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Original Article

Pharmacokinetic study of indocyanine Green after intravenous administration by UPLC-MS/MS

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Abstract: Indocyanine Green is widely used in medical diagnosis and to evaluate liver function and other regional blood flows in clinical application or animal experiments. In this work, a sensitive and selective ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for determination of Indocyanine Green in rat plasma was developed and validated. After addition of rutin as an internal standard (IS), protein precipitation by acetonitrile-methanol (9:1, *v/v*) was used to prepare samples. Chromatographic separation was achieved on a UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) with 0.1% formic acid and acetonitrile as the mobile phase with gradient elution. An electrospray ionization source was applied and operated in positive ion mode; multiple reactions monitoring (MRM) mode was used for quantification using target fragment ions *m/z* 753.4→330.2 for Indocyanine Green, and *m/z* 611.1→303.1 for IS. Calibration plots were linear throughout the range 20-5000 ng/mL for Indocyanine Green in rat plasma. Mean recoveries of Indocyanine Green in rat plasma ranged from 79.5% to 85.4%. RSD of intra-day and inter-day precision were both < 12%. The accuracy of the method was between 95.9% and 113.9%. The method was successfully applied to pharmacokinetic study of Indocyanine Green after intravenous administration.

Keywords: Indocyanine Green, UPLC-MS/MS, pharmacokinetics, rat

Introduction

Indocyanine Green (ICG, **Figure 1**) is a water-soluble organic anion synthesized in the Kodak Research laboratories in 1955 which was used as tricarbocyanine dye incipiently [1-3]. In addition, ICG is widely used in medical diagnosis [1] and to evaluate liver function and other regional blood flows in clinical application or animal experiments [4-6]. Recent research also showed that Near-infrared fluorescence imaging with intracervical ICG injection using the robotic platform has a high bilateral sentinel lymph node detection rate [7]. Because of wide range of functions of ICG, it is necessary to establish analytical methods for the determination of ICG in biological fluids for its further pharmacological and pharmacokinetic studies.

Until now, all analytical methods reported in the literature for determination of Indocyanine Green in biological and other matrices have spectrophotometry [8], high performance liquid chromatography (HPLC) [9], Size-exclusion high performance liquid chromatography (SE-HPLC) [10], liquid chromatography mass spectrometry (LC-MS) [11] and liquid chromatography-tandem mass spectrometry(LC-MS/MS) [2]. In this study, a newly UPLC-MS/MS method was developed and validated for the quantification of Indocyanine Green in rats plasma after intravenous administration of 5.0 mg/kg, with protein precipitation by acetonitrile-methanol (9:1, v/v) using 100 μL plasma as sample treatment. The column was packed with C18 articles of 1.7 mm, which contribute to higher column performance, efficient separation and a short analysis time [12, 13]. The results of study would provide some help to the apprehension of the action mechanism and further pharmacological study of Indocyanine Green.

Experimental

Chemicals and reagents

Indocyanine Green (purity > 98%) and rutin (IS, purity > 98%) were purchased from the Beijing Sunflower and Technology Development CO.

