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Short Communication

Development and full validation of an UPLC-MS/MS method for the quantification of the plant-derived alkaloid indirubin in rat plasma

Evelyn A. Jähne[1], Chethan Sampath[2], Veronika Butterweck[2,3], Matthias Hamburger[1], and Mouhssin Oufir[1*]

[1]Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland

[2]Department of Pharmaceutics, College of Pharmacy, University of Florida, 1345 Gainesville, Florida, USA

[3]School of Life Sciences Institute for Pharma Technology, Gründenstrasse 40, CH-4132 Muttenz, Switzerland

[*]Corresponding author: Dr. Mouhssin Oufir, Institute of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland, Phone: +41 61 267 1544 Fax: +41 61 267 1474

E-Mail: mouhssin.oufir@unibas.ch

Abstract

An UPLC-MS/MS method for the quantification of indirubin in lithium heparinized rat plasma was developed and validated according to current international guidelines. Indirubin was extracted from rat plasma by using Waters Ostro™ pass-through sample preparation plates. The method was validated with a LLOQ of 5.00 ng/mL and an ULOQ of 500 ng/mL. The calibration curve was fitted by least-square quadratic regression, and a weighting factor of 1/X was applied. Recoveries of indirubin and I.S. were consistent and ≥ 75.5%. Stability studies demonstrated that indirubin was stable in lithium heparinized rat plasma for at least 3 freeze/thaw cycles, for 3 hours at RT, for 96 hours in the autosampler at 10°C, and for 84 days when stored below -65°C. Preliminary pharmacokinetic (PK) data were obtained from Sprague Dawley rats after intravenous administration of indirubin (2 mg/kg b.w.) and blood sampling up to 12 hours after injection. PK parameters were determined by non-compartmental analysis**.** Indirubin had a half-life ($t_{1/2}$) of 35 min, and a relatively high clearance (CL) of 2.71 L/h/kg.

Keywords: Indirubin, Pharmacokinetics, Rat plasma, UPLC-MS/MS, Method validation, Phospholipid removal plate

Abbreviations

AUC	area under the curve
C_0	concentration at time zero
Cal	calibrator
CV%	coefficient of variation
ESI	electrospray ionization
I.S.	internal standard
LLOQ	lower limit of quantification
LTS	long-term stability
MRM	multiple reaction monitoring
MRT	mean residence time
PK	pharmacokinetic
QC	quality control
QCH	quality control high
QCL	quality control low
QCM	quality control medium
SS	stock solution
$t_{1/2}$	half-life of elimination
TFA	trifluoroacetic acid
TQD	tandem quadrupole detector
ULOQ	upper limit of quantification
UPLC-MS/MS	ultra-high performance liquid chromatography with tandem mass spectrometric detection
V_z	volume of distribution
WS	working solution

