A liquid chromatography tandem mass spectrometry method for the quantification of indocyanine green in dog plasma and bile

Cliff Y. Chen [*], R. Marc Fancher, Qian Ruan, Punit Marathe,
A. David Rodrigues, Zheng Yang

*Metabolism and Pharmacokinetics, Pharmaceutical Candidate Optimization, Bristol-Myers Squibb Co.,
P.O. Box 4000, Rt. 206 and Province Line Road, Princeton, NJ 08543, United States*

Abstract

A sensitive and specific liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) method was developed and validated for the determination of indocyanine green (ICG) in dog plasma and bile. An ICG analog (IR-820) was used as an internal standard. A protein precipitation method was used with acetonitrile for plasma sample preparation, whereas bile samples were diluted with water (120-fold) prior to analysis. Using MS/MS in the multiple reaction monitoring mode, ICG and IR-820 were detected in both matrices without interference. The lower limit of quantitation for ICG in dog plasma was 3 ng/mL, with an intra- and inter-day accuracy (%Bias) and precision (%CV) of less than 15%, so it was possible to study the pharmacokinetics of ICG in bile duct-cannulated dogs and assess their liver function after surgery. The method described herein is sensitive, selective and faster than other existing methods (e.g., spectrophotometry, HPLC/UV–vis detection, or HPLC/fluorescence detection).
© 2008 Elsevier B.V. All rights reserved.

Keywords: Indocyanine green; LC/MS/MS; Plasma; Bile; Pharmacokinetics

1. Introduction

Indocyanine green (ICG) is a synthetic organic anion that has been widely used to evaluate liver function and to estimate organ (e.g., liver and heart) and other regional blood flows in humans and laboratory animals [1–6]. This is because ICG is restricted largely in plasma volume after dosing and is cleared extensively by the liver as unchanged into the bile. A number of analytical assays have been reported in the past for the determination of ICG concentrations in plasma, which include spectrophotometry [7,8], HPLC/UV–vis detection [9,10], and HPLC/fluorescence detection [11]. However, these methods generally suffer from low assay sensitivity (0.4–2 µg/mL) and require long chromatographic run times (10–15 min). In addition, the spectrophotometric method, which

is based on the absorbance of ICG at 775–800 nm, is subject to interference by ICG impurities (only 86% pure from Sigma) [10,12], degradation and/or biotransformation products [9,11], and background turbidity from plasma or other biological samples [13].

To overcome the aforementioned issues associated with previous ICG assays, a rapid, sensitive and specific LC/MS/MS-based method was developed for the quantitation of ICG in dog plasma and bile. It was possible to evaluate the pharmacokinetics of ICG in bile duct-cannulated dogs and to assess their liver function after surgery.

2. Experimental

2.1. Reagents and chemicals

ICG and the internal standard (IS) IR-820 (Fig. 1) were purchased from Sigma (St. Louis, MO, USA). The purity of ICG and IR-820 was 86 and 90%, respectively. All solvents used

---

[*] Corresponding author. Tel.: +1 609 252 5679; fax: +1 609 252 6802.
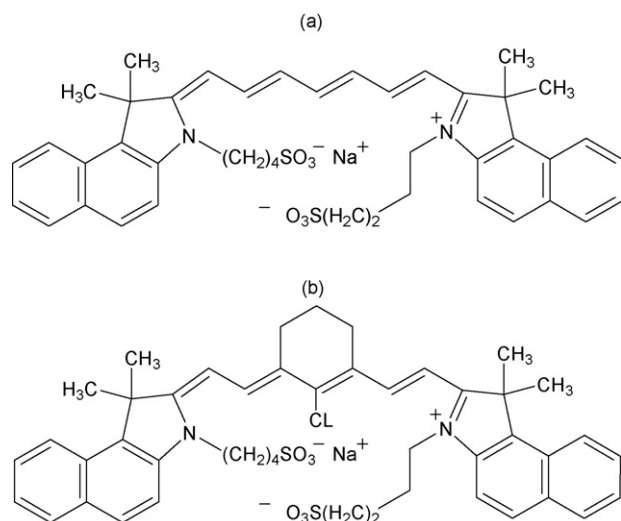*E-mail address:* cliff.chen@bms.com (C.Y. Chen).

Fig. 1. Chemical structures of indocyanine green (a) and IR-820 (b).

were HPLC grade and were obtained from EM Science (Gibbstown, NJ, USA). Blank dog plasma and bile were ordered from Bioreclamation Inc. (Hicksville, NY, USA).

2.2. LC–MS/MS conditions

LC–MS/MS analysis was performed using a Micromass Quattro LC triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with electrospray ionization interface. The HPLC system consisted of a pair of LC 10AD pumps, a SCL-10A controller (Shimadzu, Columbia, MD, USA), and an HCT PAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a cooling stack maintained at 10 °C. The HPLC column used was an Atlantis® dC18 (3 µm particle size, 2.1 mm × 100 mm) purchased from Waters (Milford, MA, USA). The mobile phase, pumped at a flow rate of 0.5 mL/min, consisted of 10 mM ammonium acetate (A) and acetonitrile (B), with pH adjusted to 3.0 using formic acid. The initial mobile phase composition was 60% A/40% B. After sample injection, the mobile phase composition was held constant for 2.0 min and then changed linearly to 10% A/90% B over 0.5 min and held at that composition for an additional 1.0 min. The mobile phase was then returned to initial conditions over 0.5 min. The total analysis time was 4.0 min.