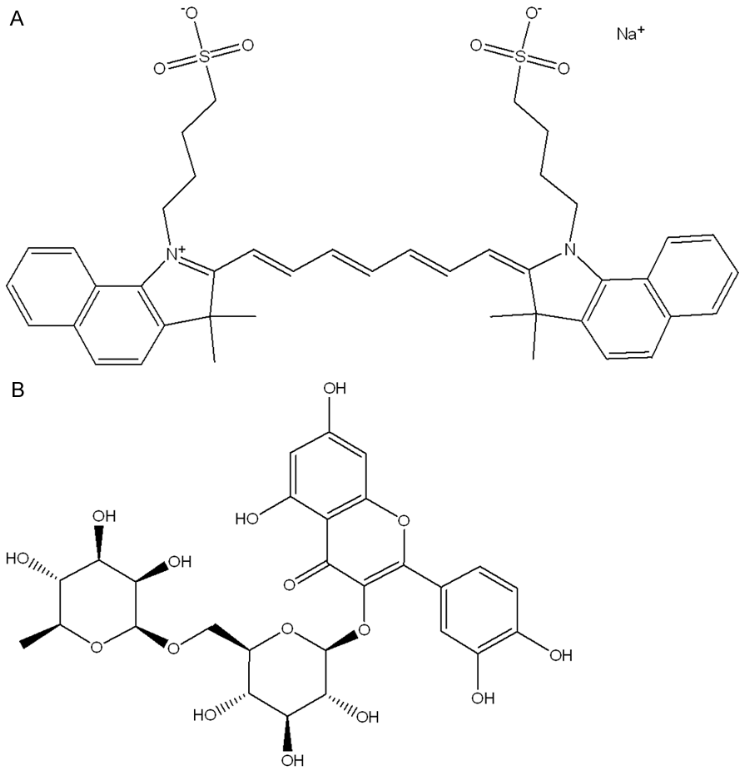

Figure 1. Chemical structure of Indocyanine Green (A) and rutin (IS, B).

LTD. (Beijing, China). LC-grade acetonitrile and methanol were purchased from Merck Company (Darmstadt, Germany). Ultra-pure water was prepared by Millipore Milli-Q purification system (Bedford, MA, USA). Rat blank plasma samples were supplied from drug-free rats.

Instrumentation and conditions

A UPLC-MS/MS system with ACQUITY I-Class UPLC and a XEVO TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA), equipped with an electrospray ionization interface, was used to analyze the compounds. The UPLC system was comprised of a Binary Solvent Manager and a Sample Manager with Flow-Through Needle. Masslynx 4.1 software (Waters Corp., Milford, MA, USA) was used for data acquisition and instrument control.

Indocyanine Green and rutin (IS) were separated on an UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) maintained at 40°C. The initial mobile phase consisted of 0.1% formic acid and acetonitrile with gradient elution at a flow rate of 0.4 mL/min and an injection volume of 2 μL. Elution was in a linear gradient, where the acetonitrile increased from 40% to 90% between 0 and 2.0 min, maintained at 90% for 0.5 min, then decreased to 40% within 0.1 min, then maintained at 40% for 0.4 min. The total run time of the analytes was 3 min. After each injection, the sample manager underwent a needle wash process, including both a strong wash (methanol-water, 50/50, *v/v*) and a weak wash (methanol-water, 10/90, *v/v*).

Nitrogen was used as the desolvation gas (1000 L/h) and cone gas (50 L/h). Ion monitoring conditions were defined as capillary voltage of 1.5 kV, source temperature of 150°C, and desolvation temperature of 500°C. MRM modes of *m/z* 753.4→330.2 for Indocyanine Green, and *m/z* 611.1→303.1 for IS were utilized to conduct quantitative analysis, **Figure 2**.

Calibration standards and quality control samples

The stock solutions of Indocyanine Green (1.0 mg/mL) and rutin (IS) (100 μg/mL) were prepared in methanol-water (50: 50, *v/v*). The 0.25 μg/mL working standard solution of the IS was prepared from the IS stock solution by dilution with methanol; working solutions for calibration and controls were prepared from stock solutions in the same manner. All of the solutions were stored at 4°C and were brought to room temperature before use.

Indocyanine Green calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the working solutions. Calibration plots were offset to range between 20-5000 ng/mL for Indocyanine Green in rat plasma (20, 50, 100, 200, 500, 1000, 2000, 4000 and 5000 ng/mL). Quality-control (QC) samples were prepared in the same manner as the calibration standards, in three different plasma concentrations (40, 2500, and 4500 ng/mL). The analytical standards and QC samples were stored at -20°C.

