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A Simple and Sensitive LC-MS/MS for Quantitation of ICG in Rat Plasma: Application to a Pre-Clinical Pharmacokinetic Study

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Abstract: A selective, sensitive, and rapid liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method was developed and validated for the quantitation of ICG in rat plasma. The chromatographic separation was achieved using an ACE excel C18 (3 µm, 50 × 3.0 mm) column, with a mobile phase composition of 0.1% formic acid and 0.1% formic acid in acetonitrile, using a gradient flow at a rate of 0.3 mL/min. The MS was operated at a unit resolution in the multiple reaction monitoring mode, using the precursor ion → product ion combinations of 753.3 → 330.2 *m/z* (ICG) and 747.45 → 717.50 (Cy7.5 amine) with a run time of 5 min. The assay was linear over a concentration range of 1–1000 ng/mL with a regression coefficient (r^2) of 0.998 or better. The inter and intra-batch precision (% relative standard deviation, %RSD) was lower than 13.5%, with accuracy (%Bias) between −10.03% and 11.56%. The ICG was stable under laboratory storage and handling conditions. The validated method was successfully applied to preclinical pharmacokinetic (PK) studies of ICG at a dose of 0.39 mg/kg in rats. PK parameters suggested the highest plasma concentration within 2 min of intravenous dosing with restricted systemic distribution and rapid clearance.

Keywords: ICG; LC-MS/MS; rat plasma; validation; pharmacokinetics; intravenous

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1. Introduction

Indocyanine green (ICG, Figure 1) is an amphiphilic, non-toxic, tricarbocyanine iodide dye composed of hydrophobic polycyclic chains and hydrophilic sulfate groups [1]. Being amphiphilic in nature, ICG readily binds to plasma proteins such as lipoproteins and serum albumin followed by rapid hepatic clearance [2].

ICG, near-infrared (NIR) dye, has served as a valuable diagnostic tool since the mid-1950s in the anatomical determination of tumors and the implementation of safer surgical procedures. It was FDA-approved for (1) the determination of cardiac output, hepatic function, and liver blood flow, (2) ophthalmic angiography, and (3) neurosurgical research [2,3]. Although there has been considerable interest in the off-label use of ICG as an optical contrast agent in surgical oncology, the use of ICG alone is limited due to extensive plasma protein binding, poor tumor retention, low contrast, and rapid elimination [4,5]. To overcome these shortcomings, the fluorescence-guided surgery (FGS) field has been

inclined to develop novel formulations of ICG to improve tumor penetration and surgical contrast [6–11].

Several analytical techniques for the quantification of ICG have been identified, including spectrophotometry [12], high-performance liquid chromatography (HPLC) [13], size-exclusion chromatography (SEC) [14], liquid chromatography mass spectrometry (LC-MS) [15], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [16], and most recently, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [17]. To date, various LC-MS/MS methods have been described in the literature using 90–100 µL of a biological matrix with a lower limit of quantification of ≥10 ng/mL [17–20]. However, because mice and rats are small animal models, there is a limited availability of biological matrix. A reliable, sensitive, and accurate analytical method is therefore required to sufficiently assess the utility, pharmacokinetics, and biodistribution of this novel agent.

We build upon these findings by the development and validation of an LC-MS/MS method for the quantification of ICG in rat plasma. This analytical method was applied for the determination of an ICG plasma concentration time profile after a single intravenous dose (IV) of ICG (0.39 mg/kg). Based on these findings, the described LC-MS/MS method can be added to the armamentarium of ICG analytical methods and utilized in a variety of preclinical and clinical applications.