1. Introduction

The bis-indole indirubin is the red isomer of the ancient blue dye indigo. Both indigoids derive from colorless precursors found in various plants including *Baphicacanthus cusia* (Acanthaceae), *Polygonum tinctorium* (Polygonaceae), *Indigofera tinctoria* (Fabaceae) and *Isatis tinctoria* (Brassicaceae), and in some marine mollusks (Muricidae) [1,2]. The clinical interest in the compound was aroused in the early 1980s when indirubin was found to be the active ingredient of Danggui Longhui Wan, a mixture of 11 herbals used in the traditional Chinese medicine to treat chronic myelocytic leukaemia (CML) [1,3]. Indirubin showed potent inhibition of cyclin-dependent kinases (CDKs) via interaction with the ATP-binding site of the kinase [1]. The compound induced a cell cycle arrest mainly in G2 and/or G2/M phase leading to apoptosis of the cell [1,4]. In addition to CDK inhibition, indirubin was shown to block other kinases, such as the glycogen synthase kinase-3β (GSK-3) and the c-Src kinase [5]. Moreover, the alkaloid was reported to inhibit the cell cycle via activation of the aryl hydrocarbon receptor (AhR) [6,7]. Indirubin was also shown to possess anti-inflammatory properties, via inhibition of interferon γ and interleukin 6 production [8,9]. The compound was found to suppress the NF-κB signaling pathway, and the expression of NF-κB regulated-gene products involved in tumorigenesis [9]. Due to the potent anti-proliferative and anti-inflammatory activities, low toxicity, and reasonably drug-like properties indirubin served as a lead for medicinal chemistry efforts [3,10]. Considering the high interest in the compound, it is somewhat surprising that the pharmacokinetic (PK) properties of indirubin and indirubin derivatives have not been evaluated. Up to now, only one bioanalytical method for quantification of indirubin in rat plasma has been published. [11]. However, the assay was using HPLC-UV and, therefore, does not meet current requirements for bioanalytical methods in terms of selectivity, specificity, and sensitivity. Therefore, we developed and validated a quantitative UPLC-MS/MS method for indirubin in rat plasma according to the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for industry [12,13], and applied it to a pilot PK study with intravenous administration of indirubin in male Sprague Dawley rats.

2. Experimental

2.1. LC-MS/MS analysis

2.1.1. Chemicals and reagents

(Z)-[2,3'-biindolinylidene]-2',3-dione (indirubin) (PubChem CID: 5359405) (Fig. 1A) and the internal standard (I.S.) (*E*,*Z*)-3-(benzylidenyl)-indolin-2-one (Fig. 1B) were synthesized according to published procedures [1,14]. Both compounds showed a purity of ≥ 99%, as determined by HPLC and NMR [15]. All used solvents were of HPLC grade. Acetonitrile and dimethyl sulfoxide (DMSO) were from Scharlau (Barcelona, Spain). Methanol was supplied by Lab-Scan (Gliwice, Poland). Formic acid and trifluoroacetic acid (TFA) were purchased from BioSolve (Valkenswaard, Netherlands). HPLC grade water was obtained by a Milli-Q (Merck Millipore, Darmstadt, Germany) water purification system.

Ostro™ 96-well plates were provided by Waters (Milford, MA, USA), and blank male Sprague Dawley rat plasma in lithium heparin batches were from Seralab (Haywards Heath, UK).

2.1.2. LC-MS/MS instrumentation and chromatographic conditions

Sample analysis was performed on an Acquity UPLC system coupled to an Acquity tandem quadrupole detector (TQD) (all Waters Corp.). Chromatographic separation was performed on a UPLC HSS T3 column (100 mm x 2.1 mm; 1.8 µm particle size) (Waters Corp.). Column temperature was set at 45°C, and autosampler temperature at 20°C. Mobile phase was delivered at a flow rate of 0.5 mL/min. The mobile phase consisted of water containing 0.1% formic acid (Eluent A) and acetonitrile containing 0.1% formic acid (Eluent B). The following gradient was used: 30% of B (0-1 min); 30-77% of B (1-5 min); 77-100% of B (5-5.01 min); 100% of B (5.01-6 min); 100-30% of B (6-6.01 min); 30% of B (6.01-7 min). Total run time was 7 min. Weak and strong wash solvents were water-acetonitrile (50:50, v/v) containing 0.2% TFA, and acetonitrile-isopropanol-acetone (40:40:30, v/v/v) containing 0.2% TFA, respectively. The seal wash solvent consisted of a water-acetonitrile mixture (90:10, v/v). Extracted samples were dissolved in DMSO and injected into the UPLC-MS/MS system in full loop mode (2 µL).