The electrospray interface was maintained at 350 °C. Nitrogen nebulization was performed at a nitrogen flow of 90 L/h. Argon was used as the collision gas. ICG and the IS were detected in the multiple reaction monitoring (MRM) mode with a positive ion detection. The parameters were set up as follows: capillary voltage at 3.0 kV, cone voltage at 35 V, extractor at 9 V, RF lens at 0.6 V, source temperature at 120 °C, collision cell entrance potential at −1.0 V, collision energy at 35 eV, collision cell exit at 5 V, multiplier at 700 V and dwell time of 0.20 s. Ions representing the $(M + H)^+$ species for ICG and the IS were selected in MS1 and collisionally dissociated with UHP argon at a pressure of 2×10^{-3} Torr to form specific product ions which were subsequently monitored by MS2. The transitions moni-

tored were m/z 753 → 330 (ICG) and m/z 827 → 330 (IS). Cone voltage and collision energy were optimized for ICG at 35 volts. For the IS, the two parameters were 35 and 45 V, respectively.

2.3. Preparation of standard and quality control samples

To minimize the potential of ICG degradation in aqueous media, a stock solution of ICG was prepared in methanol (1 mg/mL). From the stock solution, working standard solutions of ICG ranging (0.1–80 µg/mL) were prepared by sequential dilution with acetonitrile. The calibration curve in plasma consisting of 10 standards was then prepared from working standard solutions at ICG concentrations of 1, 5, 10, 20, 50, 100, 200, 400, and 800 ng/mL. Similarly, the calibration curve in bile was prepared at the same concentrations as the plasma standards after dilution with water (120-fold). Four levels of QC samples (3, 15, 150, and 600 ng/mL) in plasma or bile were prepared separately in the same fashion. In addition, a stock solution of the IS was prepared in methanol (1 mg/mL) and subsequently diluted with acetonitrile to make a working solution concentration of 100 ng/mL.

2.4. Sample preparation

Aliquots of dog plasma (150 µL) were placed into vials inserted in a 96 (deep) well plate, followed by addition of acetonitrile containing the IS (450 µL). The mixtures were vortexed for 20 s and centrifuged at 5000 × g for 10 min. The clear supernatants were then transferred to vial inserts and placed onto autosampler trays for injection. For bile samples, they were diluted with water (120-fold) and then treated in the same fashion as the plasma samples.

2.5. Method validation

The linearity of the assay was assessed by analyzing the calibration curves (1–800 ng/mL) in plasma and bile using a weighted ($1/y^2$) linear regression of the peak area ratios of ICG to the IS versus the nominal concentrations of the calibration standards.

To assess the intra-day accuracy and precision of the method, 6 concentrations of ICG (3, 5, 10, 50, 200, and 500 ng/mL) were spiked into plasma, with 6 replicates independently prepared at each concentration. In addition, ICG was spiked into bile at 5 different concentrations followed by dilution with water, so that the final concentrations of ICG were 5, 10, 50, 200, and 500 ng/mL. Similarly, the inter-day accuracy and precision was evaluated on 3 separate occasions, with 6 replicates at each aforementioned concentration (2 replicates per occasion). The accuracy was expressed as %Bias and calculated as [(mean observed concentration)–(nominal concentration)]/(nominal concentration) × 100%. The precision was expressed as %coefficient of variation (%CV) and calculated as (standard deviation of observed concentrations)/(mean of observed concentrations) × 100%.

The limit of detection (LOD) was defined as the concentration at which the signal-to-noise ratio (S/N) of the instrument

response was 10. The lower limit of quantification in plasma was the lowest concentration of ICG that could be measured with a precision (%CV) of 20% and accuracy (%Bias) of ± 20%.

The recovery of ICG (3 and 600 ng/mL) from plasma was determined in two occasions by comparing the peak area ratios of ICG relative to the IS from extracted samples with those in post-extraction blank matrix samples spiked with ICG and the IS at the same concentration.

The matrix effect in plasma was evaluated at 3 concentrations of ICG (15, 150, and 600 ng/mL) by the comparison of the ICG-IS peak area ratios of samples spiked in plasma relative to samples spiked in the aqueous mobile phase (10 mM ammonium acetate). In addition, a post-column infusion experiment was conducted to examine the matrix effect using the procedure described by Bonfiglio et al. [14]. Briefly, a syringe pump (Harvard Apparatus Model 11, South Natick, MA, USA) was connected with the HPLC column and electrospray interface using a tee union. ICG, dissolved in acetonitrile at 10 μg/mL, was infused into the electrospray interface via the syringe pump post-column at a flow rate of 0.05 mL/min along with the mobile phase pumped at a flow rate of 0.5 mL/min. A post-extraction blank plasma sample (10 μL) was then injected onto the column and separated using the sample analysis gradient as described above. The chromatogram generated was compared with that after the injection of the aqueous mobile phase. In this setting, a difference in the baseline at the retention time of the analyte of interest between the injections of the post-extraction blank plasma sample and aqueous mobile phase would indicate a matrix effect. No experiments were performed to assess the recovery and matrix effect of ICG in bile samples, as samples were substantially diluted with water (120-fold) prior to sample analysis.

Stability studies in plasma and bile samples were also conducted at 2 concentrations of ICG (3 and 600 ng/mL) to evaluate stability after 3 freeze–thaw cycles, short-term storage stability at (−20 °C for a week), and post-preparative stability in the autosampler (10 °C for 12 h). In addition, bench-top stability was evaluated for the stock solutions for up to 3 h and was also performed for plasma and bile samples for up to 6 h.