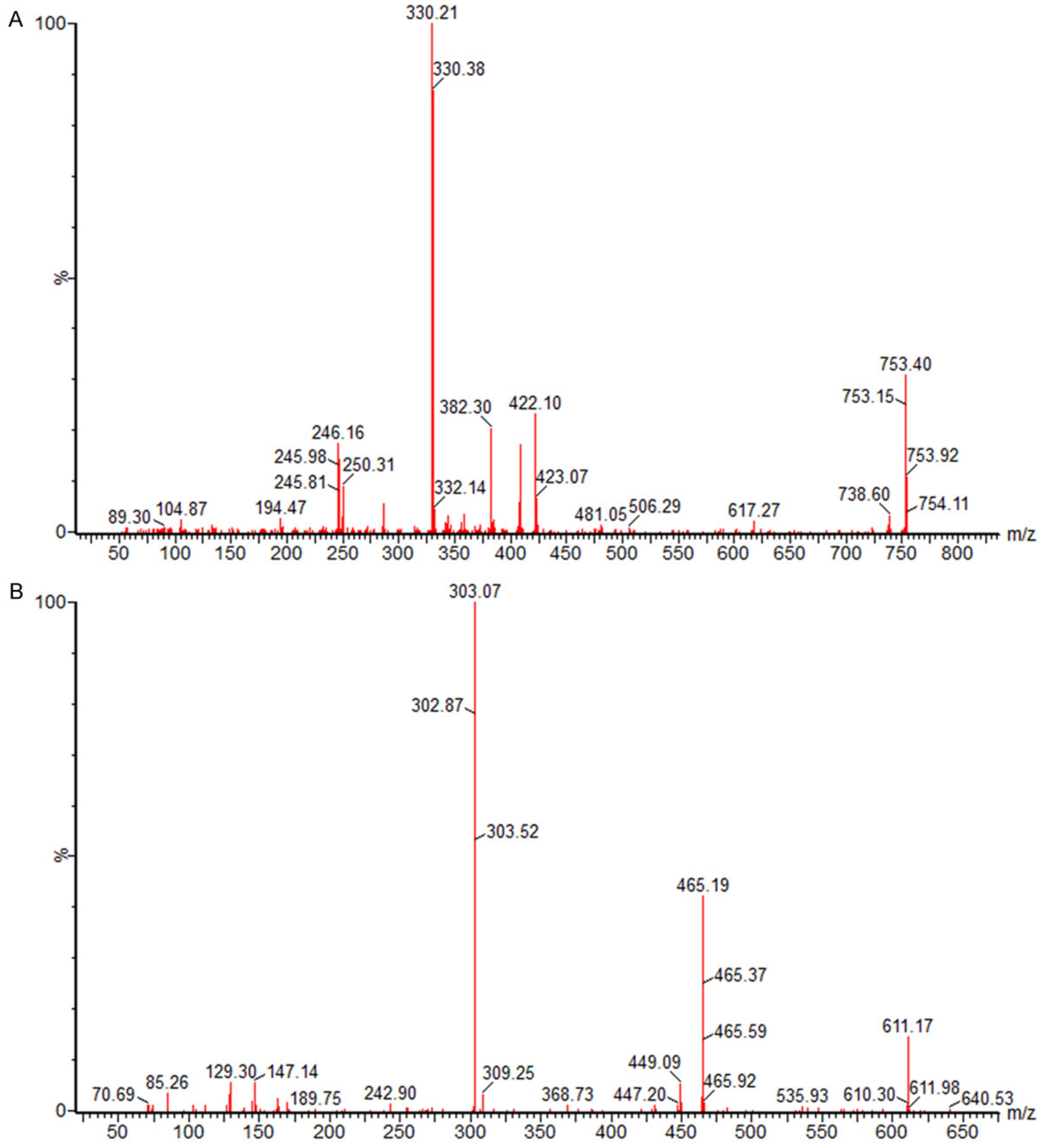

Figure 2. Mass spectrum of Indocyanine Green (A) and rutin (IS, B).

Sample preparation

Before analysis, the plasma sample was thawed to room temperature. An aliquot of 10 μL of the IS working solution (0.25 μg/mL) was added to 50 μL of the collected plasma sample in a 1.5 mL centrifuge tube, followed by the addition of 150 μL of acetonitrile-methanol (9:1, v/v). The tubes were vortex mixed for 1.0 min. After centrifugation at 14900 × *g* for 10 min, the supernatant (2 μL) was injected into the UPLC-MS/MS system for analysis.

