Development and validation of indocyanine green assay in human plasma using High-Performance Liquid Chromatography with PDA detector

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**ABSTRACT**
A robust and economical assay for routine determination of indocyanine green pharmacokinetics was developed and validated using high-performance liquid chromatography with a photodiode array detector. Plasma specimens from critically ill patients and those with hepatitis on various co-medications were used as blanks for validation of this assay. Extraction of indocyanine green was performed by simple protein precipitation with acetonitrile, and the supernatant was separated using an octadecyl column with detection at 784 nm. Blanks were found to have no interference for 40 blanks of patients who were on 56 different medications. The precision for LLOQ (0.5 µg/ml) as determined by the percentage coefficient of variation was 1.19. Stability of plasma calibration standards and stock were determined over a period of 61 days, and ICG was found to be stable for 20 days. Stability of whole blood specimens containing ICG was determined at 4°C for a period of 4 hours.

**INTRODUCTION**
Indocyanine green (ICG) is a cyanine dye given intravenously, the concentration-time profile of which is determined to assess the cardiac output (Iijima et al., 1997), liver function (De Gasperi et al., 2016) and is used for ophthalmic angiography (Agrawal et al., 2013). Global prevalence of cirrhosis is 4.5% to 9.5% of the general population as reported by Melato M et al., Graudal N and Lim YS (Graudal et al., 1991; Melato et al., 1993; Lim and Kim 2008). ICG plasma disappearance rate can be used to assess the dynamic liver function in critically ill patients (Halle et al., 2014).

Spectrophotometric methods are commonly used to determine the concentrations of ICG in plasma. But the specificity of this method is questioned (Rappaport and Thiessen 1982; Grasela et al., 1987) and measuring ICG concentrations in critically ill patients who are on multiple medications may further compromise the specificity of the spectrophotometric assay.

Rappaport et al., reported that ICG in plasma was stable when stored for 10 weeks at 6°C (Rappaport and Thiessen 1982). In another study performed by Awni WM et al., the ICG in plasma was found to be stable at room temperature for up to 24 hours (Awni and Bakker 1989). In another study, March et.al showed that the concentration of ICG decreased significantly over a period of 1 week after storage in -20°C (March et al., 1994; Vicient et al., 2017) and this instability of ICG in plasma specimens was attributed to the preservative effect of the anticoagulant that was used in these specimens.
Elimination of ICG follows an exponential curve, and the half-life of ICG is ~ 150 – 180 seconds (Henschen et al., 1993). This implies the need for multiple blood specimen collection over a period of 30 minutes to determine liver function. The difficulty in the immediate transfer of whole blood specimens and its processing mandates the need for stability of ICG in whole blood specimens.

The purpose of this study was to develop and validate an HPLC assay for ICG in human plasma which is simple, accurate, precise, highly selective, sensitive, and robust whilst being economical and a feasible option for clinical use. To improve the selectivity of ICG detection, wavelengths of more than 700 nm was screened. This assay would be routinely utilized in patients with liver pathology for studying the plasma disappearance rate of ICG prior to surgical intervention. The stability of ICG in plasma at different storage conditions was also determined.

MATERIALS AND METHODS
Reagents and chemicals
ICG was purchased from Adooq Bioscience, USA. Voriconazole (internal standard) and acetonitrile were purchased from Sigma Aldrich, and ammonium acetate was purchased from Fisher Scientific (Mumbai, India). ICG, acetonitrile and ammonium acetate were HPLC grade. Double distilled water was prepared in the laboratory from Milli-Q Gradient water purification system.

Instrumentation and Chromatographic Conditions
HPLC with Photodiode array detector (Shimadzu Quaternary Prominence LLC-2030C plus) was used for the assay development. Separation was carried out with Luna® 5µm C18(2) column of dimensions 250 × 4.6 mm with a particle size of 5 µm and pore size of 100 Å. An isocratic method was used with a mixture of 20 mM ammonium acetate and acetonitrile as the mobile phase at the ratio of 55:45, detected at 784 nm for ICG and 254 nm for the internal standard. The column was maintained at 30°C, and the auto-sampler was maintained at ambient temperature throughout the analysis.