2.6. Spectrophotometric method for determination of ICG in dog plasma

For the purpose of comparison, a spectrophotometric method described by Kawasaki et al. [7] was used to measure ICG concentrations in dog plasma. Briefly, aliquots of dog plasma samples (500 μL) were diluted with de-ionized water (6-fold) and centrifuged at 3000 × *g* for 10 min (Allegra 25R Centrifuge, Beckmann Coulter, Fullerton, CA, USA). The supernatant was analyzed at a wavelength of 800 nm using a Safire^2 spectrophotometer (Tecan US, Research Triangle Park, NC, USA).

2.7. Pharmacokinetic study and data analysis

ICG, prepared at 2.0 mg/mL in sterilized water, was administered intravenously via the femoral vein as a bolus dose of 0.5 mg/kg (0.4 mL/kg) to bile duct-canulated (BDC) dogs

(12.3 ± 1.3 kg, *n* = 3). Care was taken to prevent the exposure of the dosing solution to light prior to the study. Blood samples were taken in K2-EDTA vacutainers at pre-dose, 1, 2, 5, 10, 20, 30, 45 min, and 1, 1.5, 2, and 4 h after intravenous dosing. Bile samples were collected in 30 min intervals for 4 h, and the bile flow rate was determined by averaging bile flow collected at each interval. Harvested plasma samples were stored at −20 °C and analyzed within a week.

The pharmacokinetic parameters of ICG were obtained by the non-compartmental analysis of plasma concentration vs. time data (KINETICA^{TM} software, Version 4.2, InnaPhase Corporation, Philadelphia, PA, USA). The elimination rate constant (kel) was estimated from the terminal phase of the profile by nonlinear regression using an exponential equation. The total areas under the curve (AUC$_{0\text{-inf}}$) and under the first moment curve (AUMC$_{0\text{-inf}}$) were calculated using a log-linear trapezoidal summations to the last measured concentration (C$_{p,last}$), with an extrapolation to infinity using C$_{p,last}$/k$_{el}$ for AUC$_{t\text{-inf}}$ and C$_{p,last}$*t/k$_{el}$ + C$_{p,last}$/k$_{el}^2$ for AUMC$_{t\text{-inf}}$. The total plasma clearance (CLtot), steady-state volume of distribution (Vss), terminal half-life (T1/2) and mean residence time (MRT) were also estimated using the following equations: CLtot = dose/AUC; Vss = dose*AUMC/AUC^2; T1/2 = 0.693/k$_{el}$; MRT = AUMC/AUC.

3. Results and discussion

3.1. Mass spectrometry

Under the electrospray ionization conditions chosen, both ICG and IR-820 (IS) exhibited higher sensitivity in the positive ion mode than in the negative ion mode, with the protonated molecular ion [*M* + H]$^+$ as the major species. Fig. 2 shows the full-scan positive ion spectrum of ICG and IR-820 at the first quadrupole and daughter ion spectrum at the third quadrupole. For both compounds, the most abundant product ion was *m/z* 330 and was selected for the MRM analysis.

3.2. Chromatography

A reverse-phase chromatographic method was developed with ammonium acetate and acetonitrile as the mobile phase. Ammonium acetate was used because it was easily miscible with organic solvents and led to improved peak symmetry and ionization. Indeed, much higher ion intensities were achieved in the presence of ammonium acetate. The amount of acetonitrile in mobile phase was optimized at 40–90%, with the pH of the mobile phase adjusted to 3.0. Under these conditions, ICG and its IS were well separated chromatographically (Fig. 3). The retention time for ICG and IS was 2.8 and 2.9 min, respectively.