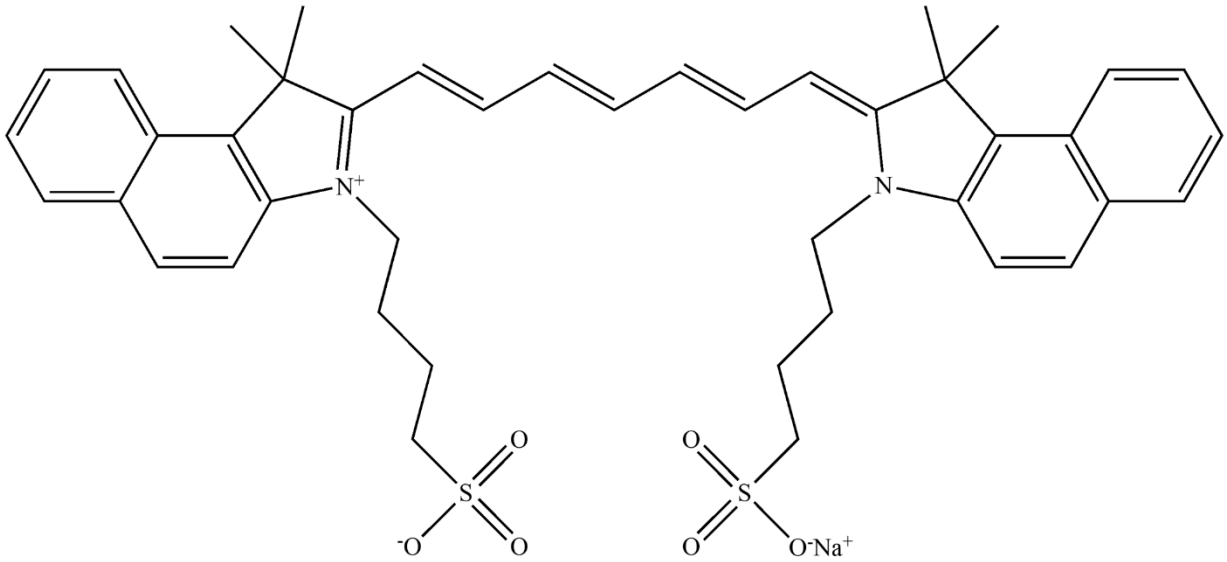


Figure 1. Chemical structure of indocyanine green dye.

2. Materials and Methods

2.1. Chemicals and Materials

ICG (purity: ≥98%) was purchased from Sigma-Aldrich (St Louis, MO, USA) and Cyanine 7.5 (Cy7.5) amine (purity: ≥99%) was utilized as an internal standard (IS) and was purchased from Lumiprobe Corporation (Hallandale Beach, FL, USA). LCMS grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ultrapure water was generated via a GenPUre xCAD plus water purification system (Thermo Fisher Scientific). Rat plasma was purchased from Equitech-Bio, Inc. (Kerrville, TX, USA). All other materials and reagents were of analytical grade or higher and purchased from standard chemical suppliers.

2.2. Liquid Chromatographic and Mass Spectrometric Conditions (LC-MS/MS)

The chromatographic analysis was performed on a Shimadzu HPLC system equipped with a binary pump system (LC-30 AD), a column oven (CTO-30AS), an auto-sampler (SIL-30AC), a controller (Shimadzu cbm-30alite), and a degassing unit (Shimadzu DGU-30AC) maintained at 4 °C, and a coupled 8060 MS/MS with a dual ion source (DUIS), electrospray

ionization (ESI), and atmospheric pressure chemical ionization (APCI), was used for the quantification of the dye (Shimadzu Scientific Instruments, Columbia, MD, USA). The chromatographic separations were performed with an ACE excel C18 (3 µm, 50 × 3.0 mm, Advance Chromatography Technologies, Ltd., Aberdeen, UK) column equipped with a C18 guard column (Thermo Scientific Inc., Waltham, MA, USA) at a flow rate of 0.3 mL/min with a total run time of only 5 min. Mobile phases A and B were composed of 0.1% formic acid and 0.1% formic acid in acetonitrile, respectively. The column temperature was maintained constant at 37 °C. The separation gradient started with a mobile phase concentration of 35% B at 0 min, increased gradually from 35% to 90% of B in 3 min, then held constant at 90% B for 1.0 min, and ultimately brought back to the initial condition of 35% B in 0.1 min followed by 1 min of re-equilibration. The injection volume of 10 µL was constant for all the samples.

The analytes were detected by a Shimadzu 8060 mass spectrometer in positive ESI mode and multiple reaction monitoring (MRM) modes. The MS/MS system was operated at unit resolution in the MRM mode, using the precursor ion > product ion combinations of 753.3 > 330.2 m/z for ICG and 747.45 > 717.50 for Cy7.5 amine. The following MS source settings were used for detection: nebulizer gas: 2 L/min, drying gas: 10 L/min, heating gas: 10 L/min, interface temperature: 300 °C, desolvation line temperature: 250 °C, and heat block temperature: 350 °C. The MRM transitions for the analyte (quantifier: 330.3 and qualifier: 422.3) and IS (quantifier: 717.5) with their respective optimized MS parameters, such as Q1 and Q3 voltage potential and collision energy (CE), are given in Table 1. Data acquisition and quantification were performed by using LabSolutions Version 5.8 (Shimadzu Scientific, Inc.).