Method validation

Rigorous tests for selectivity, linearity, accuracy, precision, recovery, and stability, according to the guidelines set by the United States Food and Drug Administration (FDA) [14] and European Medicines Agency (EMA) [15], were

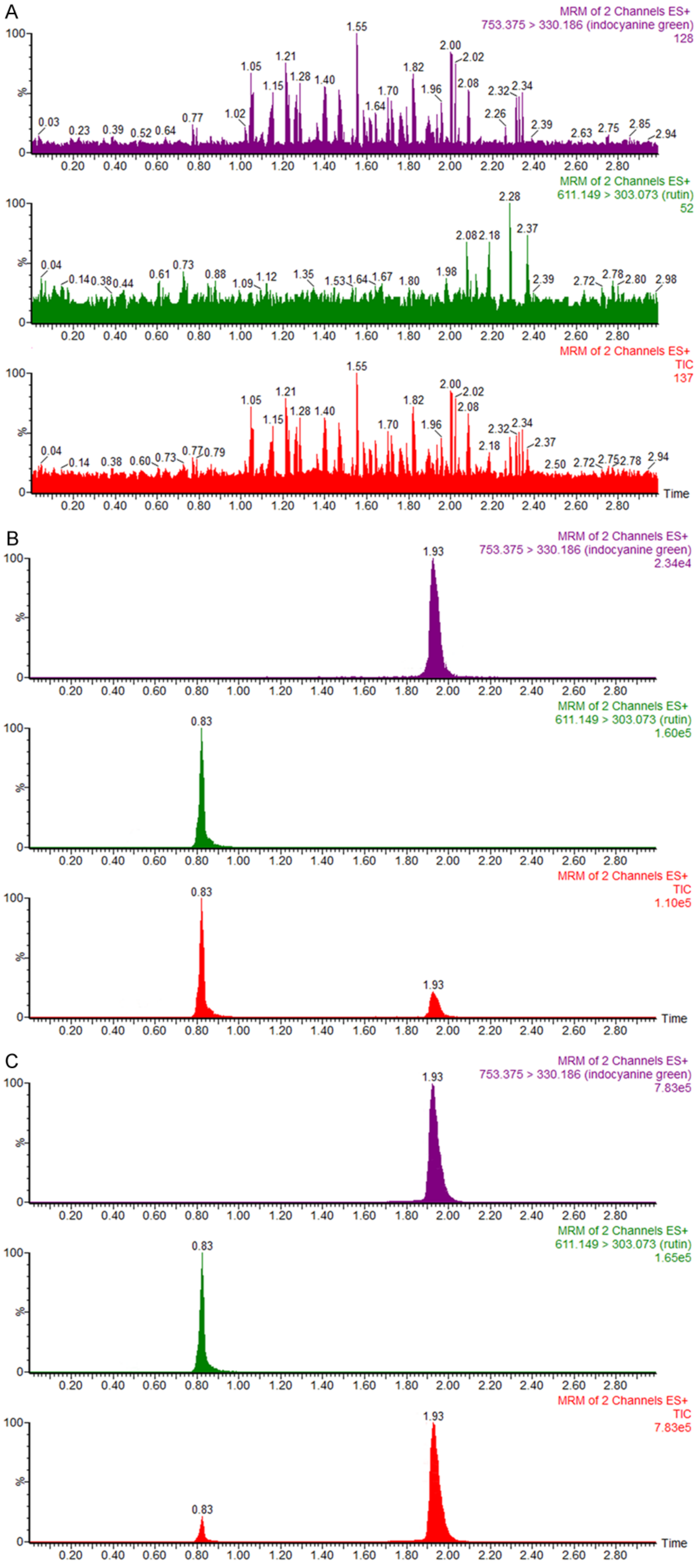

Figure 3. Representative UPLC-MS/MS chromatograms of Indocyanine Green and rutin (IS). A. Blank plasma; B. Blank plasma spiked with Indocyanine Green (20 ng/mL) and IS (50 ng/mL); C. A rat plasma sample 15 min after intravenous administration of single dosage 5 mg/kg Indocyanine Green.

conducted in order to thoroughly validate the proposed bioanalytical method. Validation runs were conducted on three consecutive days. Each validation run consisted of one set of calibration standards and six replicates of QC plasma samples.