3.3. Method validation

3.3.1. Selectivity

Fig. 4 shows blank vs. spiked samples at the ICG concentration of 1 ng/mL in dog plasma and bile. There were
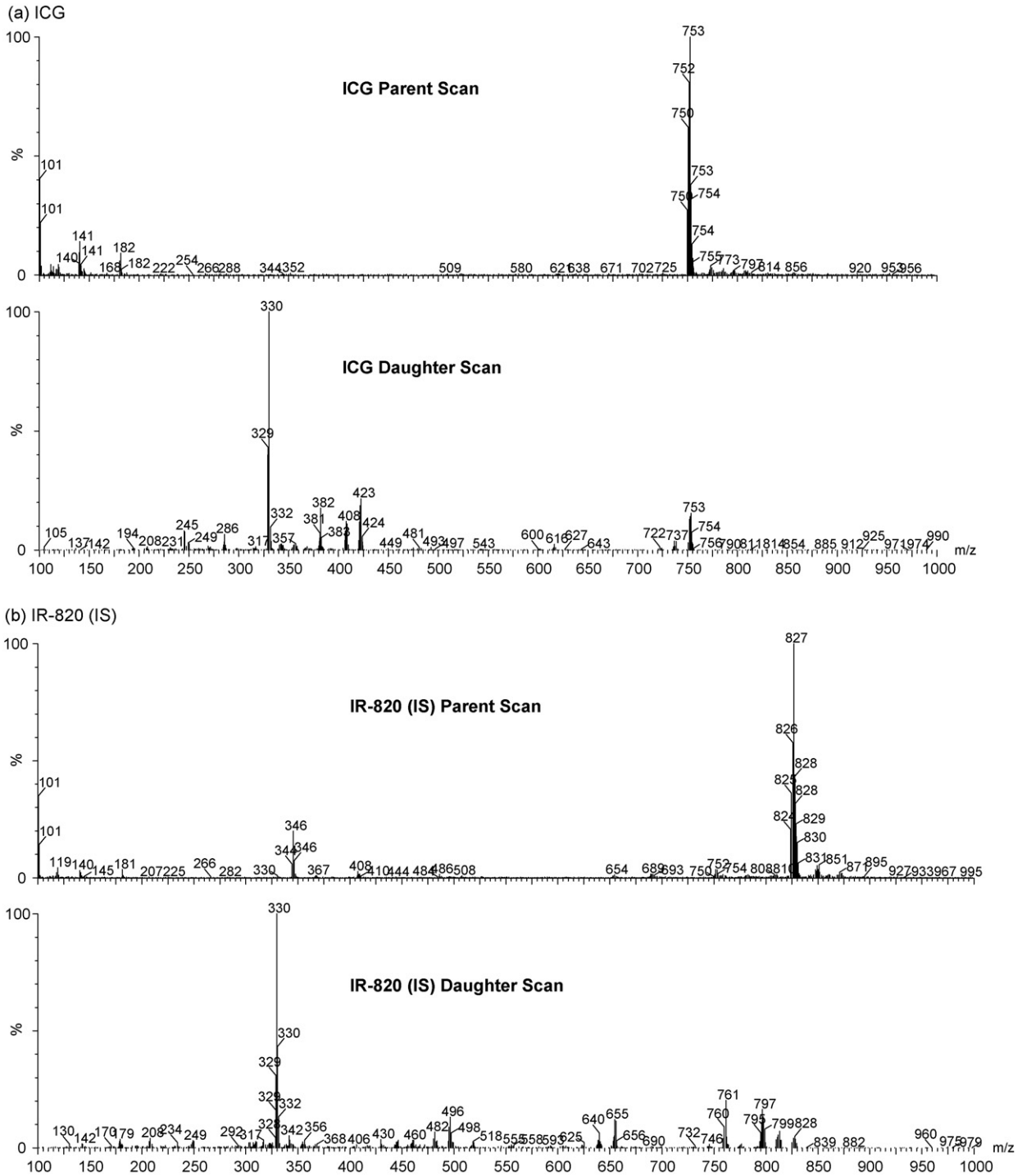
Fig. 2. Full-scan positive ion spectrum of precursor at the first quadrupole and product ion spectrum at the third quadrupole for ICG (a) and IR-820 (b).

no apparent endogenous interferences from the plasma and bile matrices, demonstrating satisfactory selectivity of the present method for determination of ICG in dog plasma and bile.

3.3.2. Accuracy, precision, recovery, and matrix effects

Tables 1 and 2 summarize the intra- and inter-day accuracy and precision for the determination of ICG in dog plasma and bile, respectively. For plasma samples, the intra- and inter-day accuracy (%Bias) at 6 concentrations (3 to 500 ng/mL) ranged from −7.8 to 11.1% and −10.2 to 11.9%, respectively (Table 1). The intra- and inter-day precision (%CV), based on six replicates at each concentration level, ranged from 2.2 to 9.2% and 1.5 to 14.2%, respectively (Table 1). Similarly, the intra- and inter-day accuracy (%Bias) in dog bile samples at 5 concentrations (5–500 ng/mL) ranged from −4.5 to 8.6% and −2.8 to 9.3%,
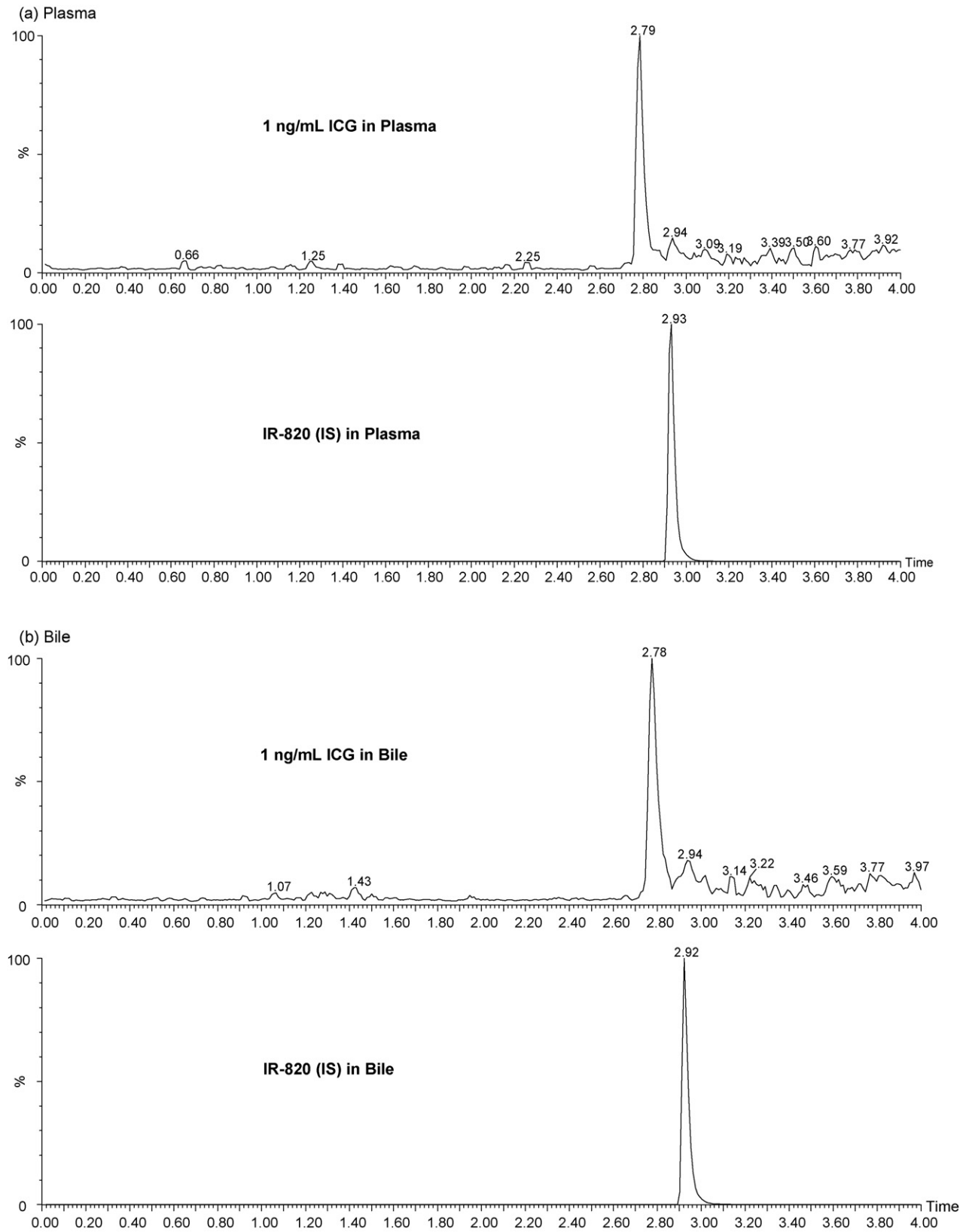
Fig. 3. Representative MRM chromatograms in dog plasma (a) and bile (b) spiked with ICG (1 ng/mL) and the internal standard IR-820.