Table 1. Summary of the MS/MS parameters: precursor ion, product ions, voltage potentials (Q1 and Q3), and collision energy (CE) for ICG and Cy7.5.

Analytes	MRM Transition *m/z* (Q1 > Q3)	Q1 (V)	Q3 (V)	CE (V)	Retention Time (min)
ICG	753.30 > 330.20	−24	−43	−24	2.9
	753.30 > 422.30	−24	−34	−22	
IS (Cy7.5)	747.45 > 717.50	−26	−45	−28	3.1

2.3. Preparation of Stock, Calibration Standards, and Quality Control Samples

Primary stock solutions of ICG and Cy 7.5 were prepared in methanol:water (50:50, v:v) at a concentration of 1 mg/mL. Working stock solutions (WS) of ICG at various concentrations were prepared by diluting the primary stock using methanol:water (50:50, v:v). On the other hand, the working stock of Cy 7.5 was prepared at a concentration of 0.5 µg/mL with 50% methanol to ensure consistent IS concentration throughout the assay. Blank rat plasma (50 µL) was spiked with 5 µL of ICG WS to prepare calibration curve (CC) standards and quality control (QC) samples. Along with the ICG working stock, the samples were spiked with 10 µL of 0.5 µg/mL Cy 7.5 solution as an IS. The final concentration of Cy 7.5 was constant (~77 ng/mL) for CCs and QCs with an ICG concentration ranging between 1 and 1000 ng/mL (1, 2, 5, 10, 50, 100, 500, 875, and 1000 ng/mL) in biological matrix. The lower limit of quantitation (LLOQ), lower QC (LQC), medium QC (MQC), and high QC (HQC), for ICG were 1, 3, 200, and 750 ng/mL, respectively. During each study, ICG concentrations were measured using a set of freshly prepared CCs and at least three sets of QCs. All the primary stocks and WS stocks were stored at −20 °C until further use.

2.4. Plasma Sample Preparation

A simple and rapid plasma protein precipitation (PPT) method using ice-cold acetonitrile (ACN) was carried out for all samples. For CCs, QCs, and PK study samples, 50 µL of the respective samples were mixed with 10 µL of IS (0.5 µg/mL solution) working solution and vortexed for 30 s. Next, all samples were quenched with 200 µL of ACN, followed by vortex mixing for 2 min and centrifugation at 17,950× g for 15 min at 4 °C. Thereafter,

100 µL of the supernatant was carefully transferred to an auto-sampler vial and injected (10 µL) into LC-MS/MS.

2.5. Assay Validation

The assay validation method was derived from the previously published literature by our group [21,22]. The assay was developed and validated as per FDA guidelines (Bioanalytical Method Validation: Guidance for Industry, 2022). Selectivity and specificity in blank rat plasma (n = 6) were evaluated by comparing the chromatograms of blank plasma with that of an ICG or IS spiked plasma sample to assess the potential interference of any endogenous compounds with the retention time of ICG or Cy 7.5 amine. The sensitivity of the assay was estimated using the signal-to-noise ratio (S/N) of the analyte in the CCs. The LOD and LLOQ for the current method were defined as the peak areas with three and ten-fold greater S/N ratios, respectively.

A linear ($1/x^2$) least square regression method was implied to construct the CC by plotting the peak area ratio (area of analyte/area of IS) versus the nominal plasma concentration of the analyte. The CC solutions prepared were used as non-zero calibration points to evaluate the CC at different concentrations of ICG (1, 2, 5, 10, 50, 100, 500, 875, and 1000 ng/mL) in plasma. The LLOQ with an S/N ratio greater than ten was described as the lowest concentration point in the calibration curve. The limit of detection (LOD) S/N was required to be greater than three. For the analyte, the regression coefficient (r^2) was expected to be $\geq$ 0.99 to confirm the linearity in the calibration curve. The carryover of the analyte was evaluated by analyzing three successive injections of blank plasma matrix after analyzing the HQC sample. The carryover area was required to be less than 15% of the LLOQ area. The concentrations of CC and QC were required to be ±15% standard deviation (SD) of their nominal concentration value, except for the LLOQ which was acceptable at ±20% of the nominal concentration.