The selectivity of the method was evaluated by analyzing blank rat plasma, blank plasma-spiked Indocyanine Green and IS, and a rat plasma sample.

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of Indocyanine Green-to-IS were plotted against analyte concentrations. Resultant standard curves were well fitted to the equations by linear regression, with a weighting factor of the reciprocal of the concentration (1/*x*) in the concentration range of 20-5000 ng/mL. The lower Limit of quantitation (LLOQ) was defined as the lowest concentration on the calibration curves.

To evaluate the matrix effect, blank rat plasma was extracted and spiked with the analyte at 40, 2500, and 4500 ng/mL concentrations. The corresponding peak areas were then compared to those

Table 1. Precision, accuracy, and recovery for Indocyanine Green of QC samples in rat plasma (n = 6)

Concentration (ng/mL)	RSD (%)		Accuracy (%)		Recovery (%)
	Intra-day	Inter-day	Intra-day	Inter-day	
40	11.5	10.5	106.3	113.9	85.4
2500	7.2	6.9	100.4	102.8	79.5
4500	6.3	8.1	98.2	95.9	83.4

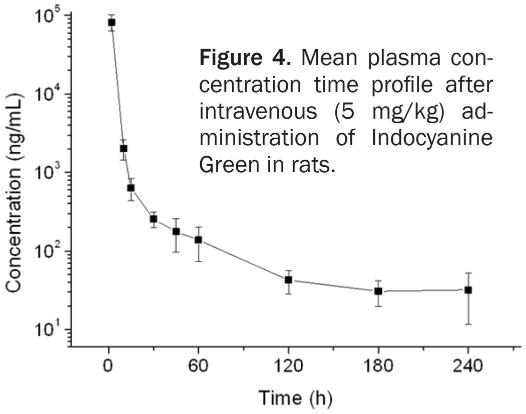

Figure 4. Mean plasma concentration time profile after intravenous (5 mg/kg) administration of Indocyanine Green in rats.

of neat standard solutions at equivalent concentrations, this peak area ratio is defined as the matrix effect. The matrix effect of the IS was evaluated at a concentration of 50 ng/mL in a similar manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (40, 2500, and 4500 ng/mL) over three days of validation testing. The precision is expressed as RSD.

The recovery of Indocyanine Green was evaluated by comparing the peak area of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts (n = 6). The recovery of the IS was determined in the same way.

Carry-over was assessed following injection of a blank plasma sample immediately after 3 repeats of the upper limit of quantification (ULOQ), after which the response was checked for accuracy [16].

Stability of Indocyanine Green in rat plasma were evaluated by analyzing three replicates of plasma samples at concentrations of 40 and 4500 ng/mL which were all exposed to different conditions. These results were compared with the freshly-prepared plasma samples. Short-term stability was determined after the exposure of the spiked samples to room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) in the HPLC autosampler at room temperature for 24 h. Freeze/thaw stability was evaluated after three complete freeze/thaw cycles (-20 to 25°C) on consecutive days. Long-term stability was assessed after storage of the standard spiked plasma samples at -20°C for 20 days. The stability of the IS (50 ng/mL) was evaluated similarly [17, 18].

Pharmacokinetic study

All six Male Sprague-Dawley rats (200-220 g) were obtained from the Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China) to study the pharmacokinetics of Indocyanine Green. The ethical number of the experiment animals was wydw 2013-0071. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical University. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples (0.2 mL) were collected from the caudal vein into heparinized 1.5 mL tapered plastic centrifuge tubes at 2, 10, 15, 30, 45, 60, 120, 180, and 240 min after intravenous (5 mg/kg) administration of Indocyanine Green. The caudal vein of rat was cleaned by 75% alcohol, after that the end of caudal vein was cut by scissors. A 1.5 mL tapered plastic centrifuge tube was used to collect the blood which dropped from the end of caudal vein by squeezing and massaging gently. The samples were immediately centrifuged at 3000 × g for 10 min. The plasma as-obtained (50 μL) was stored at -20°C until UPLC-MS/MS analysis. Plasma Indocyanine Green concentration versus time data for each rat was analyzed by DAS (Drug and Statistics) software (Version 2.0, Wenzhou Medical University, China).