Table 1a
Intra-day accuracy and precision for the determination of ICG in dog plasma

Nominal concentration (ng/mL)	Calculated concentration (ng/mL)[a]	Accuracy (%Bias)	Precision (%CV)
3	3.33 ± 0.308	11.1	9.2
5	4.61 ± 0.38	−7.8	8.4
10	9.83 ± 0.54	−1.6	5.5
50	50.7 ± 2.3	1.4	4.6
200	188.9 ± 8.8	−5.5	4.7
500	511.6 ± 11.6	2.3	2.2

[a] Mean ± S.D., $n = 6$.

Table 1b
Inter-day accuracy and precision for the determination of ICG in dog plasma

Nominal concentration (ng/mL)	Calculated concentration (ng/mL)[a]	Accuracy (%Bias)	Precision (%CV)
3	3.35 ± 0.475	11.9	14.2
5	4.48 ± 0.45	−10.2	10.2
10	9.70 ± 0.74	−3.0	7.7
50	54.3 ± 2.2	8.7	4.1
200	196.2 ± 8.0	−1.8	4.0
500	501.9 ± 7.6	0.38	1.5

[a] Mean ± S.D., $n = 6$.

respectively (Table 2). The intra- and inter-day precision (%CV) in dog bile samples ranged from 2.4 to 8.2% and 1.6 to 9.2%, respectively (Table 2).

The recovery of ICG from dog plasma was determined in two separate experiments (3 replicates per experiment) and was found to be 94–100% at 3 ng/mL and 84.3–85.5% at 600 ng/mL, indicating that the recovery is constant between experiments.

The matrix effect in plasma samples was examined using two methods. In the first method, the instrument response of ICG spiked in plasma samples (measured as the peak area ratio of

Table 2a
Intra-day accuracy and precision for the determination of ICG in dog bile

Nominal concentration (ng/mL)	Calculated concentration (ng/mL)[a]	Accuracy (%Bias)	Precision (%CV)
5	5.43 ± 0.27	8.6	5.0
10	9.55 ± 0.75	−4.5	7.9
50	50.1 ± 4.6	−0.7	8.2
200	190.8 ± 7.8	−4.6	4.1
500	514.8 ± 12.7	2.9	2.4

[a] Mean ± S.D., $n = 6$.

Table 2b
Inter-day accuracy and precision for the determination of ICG in dog bile

Nominal concentration (ng/mL)	Calculated concentration (ng/mL)[a]	Accuracy (%Bias)	Precision (%CV)
5	5.46 ± 0.50	9.3	9.2
10	10.98 ± 0.52	9.8	4.8
50	53.3 ± 2.9	6.5	5.5
200	194.4 ± 7.1	−2.8	3.7
500	504.4 ± 8.0	0.9	1.6

[a] Mean ± S.D., $n = 6$.

Table 3
Pharmacokinetic parameters of ICG following an IV bolus of 0.5 mg/kg to bile duct-cannulated dogs

PK parameter	Mean ± S.D., $n = 3$
CLtot (mL/min/kg)	7.1 ± 1.5
Vss (L/kg)	0.16 ± 0.06
Terminal T1/2 (h)	0.71 ± 0.078
MRT (h)	0.38 ± 0.064
% Dose excreted unchanged in the bile[a]	74 ± 11

[a] Over a 4-h period.

ICG vs. the IS) was compared with that spiked in the aqueous mobile phase. The percentage of the peak area ratio of ICG spiked in plasma relative to that in the aqueous mobile phase was 98.7 ± 8.0, 110.5 ± 6.1, 114.5 ± 7.9% at 15, 150, and 500 ng/mL, respectively ($n = 3$), indicating no significant matrix effect. This was confirmed with the second method, where no significant change in the baseline was observed at the retention time of ICG (2.8 min) in the presence of a post-extraction blank plasma sample (Fig. 5).

