa rhodamine), plasma proteins such as albumin (53), and, consequently, tracers such as ICG and Evans blue, which bind rapidly to those plasma proteins. This fact underlines the statement of Tschaikowsky and co-workers (51) that “the smaller the indicator molecule, the bigger the [measured] plasma volume.” However, it is probably not a faster vascular clearance of small molecules during the measurements that causes a “falsely too high” PV. The more likely reason for the finding of Tschaikowsky and co-workers is that, by using large molecules or particles that do not enter the ESL for PV measurement (e.g., stained red blood cells), only the circulating part of PV can be determined, which is lower than the total PV determined with tracers entering the ESL quickly (53). It may be expected that other large molecules, such as hydroxyethyl starch and large dextrans, do not enter the ESL within the few minutes taken for PV measurement and, therefore, should exhibit a distribution space more like stained red blood cells (Fig. 8) (53). Indeed, it has been demonstrated that...
there is no significant difference between the PV determined by using the tracer “10% hydroxyethyl starch 200,000/0.5%” and that by using labeled red blood cells (51). Since the result should represent PVcirc directly, no fcell correction factor is necessary to calculate RCV from this PV and HctLV (26, 51). On the other hand, if it is assumed that the distribution space of albumin is PVcirc and the ESL, it is not at all surprising that the PV determined with radioactively labeled albumin is higher than the PV determined by red blood cells (50). Consequently, this phenomenon cannot be taken as a sign that the ICG-DT lacks precision.

Transcapillary leakage of albumin. Distrust in the ability of the ICG-DT to determine PV was also based on an indisputable transcapillary leakage of albumin (20, 23, 29). This exists even in normal subjects. Fleck et al. (9) reported a “transcapillary escape rate” of albumin of approximately 5%/h in healthy volunteers that can increase to 15%/h in the case of septic shock. On the basis of these values, “escape” of ICG attached to albumin molecules from blood vessels within our period of interest for PV measurements (0–5 min after injection of ICG) is low, normally ~0.4%, and rises to 1.25% under the worst clinical conditions (9). It is assumed that this loss represents an increase in distribution volume, PV would be overestimated in a young man [180 cm height, 75 kg body wt, normal PV = 3,050 ml (36)] by only 12 ml, which would rise to 38 ml in the worst case. Obviously, this is not a relevant methodological pitfall for the ICG-DT.

Additionally, it is likely that there is no increased initial distribution volume of ICG due to the transcapillary escape rate together with albumin. Escape forced by attachment to albumin, even under the conditions of an increased capillary leak [e.g., caused by an increase of the capillary surface area (29)], should only add to the elimination rate normally dictated by the liver (18, 35). If the capillary leak of albumin does not change markedly within the 5 min of the PV measurement, the escape of ICG after the initial, rapid binding to plasma proteins (7, 34) and the initial distribution of the labeled proteins within the intravascular space will proceed at a monoexponentially constant rate. Consequently, monoexponential extrapolation, also under the conditions of a raised transcapillary escape rate, should lead to a quite correct estimation of the theoretical concentration of ICG at t0, although the elimination curve appears more steep in the monoexponential presentation.

Intraindividual inconstancy of PV. PV is not a fixed individual quantity but, rather, varies due to physiological and pathophysiological stimuli. We found PV to be quite unstable shortly after induction of anesthesia or after an infusion therapy and surgical procedure (39). Changes in PV have been reported under the influence of different anesthetic drugs (32). As a result, a change in PVICG after an intervention such as induction of anesthesia is no legitimate basis for concluding that the ICG-DT is principally inaccurate (29).

Validation of the method. Validation and testing of restest reliability are serious problems if the target of a method is intraindividually inconstant and each tracer has an individual distribution space. Nevertheless, the ICG-DT has been validated successfully. Comparison of two PV values derived from two different tracer dilutional methods is possible, if the respective tracers have a comparable distribution space. Because ICG rapidly binds to plasma proteins, validation of its distribution space using the distribution space of these proteins is justified. Validation with the reference method, radiiodinated albumin, was performed in 10 healthy volunteers and 21 patients after elective open-heart surgery, with excellent results even for peripheral ICG injection (28). In vitro and in vivo validation was performed by another group; however, they used a questionable reference tracer (radiochromium-labeled red blood cells) (5). This principal error in reasoning logically should not influence the in vitro results, inasmuch as there is no EG dividing PV into two compartments. The authors “saved” their mean in vivo results by entering a correction factor, namely, an assumed fcell ratio. In a comparison of ICG with Evans blue in 7 dogs and 16 patients, Haneda and Horiuchi (16) found a correlation coefficient of 0.98 between these two dyes. Although they erroneously assumed that they were measuring “total circulating blood volume,” this misconception, inasmuch as the assumption was made for both markers, did not influence the information concerning validity of the ICG-DT.

Fig. 8. Schematic drawing of distribution space (DS) of artificial colloids (e.g., hydroxyethyl starch or dextran) and natural plasma proteins (e.g., albumin) at the vessel wall within the first minutes after injection, i.e., within the time window required for plasma volume measurement.
Repeatability has been demonstrated for the plasma method and the whole blood method (14). A prerequisite is a steady state between the two measurements that are to be compared. Indeed, for assessment of validity or repeatability, at best, general anesthesia and full relaxation, or, at least, supine position for an extended period of time of the awake volunteer, should be implemented.

In conclusion, the ICG-DT can be a precise tool for measuring PV if it is completely understood by the investigator. In particular, one needs to recognize that the result obtained from this technique is, due to the tracer’s distribution space, the total PV. Since this comprises the ESL, in addition to the circulating PV, it is larger than the distribution space of the red blood cells and large molecules. Consequently, direct comparisons with other methods to evaluate the accuracy of the ICG-DT are not applicable in most cases. In combination with direct measurement of RCV, the technique affords impressive insights concerning the integrity of the blood-tissue barrier. Besides routinely measuring total PV, the real target of infusion therapy should be implemented.

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