Results and discussion

Method development

The mobile phase played a critical role in achieving good chromatographic behavior and

Table 2. Primary pharmacokinetic parameters after oral or intravenous administration of Indocyanine Green in rats (n = 6)

Parameters	Unit	Mean	SD
		Intravenous, 15 mg/kg	
$AUC_{(0-t)}$	ng/mL *min	501111.2	256317.6
$AUC_{(0-\infty)}$	ng/mL *min	503070.1	255807.0
$t_{1/2}$	min	79.5	29.2
$MRT_{(0-t)}$	min	5.8	4.7
$MRT_{(0-\infty)}$	min	9.1	9.5
CL	L/min/kg	0.02	0.03
V	L/kg	2.7	4.1
C_{max}	ng/mL	68095.1	37439.8

appropriate ionization [19-25]. Acetonitrile and water (containing 0.1% formic acid) were chosen as the mobile phase because the combination provides harper peak shape, better sensitivity and proper retention time. Efficient removal of proteins and other potential interference in the bio-samples prior to LC-MS analysis was a crucial step in the development of this method [26-30]. Then the simple protein precipitation was employed in our work, acetonitrile-methanol (9:1) was chosen as the protein precipitation solvent because it exhibited acceptable recovery (between 79.5% and 85.4%).

Selectivity and matrix effect

Figure 3 shows typical chromatograms of a blank plasma sample, a blank plasma sample spiked with Indocyanine Green and IS, and a plasma sample. There were no interfering endogenous substances observed at the retention time of the Indocyanine Green and IS.

The matrix effect for Indocyanine Green at concentrations of 40, 2500, and 4500 ng/mL were measured to be 104.5%, 96.1% and 98.2% (n = 6). The matrix effect for IS (50 ng/mL) was 103.6% (n = 6). As a result, matrix effect from plasma was considered negligible in this method.

Calibration curve and sensitivity

Linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 20-5000 ng/mL for Indocyanine Green in rat plasma. The equation utilized to express the calibration curve was: $y = 0.00003265*x+0.000148375$, $r = 0.9979$, where y represents the ratios of Indocyanine Green peak area to that of IS, and x represents the plasma concentration. The LLOQ for the determination of Indocyanine Green in plasma was 20 ng/mL. The precision and accuracy at LLOQ were 17.7% and 84.7%.

Precision, accuracy and recovery

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three days of validation tests. Intra-day precision was 12% or less, and inter-day precision was 11% or less at each QC level. The accuracy of the method ranged from 95.9% and 113.9% at each QC level. Mean recoveries of Indocyanine Green were higher than 79.5%. The recovery of the IS (50 ng/mL) was 80.7%. Assay performance data was presented below in **Table 1**.

Carry-over

None of the analytes showed any significant peak (≥ 20% of the LLOQ and 5% of the IS) in blank samples injected after the ULOQ samples. Adding 0.4 extra minutes to the end of the gradient elution effectively washed the system between samples, thereby eliminating carry-over [16].

Stability

Results from the auto-sampler showed that the Indocyanine Green was stable under room temperature, freeze-thaw, and long-term (20 days) conditions, confirmed because the bias in concentrations were within 90% and 115% of their nominal values. To this effect, the established method was suitable for pharmacokinetic study.

Application

The method was applied to a pharmacokinetic study in rats. The mean plasma concentration–time curve after intravenous (5 mg/kg) administration of Indocyanine Green was shown in **Figure 4**. Primary pharmacokinetic parameters, based on non-compartment model analysis, were summarized in **Table 2**.

Conclusion

The developed and validated UPLC-MS/MS method, utilizing only 50 μL of plasma with an LLOQ of 20 ng/mL, was successfully applied to a pharmacokinetic study of Indocyanine Green after intravenous administration.

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Disclosure of conflict of interest

None.

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