3.3.3. Calibration curve

The linearity of the instrument response in blood and bile samples was demonstrated over a concentration range of 1–800 ng/mL, with a coefficient of determination (R^2) greater than 0.998. For dog plasma, a typical regression equation of the calibration curve was $Y = 6.78 \times 10^{-4} + 3.11 \times 10^{-4} X$, with the standard error of the intercept and slope equal to 1.06×10^{-3} and 3.43×10^{-6}, respectively. For dog bile, it was $Y = 8.76 \times 10^{-5} + 9.13 \times 10^{-5} X$, with the standard error of the intercept and slope equal to 4.67×10^{-4} and 1.52×10^{-6}, respectively.

On the basis of a signal-to-noise ratio (S/N) of 10, the limit of detection for ICG was found to be 1 ng/mL on an injection of 20 μL of sample into the LC–MS/MS system, and the lower limit of quantitation (LLOQ) for ICG in dog plasma was found to be 3 ng/mL, with the %Bias and %CV less than 15%. This is a significant improvement over other quantitation methods where the LLOQ is in a range of 400–2000 ng/mL [7–11].

3.3.4. Stability

The stability of ICG was evaluated at 3 and 600 ng/mL in dog plasma and bile. ICG were stable at −20 °C for at least 1 week and its concentration did not show any significant change after 3 freeze and thaw cycles. The prepared samples in acetonitrile with the IS in the autosampler was also stable at 10 °C for at least 12 h. In addition, the bench-top stability of ICG was good for at least 3 h in stock and working solutions and was good for at least 6 h in dog plasma and bile samples.

It has been reported that ICG was not stable in aqueous solutions at room temperature in ambient light, with a degradation half-life of 14 h [15]. Care was taken to examine the stability of the dosing solution covered from light and stored at 4 °C for overnight, a condition used in the pharmacokinetic study. The
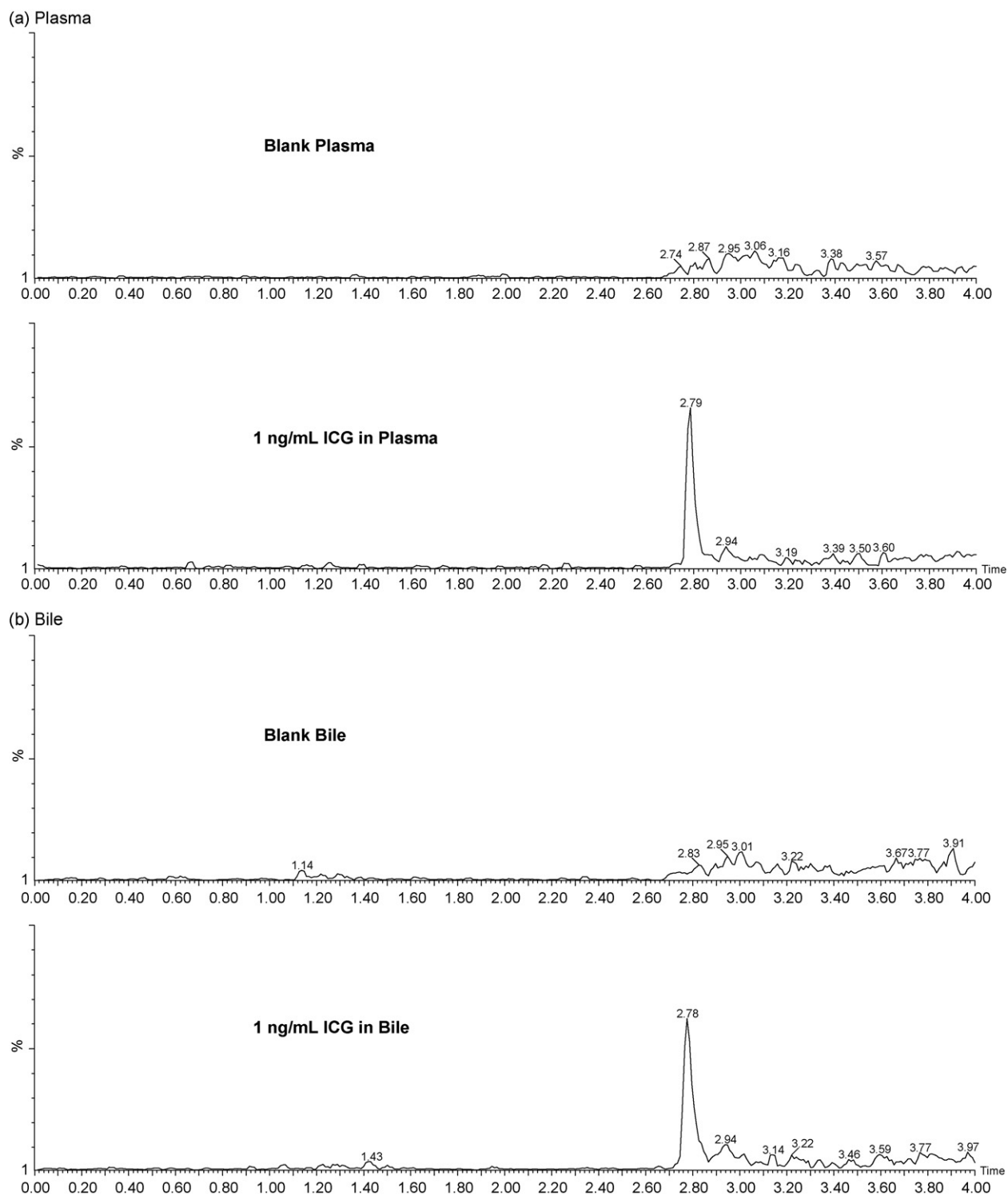
Fig. 4. Blank vs. spiked samples at the ICG concentration of 1 ng/mL in dog plasma (a) and bile (b). For comparison, the chromatograms shown have the same intensity scale in the *Y*-axis.