To evaluate the inter and intra-day accuracy and precision, four QCs with different ICG concentrations (LLOQ, LQC, MQC, and HQC, n = 5) were prepared, along with one set of calibration curve standards. CC and QC concentrations were measured using the analytical method described above and the percent coefficient of variation (%CV) was calculated. The assay validation was performed in replicates of five on three consecutive days. Precision and accuracy were defined as the percent relative standard deviation (% RSD) and percent bias (% Bias), respectively, with an acceptable limit of ±15% (except ±20% for the LLOQ) for both parameters.

2.6. Extraction Recovery and Matrix Effect

The recovery and matrix effect (ME) of the ICG and IS was estimated based on previously published articles from our group [21,22]. The extraction recovery was calculated by comparing peak areas of the ICG and Cy7.5 amine between the pre-extracted and corresponding post-extracted plasma standard. This parameter was analyzed at three concentrations (LQC, MQC, and HQC) and the recovery was acceptable, even if it was not 100%, precise, and reproducible.

The ME of rat plasma endogenous constituents with the ionization of ICG and IS was estimated by comparing the peak area of the post-extracted plasma QCs (LQC, MQC, and HQC) with corresponding QC concentrations prepared in the mobile phase. The ME and IS normalized ME were calculated using Equations (1) and (2). The acceptance limit of %CV of precision should not exceed 15% in all matrix plasma tested.

$$\text{ME} = \frac{\text{Mean Peak area of analyte spiked post} - \text{extraction}}{\text{Mean Peak area of analyte in Solvent}} * 100 \quad (1)$$

$$\text{IS normalized ME} = \frac{\text{Mean Peak area ratio of analyte/IS spiked post} - \text{extraction}}{\text{Mean Peak area ratio of analyte/IS in Solvent}} * 100 \quad (2)$$

2.7. Stability Studies

The stability of ICG in neat stocks and plasma was determined at three concentrations (LQC, MQC, and HQC). These studies were performed under various conditions, such as benchtop conditions under the presence of light (4 h and 24 h) and absence (4 h and 24 h) of room light and for long-term stability (−20 °C for 5 months). Along with the stability of ICG in neat stocks, the stability of the analyte in rat plasma was also assessed in the following conditions: bench-top storage (4 h at room temperature in light or dark conditions), three freeze-thaw cycles (−80 °C to room temperature and back to −80 °C, stored for at least 24 h), and long-term storage (30 days at −80 °C). Furthermore, a 7 day auto-sampler stability of extracted samples (at 4 °C) was performed.

3. In Vivo PK Studies and Data Analysis

The University of Nebraska Medical Center (UNMC) Institutional Animal Care and Use Committee (IACUC) approved all animal studies (protocol number 18-081-05-FC). Female Sprague Dawley rats (weight range = 200–250 g) were used for PK studies. Animals were housed in the UNMC animal facility for more than 7 days prior to the experimentation to let animals acclimatize to the laboratory conditions (temperature of 23–24 °C, relative humidity of 40–70%, and 12/12 h light/dark cycles). Prior to dosing, rats were anesthetized via inhalation of isoflurane (1–3% in O_2) (Piramel Group; Mumbai, India) and were catheterized (jugular vein) with heparinized polypropylene tubing. Being aqueous soluble, the ICG solution was prepared in water and was administered by the IV route. The standard dose of ICG for clinical use is between 0.1 and 0.5 mg/mL/kg, therefore, an IV dose of 0.39 mg/kg was used for this study. Following the dose, ~100 µL of blood was collected in a heparinized polypropylene tube from the jugular vein at 2, 5, 15, and 30 min and 1, 2, 4, and 6 h. Rats were divided into three groups of five, blood was collected at three different time points from every rat, and the rats were sacrificed at the terminal time point (five rats/group/per time point). Blood was centrifuged at 4000× *g* at 4 °C for 10 min to obtain plasma and was stored at −80 °C until analysis.

ICG PK parameters were estimated using non-compartmental analysis using Phoenix WinNonlin 8.2 (Pharsight Corporation, Mountain View, CA, USA). The concentration (C_0) at time zero was extrapolated based on the plasma concentration time profile. The area under the curve ($AUC_{0-\infty}$) was estimated using the linear trapezoidal method from 0-t_{last} and extrapolation of the observed concentration at t_{last} divided by the terminal elimination rate constant (k). The first-order elimination half-life ($t_{1/2}$) was calculated using the formula 0.693/k. For IV administration, the clearance (Cl) and the apparent volume of distribution (V_d) were described as dose/$AUC_{0-\infty}$ and dose/k*$AUC_{0-\infty}$, respectively.