Calibration curve standards and quality controls
Two separate primary stocks (1 mg/ml) in methanol was prepared for the calibration standards and the quality controls (QC). The primary stock of ICG is further diluted in methanol (1:10) to produce the secondary stock, which is again diluted in methanol (1:10) to produce the tertiary stock. Calibrators and quality controls were obtained by dilution from either primary or secondary or tertiary stocks in plasma. Calibrators included 20, 15, 10, 7.5, 2.5, 1.0, 0.75, 0.5 and 0.1 µg/ml and quality controls included 12.5 (high), 5 (Medium) and 0.8 (low) µg/ml.

Extraction method
ICG was extracted by a simple protein precipitation method. Into 300 µl of plasma, 30 µl of internal standard (1 µg/µl) was added and precipitated with 400 µl of acetonitrile. The solution was vortexed and centrifuged at 9447 g for 5 min. 50 µl of supernatant was injected into the system for the determination of ICG concentration.

Validation of ICG assay
The validation of the assay was based on the guidelines issued under the United States, Food and Drugs Administration guidance for a bioanalytical method of validation (Guidances (Drugs)).

Selectivity
Six zero standards were prepared from blood specimens of patients with liver cirrhosis, hepatitis and other hospital inpatients by spiking 30 µl of the internal standard. Serum specimens of 40 patients on various co-medications were used as blank specimens to check for interferences at the retention time of ICG and internal standard. The list of co-medications used by the 40 patients is listed in Table 1.

Accuracy, bias and precision of QC
The low, medium and high concentration quality controls were compared against the calibration curve to determine the accuracy and precision of ICG extraction. For this, five different extractions were performed from one sample, for each of the QC’s on day 0 and another five different extractions were performed on day 2. The days were counted from the time of preparation of the QC. Same calibration standards used on day 0 were used on day 2. One injection was performed for each extract. The accuracy of the assay was determined by estimating the bias for each extraction measured on the same day, in comparison to the true concentration that was spiked. The precision of the five extracts of a single concentration was also determined.

\[
\text{Bias} = \frac{\sum_{i=1}^{x} \text{Observed value} - \text{Expected value}}{x} \times 100
\]

\[
\text{Precision} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100
\]

\[
\text{Mean concentration} = \frac{\sum_{i=1}^{x} \text{Concentration (n)}}{x}
\]
Inter-day accuracy and precision were also calculated for Day 0 and day 2 for each of the concentrations.

Reinjection reproducibility was determined by estimating the precision of low, medium and high QCs, by repeatedly injecting five times from the same extracted sample.

**Accuracy and precision for LLOQ and ULOQ**

The intra-day bias and precision of LLOQ and ULOQ were estimated by comparing 5 different extractions of LLOQ and ULOQ, in the same batch and on the same day.

**Linearity of the calibration curve**

The linearity of the calibration curve was validated on six different days with calibration standards made using newly spiked calibration standards on each day. Two different primary stocks were used to prepare the standards and quality controls respectively over these 6 days.

**Process efficiency**

The process efficiency of extraction was calculated for low QC and high QC as follows:

\[
\text{Process efficiency (‰) } = \frac{\text{Pre extraction ratio}}{\text{Stock solvent extraction ratio}} \times 100
\]

Pre-extraction ratio was the ratio of chromatogram area of the drug to the internal standard with the usual extraction procedure. Stock solvent extraction ratio was obtained by mixing 300 µl of methanol stock containing 0.8 µg/ml and 12.5 µg/ml of ICG with 30 µl of 1µg/µl internal standard. It was performed with five different extractions of low QC and high QC.