The TQD system was equipped with an electro-spray ionization (ESI) interface, and measurements were performed in positive ion mode (ESI+) with multiple reactions monitoring (MRM). Nitrogen, generated by a nitrogen generator N2-Mistral (Schmidlin AG, Neuheim, Switzerland), was used both as desolvation and nebulization gas. Argon was used as collision gas. Capillary voltage was 4 kV for both analyte and I.S.. Source temperature was set at 150ºC, and desolvation temperature was 400ºC. Flow rates for desolvation gas and cone gas were 1000 L/h and 10 L/h, respectively. Data were acquired with MassLynx V4.1 software and quantified by means of QuanLynx software (Waters Corp).

2.1.3. Sample preparation

Stock solutions (SS) of analyte and I.S. were prepared in DMSO. Working solutions (WS1) of analyte (100 µg/mL) and I.S. (10 µg/mL) were obtained by further diluting the corresponding SS in methanol. SS and WS1 were stored below -65°C until use. WS2 of I.S. was prepared freshly before each analytical run by diluting WS1 with acetonitrile + 1% formic acid to a final concentration of 1000 ng/mL. Seven calibration samples (calibrators, cals) in the range of 5.00 - 500 ng/mL, and quality controls (QCs) at low, medium and high levels (QCL = 15.0 ng/mL, QCM = 250 ng/mL, QCH = 400 ng/mL) were prepared in lithium heparinized rat plasma by serial dilution of WS1 of the analyte. Calibrators and QCs were aliquoted into polypropylene tubes and stored below -65°C until analysis.

2.1.4. Extraction of plasma samples

Indirubin was extracted from rat plasma using Waters Ostro™ pass-through sample preparation plates. For processing, the Ostro™ plate was placed onto a 96-deep well plate (96-DWP) serving as collection plate. To 50 µL lithium heparinized rat plasma, 150 µL of I.S. WS2 at 1000 ng/mL were added. The Ostro™/collection plate assembly was shaken for 10 min at RT on an Eppendorf Thermomixer (1000 rpm). After mixing, the plate assembly was placed onto a positive pressure processor (Biotage® PRESSURE+, Uppsala, Sweden), and a pressure of 30 - 40 psi was applied for 10 min. The Ostro™ plate was discarded, and the filtrate in the collection plate was dried under nitrogen (Evaporex EVX-96, Apricot Designs, Monovia, CA, USA). Dried extracts were reconstituted with 200 µL of DMSO. The 96-DWP was shaken for 45 min at RT on an Eppendorf Mixmate and centrifuged for 2 min at 3000 rpm (Megafuge, Heraeus Instruments AG, Switzerland). Due to adsorption of the I.S. to the polypropylene of 96-DWP, the samples were transferred into 300 µL glass inserts in HPLC vials, prior to injection in full loop mode (2 µL).

2.2. Bioanalytical method validation

The method was fully validated according to FDA and EMA guidelines [12,13]. Seven calibrators ranging from 5.00 - 500 ng/mL were injected at increasing concentrations, after a blank sample (blank rat plasma) and a zero sample (rat plasma only spiked with I.S.). The calibration curve was validated through six QCs (QCL, QCM, and QCH), which were inserted randomly into each analytical run. Calibrators and QCs were analyzed in duplicates. All validation runs were performed as described earlier [16,17]. Imprecision was expressed by the coefficient of variation (CV%), and inaccuracy as the relative error (RE%).

Specificity and *selectivity* was evaluated by six blank samples and six QC samples of indirubin at the lower limit of quantification (LLOQ = 5.00 ng/mL) (duplicates, 3 different batches of lithium heparinized rat plasma), respectively.

Intra-run repeatability and *inter-run reproducibility* of the method were evaluated within one run (intra-run) and within three runs on three consecutive days (inter-run), respectively, by injecting six replicates of indirubin samples at five concentration levels (LLOQ, QCL, QCM, QCH, and ULOQ).

Carry-over was assessed by directly injecting an extracted blank after both replicates of the upper limit of quantification (ULOQ = 500 ng/mL).

Absolute recovery of indirubin was determined at three levels (QCL, QCM, and QCH) by comparing the peak areas of six extracted QC samples with six unextracted samples (= 100% recovery).