4. Results and Discussion

4.1. Liquid Chromatographic and Mass Spectrometric Conditions Optimization

Mass spectrometry detection was optimized by manual and auto optimization methods using LabSolutions software, Version 5.8 (Shimadzu Scientific, Inc., Columbia, MD, USA). The precursor and product ion search for ICG and the IS was performed using a 0.5 µg/mL solution of both ICG and IS compound in 50% aqueous ACN via an autosampler injection. The ESI and APCI conditions were tested to optimize the MS/MS conditions for the detection of ICG and IS. The MS/MS response of ICG and the IS were found to be the highest using the positive ESI mode (data not shown for APCI). The mass spectrometer was operated in a Q1 scan followed by a product ion scan, and MRM scan in positive ESI mode. The protonated precursor ion (M+H, positive ion mode) for ICG and IS was *m*/*z* 753.30 and 747.45, respectively. The resulting MRMs were used for the quantitation (precursor ion → product ion) of ICG and IS: 753.3 → 330.2 *m*/*z* for ICG and 747.45 → 717.50, respectively (Figure 2a,b).

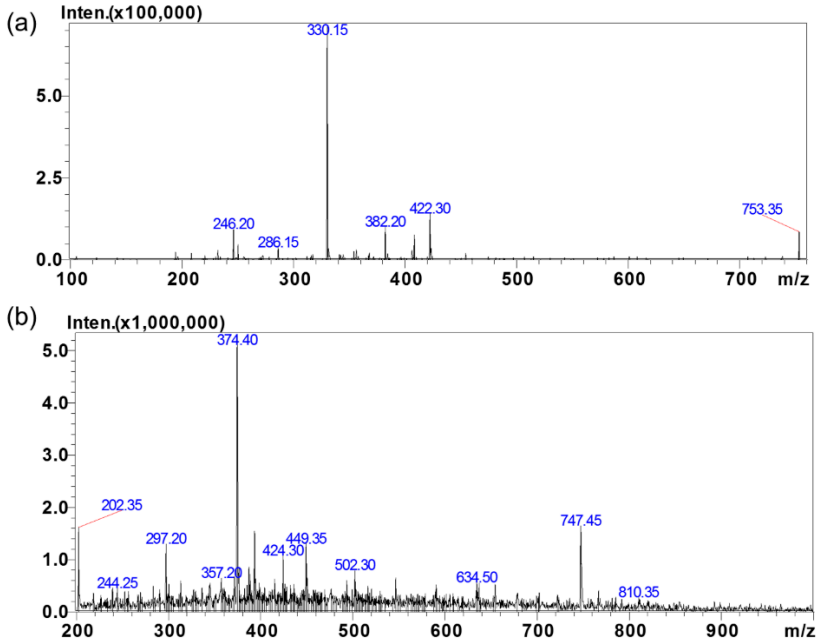


Figure 2. MS/MS product ion spectra; (**a**) precursor ion peak for ICG in positive ESI mode [M+H] at *m*/*z* 753.35 and (**b**) precursor ion peak for Cy7.5 in positive ESI mode [M+H] peak at *m*/*z* 747.45.

The chromatographic separation of the ICG and Cy 7.5 was carried out on a C18 reversed-phase column and therefore came with a few challenges such as carryover and peak tailing during the LC method optimization. To attain shorter retention times with better peak resolution, various chromatographic conditions such as different mobile phase compositions (acetonitrile, methanol, and water), as well as different additives (formic acid, acetic acid, ammonium acetate, and ammonia formate) in isocratic or gradient flow programs were tested. In addition, different analytical columns (C8, C18, and C18 PFP) were investigated. Finally, the ACE Excel C18 column (Section 2.2), with a mobile phase composition of 0.1% formic acid in water and 0.1% formic acid in acetonitrile and a gradient flow program, yielded an optimal balance of retention times between ICG (2.9 min) and Cy 7.5 (3.1 min) and peak shape with a run time of 5 min. Cy 7.5 was chosen as IS for ICG as both compounds sharing similar molecular moieties behaved similarly. Furthermore, endogenous compounds from rat plasma were not found to interfere with the analysis of either ICG or IS (Figure 3).