**Stability of ICG**

**Stability in -20 degree Celsius**

Both the stock and the plasma specimens were stored in -20°C, and were extracted on day 9, 17, 20, 41 and 61. Plasma specimens of concentration 8 and 20 µg/ml were used. The concentrations measured on each of the days were compared against the freshly prepared plasma calibration standards prepared on the day of analysis.

**Freeze-Thaw cycles**

Plasma specimens of concentration 8 and 20 µg/ml were used. Four freeze-thaw cycles were performed over a period of 5 days from the preparation of calibration standards. The concentrations measured after each freeze-thaw cycle was compared against the freshly prepared plasma calibration standards prepared on the day of analysis.

**Stability of blood specimen in the refrigerator at 4°C**

To determine the stability of ICG in the refrigerator, blood specimens were collected from a healthy volunteer and spiked with ICG with two different concentrations and mixed gently with the blood mixer. Plasma was separated at 0 hr, 0.5 hr, 1.0 hr, 2.0 hr and 4.0 hr respectively from the spiked blood samples stored at 4°C and was analyzed. All the extractions were performed in duplicate, and the mean concentration was determined. The concentrations obtained after storage of blood specimens for prolonged intervals in the refrigerator was compared with the immediately separated plasma concentration.

**Benchtop stability**

Benchtop stability was determined over a period of 5 hours at room temperature. New plasma quality controls were prepared and were compared against newly prepared calibration standards.

**Autosampler stability**

The stability of extracted QC specimens in the autosampler was evaluated against calibration standards, which were estimated immediately after extraction. The bias and precision of low, medium and high-quality controls were estimated over a period of 48 hours post extraction.

**RESULTS**

The total run time of the assay was 13 min with elution of ICG at 11.0 min and internal standard at 7.35 min respectively. The chromatogram is shown in figure 1. The optimal wavelengths for detection of ICG and standard internal detection were determined to be 784 nm and 254 nm respectively as the chromatogram had minimum baseline fluctuation and the blanks were devoid of peaks at the corresponding retention times for the drug and internal standard.

**Selectivity**: As blanks, 40 patient specimens containing 56 different drugs were tested. No peaks were detected for these blank specimens between the 6 to 11 min chromatogram run times. The signal: noise ratio of Lower Limit of Quantification (0.1 µg/ml) was 8.5.

**Accuracy and precision for QC’s, LLOQ and ULOQ**

The intra-day and inter-day bias and precision of the quality controls are reported in Table 2. The precision (CV%) of 5 injections of low, medium and high QCs from the same extract were 0.35, 0.38 and 0.27 respectively.

LLOQ and ULOQ was determined to be 0.5 µg/ml and 20 µg/ml respectively. The signal to noise ratio of LLOQ was 13.09 for ICG. The same-day and inter-day bias and precision of 5 different extractions of LLOQ and ULOQ were within acceptable limits. The accuracy and precision for QC’s, LLOQ and ULOQ are shown in Table 2.
Figure 1: Typical Chromatogram of A. ULOQ, B. LLOQ, C. Internal standard, and D. Blank

Table 1: Co-medications screened in patients to determine the selectivity of the assay for ICG