LC/MS/MS results showed no change in the dosing solution concentration. This was further confirmed using LC/UV/Ion-trap MS (Surveyor LC with a photo-diode array detector and LCQ Deca XP, Thermo Fisher Scientific, Bremen, Germany) where no formation of degradation products was found when compared with a freshly prepared dosing solution. In comparison, a posi-

tive control sample, where ICG was spiked in water and stored at room temperature under ambient light after 3 days, showed significant degradants of ICG. In addition, the same technique was used to monitor the formation of degradants of ICG for all the stability studies performed above and found no degradation in the samples.
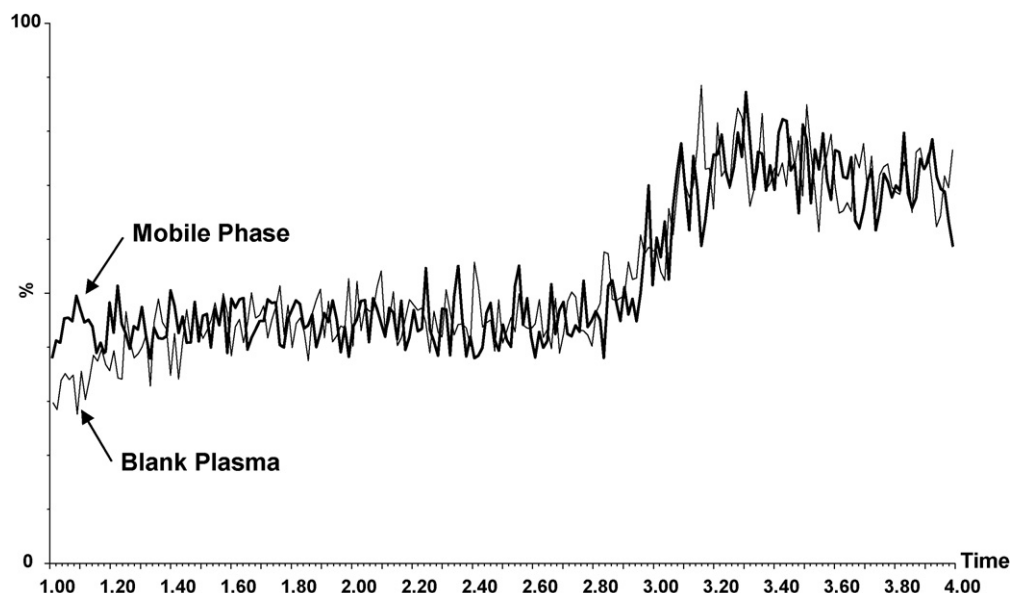
Fig. 5. Chromatograms from a post-column infusion experiment displaying no significant matrix effect upon the injection of a post-extraction blank plasma sample onto the column (chromatograms shown after the switching valve turned on at 1 min).

3.4. Pharmacokinetic study in bile duct-cannulated dogs

Using the LC/MS/MS assay established above, the pharmacokinetics of ICG was studied in bile duct-cannulated dogs in order to evaluate their liver function after surgery. Following intravenous injection, ICG exhibited a bi-exponential decline in plasma (Fig. 6), with an average terminal T1/2 of 0.71 h. The CLtot was 7.1 ± 1.5 mL/min/kg and the Vss was 0.16 ± 0.06 L/kg (Table 3). ICG was predominately secreted into the bile. Over a 4-h study period, the amount recovered unchanged in the bile was $74.2 \pm 10.7\%$ of the total dose (Fig. 7). The average bile flow rate in these dogs was determined to be 5.6 ± 1.1 µg/min/kg, comparable to the literature value [7,16]. These results demonstrate that liver function is not impaired after surgical preparations in these dogs.

The present LC/MS/MS method represents a significant improvement over the previous bioanalytical methods where the assay sensitivity is reported to be in the range of 400–2000 ng/mL [7–11]. As shown in Fig. 6, a significant portion of the terminal phase of ICG plasma concentration–time curve would have been missed had the LLOQ been 400 ng/mL. In addition to the improvement in the assay sensitivity, the current method is more selective than other methods. In a separate study, the ICG plasma concentrations in dog plasma were measured using a spectrophotometric method as described by Kawasaki et al. [7]. Although the plasma concentration–time profile was similar between the two methods, the ICG concentrations measured by the spectrophotometric method were generally higher than those determined by the LC/MS/MS method (Fig. 6). As a result, the average plasma CLtot in dogs by the spectrophotometric method was 3.3 ± 1.1 mL/min/kg, which is similar to other reported values 3.7 ± 0.6 mL/min/kg
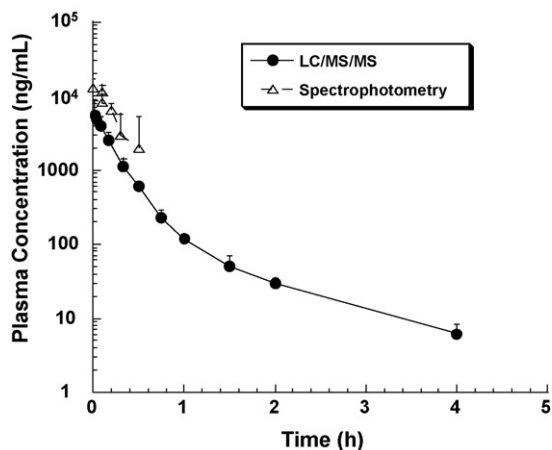