The PPT method chosen in this study is a simple and fast method that has been commonly used in the sample preparation of biological matrix for LC-MS/MS analysis. In this research, ACN was used as the precipitating solvent of choice in the PPT method due to its (1) maximum deproteinization effect, (2) extraction recovery of the analyte and IS was maximum, and (3) minimum endogenous interferences. The simple and fast PPT extraction method yielded mean recoveries greater than 85%. Cy 7.5 was the choice of internal standard due to similar chromatographic and mass spectrometric properties.

4.2. Assay Validation

4.2.1. Specificity and Selectivity

Rat plasma was obtained from three different sources to test specificity and selectivity. No endogenous interferences or any additional characteristic peaks were observed at the retention time of ICG or IS (2.9 min and 3.1 min, respectively) with an intensity of more than 5% of the baseline (Figure 3a,c).

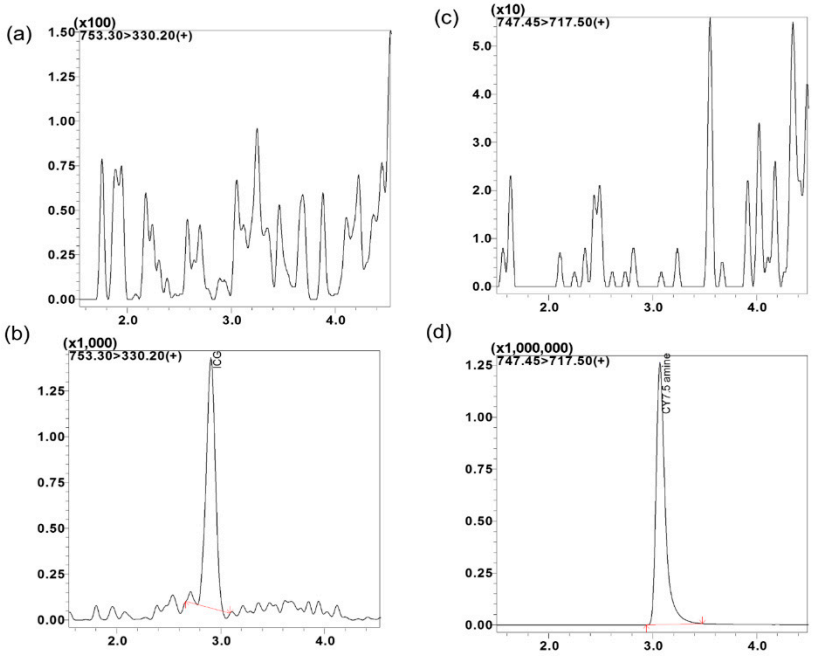


Figure 3. Representative MRM ion-chromatograms of (**a**) blank rat plasma using the conditions for ICG detection, (**b**) ICG spiked in rat plasma at LQC (rt. 2.8 min, 3.0 ng/mL), (**c**) blank rat plasma using the conditions for Cy7.5 detection, and (**d**) Cy7.5 spiked in rat plasma (rt. 3.1 min, 77 ng/mL).

4.2.2. Calibration Curve and Linearity

All the standards lying within the concentration range of 1 to 1000 ng/mL were linear for ICG ($r^2 \geq 0.998$) using a $1/x^2$ weighted linear regression. Plasma samples spiked with 1 ng/mL were found to have the lowest concentration with an RSD < 20%, and thus were defined as LLOQ (1 ng/mL). The calibration curve equation was Y = (0.000763606)X + (−0.00120790).

4.2.3. Carryover

No significant ICG peaks (≥20% of LLOQ) were seen (data not shown) by injecting blank samples after HQC samples. Thus, the carryover of ICG was not observed with the current method.

4.2.4. Accuracy and Precision

Intra-day and inter-day accuracy and precision for ICG were assessed at all four levels of QCs (LLOQ, LQC, MQC, and HQC) in rat plasma. Both accuracy and precision were found to be within the acceptance limits (FDA bioanalytical guidelines, 2022) at all QC levels (Table 2). The accuracy ranged between −11.39 and 14.56%, while the range of precision varied from 2.71 to 11.95%.

Table 2. Intra and inter-assay accuracy and precision of ICG in rat plasma (n = 5).