<table>
<thead>
<tr>
<th>Anti-infective drugs</th>
<th>Meropenem</th>
<th>Norfloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoperazone</td>
<td>Vancomycin</td>
<td>Flucloxacillin</td>
</tr>
<tr>
<td>Sulfatam</td>
<td>Posaconazole</td>
<td>Caspofungin</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>Tigecycline</td>
<td>Metronidazole</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Crystalline Penicillin</td>
<td>Amikacin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other drugs</th>
<th>Noradrenaline</th>
<th>Ephedrine Hydrochloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenaline</td>
<td>Succinylated Gelatin</td>
<td>Thiamine</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>Filgrastim</td>
<td>Propofol</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Pantoprazole</td>
<td>Vecuronium</td>
</tr>
<tr>
<td>Midazolam</td>
<td>Ferrous fumarate</td>
<td>Enoxaparin sodium</td>
</tr>
<tr>
<td>Suxamethonium Chloride</td>
<td>Ondansetron</td>
<td>Tramadol</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>Spironolactone</td>
<td>Metoprolol</td>
</tr>
<tr>
<td>Furosemide</td>
<td>Hydrochlorothiazide</td>
<td>Lansoprazole</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>Aspirin</td>
<td>Salbutamol</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>Clonidine</td>
<td>Atorvastatin</td>
</tr>
<tr>
<td>Heparin</td>
<td>Levosulpiride</td>
<td>Levetiracetam</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>Metoclopramide</td>
<td>Risperidone</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>Amlodipine</td>
<td>Theophylline</td>
</tr>
<tr>
<td>Propranolol</td>
<td>Folic Acid</td>
<td></td>
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</tbody>
</table>

Table 2: Accuracy and precision of low, medium and high QC’s, LLOQ and ULOQ

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Bias%</th>
<th>Precision (CV %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day Low QC</td>
<td>0.84</td>
<td>4.91</td>
<td>1.88</td>
</tr>
<tr>
<td>Medium QC</td>
<td>5.05</td>
<td>0.95</td>
<td>2.73</td>
</tr>
<tr>
<td>High QC</td>
<td>12.09</td>
<td>-3.26</td>
<td>3.37</td>
</tr>
<tr>
<td>LLOQ</td>
<td>0.57</td>
<td>13.51</td>
<td>1.19</td>
</tr>
<tr>
<td>ULOQ</td>
<td>18.45</td>
<td>-7.75</td>
<td>0.006</td>
</tr>
<tr>
<td>Inter-day Low QC</td>
<td>0.84</td>
<td>4.78</td>
<td>1.97</td>
</tr>
<tr>
<td>Medium QC</td>
<td>4.80</td>
<td>-4.00</td>
<td>7.51</td>
</tr>
<tr>
<td>High QC</td>
<td>12.14</td>
<td>-2.88</td>
<td>3.50</td>
</tr>
</tbody>
</table>

Low QC = 0.80 µg/ml, medium QC = 5.0 µg/ml, high QC = 12.50 µg/ml, LLOQ = 0.50 µg/ml, ULOQ = 20 µg/ml
Table 3: Stability of different plasma standard concentrations over a period of 61 days

<table>
<thead>
<tr>
<th>Conc (µg/ml)</th>
<th>8</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stability of plasma standards when stored at -20°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 9</td>
<td>5.24</td>
<td>-1.51</td>
</tr>
<tr>
<td>Day 17</td>
<td>-6.19</td>
<td>-8.64</td>
</tr>
<tr>
<td>Day 20</td>
<td>-4.79</td>
<td>-2.55</td>
</tr>
<tr>
<td>Day 41</td>
<td>-17.66</td>
<td>-8.33</td>
</tr>
<tr>
<td>Day 61</td>
<td>-30.07</td>
<td>-26.30</td>
</tr>
</tbody>
</table>

Stability of standard stock when stored at -20°C

<table>
<thead>
<tr>
<th>Day</th>
<th>8 µg/ml (% agreement)</th>
<th>20 µg/ml (% agreement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.61</td>
<td>20.4</td>
</tr>
<tr>
<td>1</td>
<td>8.81 (102.2)</td>
<td>20.22 (99.1)</td>
</tr>
<tr>
<td>2</td>
<td>8.49 (98.5)</td>
<td>20.12 (98.6)</td>
</tr>
<tr>
<td>3</td>
<td>8.68 (100.8)</td>
<td>20.05 (98.3)</td>
</tr>
<tr>
<td>4</td>
<td>8.36 (97.1)</td>
<td>20.55 (100.7)</td>
</tr>
</tbody>
</table>

Table 4: Measured concentrations of ICG over a period of 5 consecutive days to determine freeze-thaw cycle stability