Fig. 6. Plasma concentration–time profiles of ICG following an IV bolus of 0.5 mg/kg to bile duct-cannulated dogs determined by either the LC/MS/MS or spectrophotometric method (mean ± S.D., *n* = 3).
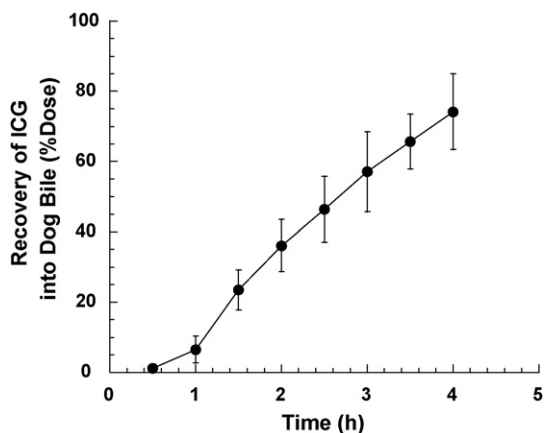

Fig. 7. Recovery (%dose) of ICG in dog bile after a 0.5 mg/kg dose over a 4-h period (Mean ± S.D., *n* = 3).

by Kawasaki et al. [7] and 3.6 ± 0.3 mL/min/kg by Skerjanee et al. [8]). However, this result is 2-fold lower than the CLtot determined from the current LC/MS/MS method. This is presumably due to the fact that the spectrophotometric method is non-specific and measurements can be greatly affected by plasma turbidity and ICG impurities. In addition, bilirubin and other bile acid salts may interfere with the spectrophotometric method and make the assay less sensitive.

The established LC/MS/MS method of ICG can be routinely applied to monitor the liver function of bile duct-cannulated animals and assess the patency of cannulas over time. In addition, this novel method works at an ICG concentration range of ng/mL, which avoids the issue of ICG aggregations at high concentrations that result in a wavelength shift of the main absorption peak and, thus, the loss of signals in the spectrophotometric, UV, or fluorescence detection [17,18].

4. Conclusions

A sensitive and specific LC/MS/MS method was developed and validated for the determination of ICG in dog plasma and bile, with a LLOQ of 3 ng/mL in dog plasma. The present method represents a significant improvement over other existing methods based on spectrophotometry, HPLC/UV–vis detection, or HPLC/fluorescence detection. Validation experiments demonstrated that good precision and accuracy over a wide concentration range was attained and that there was no interference by endogenous substances. With the assay in hand, it was possible to evaluate the pharmacokinetics of ICG in bile duct-cannulated dogs and assess their liver function after surgery.

References

[1] D.B. Hunton, J.L. Bollman, H.N. Hoffman, Gastroenterology 39 (1960) 713–724.
[2] J. Caesar, S. Shaldon, L. Chiandussi, L. Guevara, S. Sherlock, Clin. Sci. 21 (1961) 43–57.
[3] P. Sekelj, K.R. Shankar, J. Doman, I.P. Sukumar, W.H. Palmer, J. Appl. Physiol. 29 (1970) 249–253.
[4] P. Hopton, T.S. Walsh, A. Lee, J. Appl. Physiol. 87 (1999) 1981–1987.
[5] L.R. Jiao, A.A. El-Desoky, A.M. Seifalian, N. Habib, B.R. Davidson, Br. J. Surg. 87 (2000) 568–574.
[6] R. Boushel, H. Langberg, J. Olesen, M. Nowak, L. Simonsen, J. Bulow, M. Kjaer, J. Appl. Physiol. 89 (2000) 1868–1878.
[7] S. Kawasaki, N. Umekita, T. Beppu, T. Wada, Y. Sugiyama, T. Iga, M. Hanano, Tox. Appl. Pharmacol. 75 (1984) 309–317.
[8] A. Skerjanec, D.W. O'Brien, Y.K. Tam, Pharm. Res. 11 (1994) 1511–1515.
[9] P.L. Rappaport, J.J. Thiessen, J. Pharm. Sci. 71 (1982) 157–161.
[10] R. Heintz, C.K. Svensson, K. Stoeckel, G.J. Powers, D. Lalka, J. Pharm. Sci. 75 (1986) 398–402.
[11] M.B. Dorr, G.M. Pollack, J. Pharm. Sci. 78 (1989) 328–333.
[12] Sigma–Aldrich. Certificate of analysis.
[13] M.J. Luetkemeier, J.A. Fattor, Clin. Chem. 47 (2001) 1843–1845.
[14] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185.
[15] V. Saxena, M. Sadoqi, J. Shao, J. Pharm. Sci. 92 (2003) 2090–2097.
[16] B. Davies, T. Morris, Pharm. Res. 10 (1993) 1093–1095.
[17] R. Weigand, F. Rotermund, A. Penzkofer, J. Phys. Chem. 101 (1997) 7729–7734.
[18] R. Rajagopalan, P. Uetrecht, J.E. Bugaj, S.A. Achilefu, R.B. Dorshow, Photochem. Photobiol. 71 (2000) 347–350.