Nominal Concentration (ng/mL)	Accuracy		Precision	
	%Bias Intra-Assay	%Bias Inter-Assay	%RSD Intra-Assay	%RSD Inter-Assay
LLOQ (1 ng/mL)	7.32	−3.33	11.95	10.34
LQC (3 ng/mL)	−11.39	−6.34	2.71	4.56
MQC (200 ng/mL)	−2.43	2.88	9.91	5.81
HQC (750 ng/mL)	14.56	11.77	4.63	4.62

4.3. Recovery and Matrix Effect

The recovery of ICG from the spiked rat plasma was determined at three QC levels (LQC, MQC, and HQC) and for IS from plasma at a concentration of 0.5 µg/mL. The % mean recovery for ICG in LQC, MQC, and HQC were 97.7 ± 12.1, 87.6 ± 3.2, and 89.8 ± 10.1%, respectively (Table 3). The mean recovery of all three QC levels was 91.7 ± 9.3%, whereas the mean recovery of IS was 97.5 ± 8.3%. Furthermore, the ME and IS normalized ME for ICG was <±15% (93.2–111.5), which is within the acceptable range.

Table 3. Assessment of the % extraction recovery of ICG in rat plasma (Mean ± SD, *n* = 3).

Nominal Concentration (ng/mL)	% Extraction Recoveries (Mean ± SD, *n* = 3)
LQC (3 ng/mL)	97.7 ± 12.1
MQC (200 ng/mL)	87.6 ± 3.2
HQC (750 ng/mL)	89.8 ± 10.1
Internal standard (IS) (0.5 µg/mL)	97.7 ± 8.3

4.4. Stability

The stability of ICG in rat plasma and the neat stock solution was tested under various conditions. The stability results of these experiments are shown in Tables 4 and 5. For ICG, the accuracy at the investigated conditions was between 86% and 113%, indicating these compounds are stable in stock solutions for up to 24 h at room temperature under light and, therefore, stable in dark conditions, along with being stable in the freezer (−20 °C) for 5 months (Table 4).

In rat plasma, ICG was proven to be stable under all storage conditions, including autosampler stability (4 °C, up to 7 days), three freeze-thaw cycles, 4 h benchtop (at room temperature), and 30 days frozen at −80 °C (Table 5). Overall, ICG was found to be stable under the above-tested conditions as concentrations were within 15% of their nominal values, falling under the FDA's acceptable limits. These studies indicated the stability of ICG under relevant sample handling conditions and storage conditions with the appropriateness of the current method.

Table 4. Stability of ICG in stock solution under different laboratory storage conditions (mean ± SD, *n* = 3).

Storage Conditions	Nominal Concentration (ng/mL)	% Accuracy
Benchtop (20 °C, up to 4 h under room light condition)	LQC (3 ng/mL) MQC (200 ng/mL) HQC (750 ng/mL)	86.1 ± 0.5 89.7 ± 2.9 111.5 ± 1.5
Benchtop (20 °C, up to 4 h under dark condition)	LQC (3 ng/mL) MQC (200 ng/mL) HQC (750 ng/mL)	86.4 ± 9.7 90.4 ± 2.0 109 ± 8.6
Benchtop (20 °C, up to 24 h under room light condition)	LQC (3 ng/mL) MQC (200 ng/mL) HQC (750 ng/mL)	84.5 ± 6.5 88.6 ± 4.6 107 ± 1.2
Long term stability (20 °C, up to 5 months)	LQC (3 ng/mL) MQC (200 ng/mL) HQC (750 ng/mL)	94.5 ± 9.2 92.6 ± 9.2 88.9 ± 2.7

Table 5. Stability of ICG in rat plasma at different laboratory storage conditions (Mean ± SD, *n* = 3).

Storage Conditions	Nominal Conc. (ng/mL)	% Accuracy
Benchtop (20 °C, up to 4 h under dark condition)	LQC (3 ng/mL) MQC (200 ng/mL) HQC (750 ng/mL)	105.6 ± 5.6 112.0 ± 7.0 108.0 ± 4.2
Freeze-thaw stability (−80 °C, up to 3 Cycle)	LQC (3 ng/mL) MQC (200 ng/mL) HQC (750 ng/mL)	91.4 ± 10.3 94.0 ± 8.6 95.4 ± 5.0
Auto-sampler (AS) storage (4 °C, up to 24 h)	LQC (3 ng/mL) MQC (200 ng/mL) HQC (750 ng/mL)	93.5 ± 13.9 107.4 ± 11.1 113.0 ± 1.1
Auto-sampler (AS) storage (4 °C, up to 7 days)	LQC (3 ng/mL) MQC (200 ng/mL) HQC (750 ng/mL)	87.6 ± 10.6 96.4 ± 24.9 84.1 ± 6.6
Long term stability (−80 °C, up to 30 days)	LQC (3 ng/mL) MQC (200 ng/mL) HQC (750 ng/mL)	91.2 ± 7.6 87.9 ± 4.3 94.1 ± 3.9

4.5. In Vivo PK Studies: Application of Analytical Method

The validated LC-MS/MS method was successfully applied to the estimation of the PK of ICG in rats following a single IV dose of ICG (0.39 mg/kg). The mean plasma concentration vs. time profile is shown in Figure 4.