<table>
<thead>
<tr>
<th>Freeze-Thaw cycle</th>
<th>8 µg/ml (% agreement)</th>
<th>20 µg/ml (% agreement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>8.94 (100.7)</td>
<td>20.55 (100.7)</td>
</tr>
<tr>
<td>1</td>
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<td>20.55 (100.7)</td>
</tr>
</tbody>
</table>

Linearity of the calibration curve

The calibration curve was fitted using quadratic least square regression ($y = ax^2 + bx + c$) of the peak ratios (drug area/ internal standard area) ($r^2 = 0.999$) over the concentration range of 0.1 to 20 µg/ml, where $y$ is the concentration in µg/ml and $x$ is the peak ratio.

Process efficiency

The extraction process efficiency was 117.2% and 104.1% for low QC and high QC respectively.

Stability of stock and plasma specimens at -20 degree Celsius: The plasma specimens of concentration 8 and 20 µg/ml prepared from the stored primary stock was compared against the newly prepared primary stock calibration standards on day 20. The agreement between day 0 and day 61 for the primary stock for low, medium and high concentrations were 96.9 %, 108.9 % and 99.9 % respectively.

For the plasma specimens stored at -20°C, the agreement against the freshly prepared calibration curve (prepared from the fresh primary stock) was 69.9 % and 73.7 % respectively for 8 and 20 µg/ml on Day 61.

Benchtop stability: Plasma standard concentrations 8 and 20 µg/ml were extracted immediately after thawing and analyzed. The same plasma specimens were stored for 1 hour, 2.5 hour and 5 hours on the benchtop and then analyzed. No degradation was observed when stored over a period of 5 hours at room temperature.

Freeze-Thaw cycles: The average estimated concentration for 8 and 20 µg/ml obtained over the entire study duration of 5 consecutive days is described in Table 4.

Stability of blood specimen in the refrigerator at 4°C

The precision for low concentration (3.90 µg/ml) was 1.64 %, and high concentration (18.02 µg/ml) was 3.34 % respectively for whole blood ICG specimens stored in refrigerator over a period of 4 hours.

Autosampler stability

The agreement of fresh spiked concentrations of 8 and 20 µg/ml with specimens stored in the autosampler for 48 hours post extraction was 97.5%, 101.0% and 100.3%.

DISCUSSION

The method is validated in critically ill patients and those with hepatitis who were on co-medications mentioned in table 1. The extraction method is a modification of the method published by Vicient et al., (Vicient et al., 2017). The run time of the developed assay is short (13 minutes) and the chromatogram obtained is good (number of theoretical plates calculated by tangent line method was 7460 and 7496 and tailing factor was 1.2 and 1.1 for internal standard and ICG respectively), for routine therapeutic drug monitoring purpose. Process efficiency of ICG assay is more than 100% and the patient blank specimens examined for the selectivity of the assay for ICG is found to be reliable.

ICG is stable in plasma at room temperature over a period of 5 hours, and this finding is in agreement with Awni WM et al., and Rappaport P. L et al., (Rappaport and Thiessen 1982; Awni and Bakker 1989). Contrary to the findings of March C et al.,
(March et al., 1994), our study has demonstrated the stability of ICG plasma specimens over a period of 20 days in -20°C. ICG is also found to be stable in whole blood for a period of 4 hours when stored at 4°C. Stability of ICG in plasma, when stored at different temperatures for varying periods of time, helps conduct the ICG pharmacokinetic tests in settings like intensive care units.

CONCLUSION

The assay developed is economical, easy to perform, accurate, sensitive, highly selective and robust to determine the concentration-time profile of ICG in liver cirrhosis patients and this assay could even be used in critically ill patients who may be on many co-medications. The stock prepared in methanol and the plasma calibration standards are stable over a period of 20 days when stored at -20°C. The whole blood specimens containing ICG are stable when stored at 4°C for 4 hours.

Acknowledgement

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REFERENCES