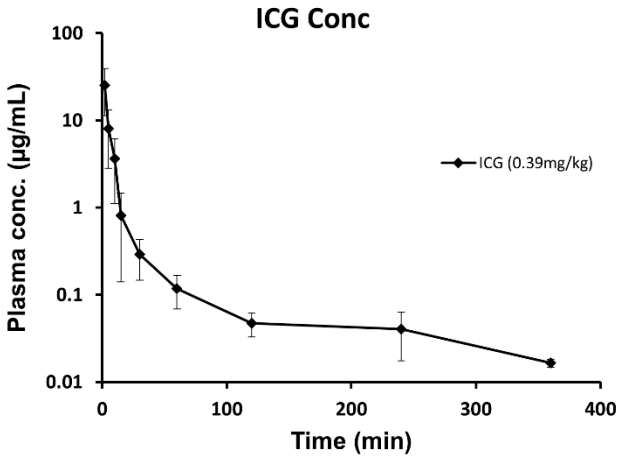


Figure 4. Plasma concentration time profile of ICG after an intravenous (IV) dose of 0.39 mg/kg ICG (mean ± SD, *n* = 3).

The PK parameters (mean ± SD) of ICG in healthy rats are given in Table 6. Following an IV dose, the plasma concentration of the dye was extremely high (56.38 ± 34.48 µg/mL) due to extensive plasma protein binding. ICG reached its highest plasma concentration within 2 min of administration, validating the dosing efficiency. The compound was exponentially cleared by the liver (>99%) in less than 30 min with an ICG concentration of less than 20 ng/mL by the end of 6 h. The validated LC-MS/MS method accurately and precisely quantified extremely high (diluted) and low ICG concentrations at the beginning and the end of the PK study, respectively. The systemic exposure of ICG ($AUC_{0–last}$) was found to be 191.53 ± 89.00 min×µg/mL with restricted systemic circulation (V_d = 1.5 ± 1.3 L/kg). As mentioned earlier, ICG was rapidly cleared from the rat body, reducing half the initial concentration ($t_{1/2}$) in 90.9 ± 41.1 min with a Cl of 351.5 ± 279.9 L/h/kg. The percentage extrapolation of AUC measured from the last time point to infinity was less than 5% (Table 6).

Table 6. Summary of ICG PK parameters after IV (0.39 mg/kg) administration (mean ± SD, n = 3).

PK Parameter	Mean ± SD
C_0 (μg/mL)	56.38 ± 34.48
T_{max} (min)	2.0 ± 0.0
$t_{1/2}$ (min)	90.9 ± 41.1
$AUC_{0-\infty}$ (min × μg/mL)	189.42 ± 89.61
AUC_{0-last} (min × μg/mL)	191.53 ± 89.00
V_d (L/kg)	1.5 ± 1.3
Cl (L/h/kg)	351.5 ± 279.9

5. Conclusions

We have developed a sensitive and reliable LC-MS/MS method for the quantitation of ICG in rat plasma. The LLOQ of 1 ng/mL will be beneficial to precisely detect dye concentrations during surgical procedures and is superior to studies reported in the literature so far. The assay performance (linearity, precision, selectivity, and specificity) with a total run time of 5 min makes this analytical method a valuable tool for high-throughput bioanalysis and application to pharmacokinetic studies of ICG and its formulations. Additionally, this method used a small volume of the biological matrix, 50 μL of plasma, which allowed for the determination of drug levels in the inherently small organs or tumors of rat models. Due to high selectivity, this method can prove to be very useful in understanding the pharmacokinetic/pharmacodynamic (PK/PD) relation of ICG, either as a single agent or in combination with other drugs. Hence, the currently validated LC-MS/MS method provides a valuable tool to access optical surgical oncology and improve our understanding of the safety of ICG therapy.

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