Dilution integrity of the samples was tested by spiking blank rat plasma with indirubin at a concentration of 2500 ng/mL, and by further diluting the obtained dilution QC (QC Dil.) with blank rat plasma in a ratio of 1:10 and 1:100. For each concentration level (25.0 and 250 ng/mL), six replicates were analyzed.

Short-term stability was assessed through six replicates at two concentration levels (QCL and QCH) after 3 hours at RT, after 3 freeze and thaw cycles, and after 96 hours storage under autosampler conditions (20°C, light protected).

Long-term stability was determined by six replicates at three concentration levels (QCL, QCM, QCH) after 84 days, when stored below -65°C. Stored samples were analyzed by a freshly prepared calibration curve.

Stock solution stability for indirubin was assessed after storage for 598 days below -65°C, and for 6 hours at RT. For this purpose, a working solution (5 µg/mL) was prepared in injection solvent from the freshly prepared and the stored stock solutions, and injected six times in the UPLC-MS/MS system. Stock solution stability of I.S. after 190 days storage below -65°C was already described by Oufir et al. [16].

2.3. Preliminary PK study

All animal studies were performed according to the policies and guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Florida, Gainesville, USA (NIH publication # 85-23), study protocol # 200802291 as previously described [16]. Indirubin was dissolved in DMSO and administered intravenously in male Sprague Dawley rats (N=4) at a dose of 2 mg/kg b.w.. Blood samples (300 µL) were collected from the sublingual vein into Vaccuette® heparinized tubes at times of 0 (prior to dosing), 2, 5, 10, 20, 30 min, 1, 2, 3, 4, 6, 8, and 12 hours. Blood samples were centrifuged at 4000 rcf for 15 min at 4 °C. The plasma samples were aliquoted into 1.5 ml tubes and stored below -65°C until analysis. PK parameters were determined by non-compartmental analysis using PKSolver 2.0 [18].

2.4. Data analysis

Mean plasma concentrations of indirubin after intravenous (i.v.) administration versus time curves were generated in Graphpad Prim (version 5.01, San Diego, CA, USA). PK parameters were determined by non-compartmental analysis using PKSolver 2.0 [18], and included the concentration at time zero (C_0), the terminal elimination half-life ($t_{1/2}$), area under the curve extrapolated to infinity ($AUC_{0-\infty}$), the elimination rate constant (K_e), the mean residence time (MRT), the volume of distribution at terminal phase (V_z), and the clearance (CL). $AUC_{0 \rightarrow last}$ was calculated using a linear/log trapezoidal method from time zero to the last detectable sampling point 240 min after administration.

3. Results and discussion

3.1. LC-MS/MS analysis

MS/MS parameters were generated using Waters IntelliStart software, and manually optimized according to Table S1. Due to the slow inter-conversion at RT of the I.S., both *E* and *Z* isomer peaks were integrated and quantified as previously described [16,17]. Quantification was achieved using

MRM transitions at *m/z* 262.70 > 219.17 (Quantifier) and 262.70 > 190 (Qualifier) for indirubin, and 221.8 > 194.0 for I.S.. The fragmentation patterns of indirubin and I.S. are shown in Fig. S3.

3.2. Method validation

The method of indirubin in rat plasma was validated with respect to specificity, selectivity, intra-run and inter-run reproducibility, carry-over, extraction yield, dilution, and short- and long-term stabilities. In accordance with FDA guidance, a run was considered to be valid if at least 75% of the calibrators were used to generate the calibration curve. Moreover, at least one duplicate of the LLOQ (Fig. 2A and B) and the ULOQ (Fig. 2C and D) had to be accepted. Among the six QCs, four replicates in total, and at least one replicate at each level had to be valid. To fulfill the acceptance criteria of FDA and EMA regulatory guidelines [12,13], imprecision (CV%) should be below 15% of the nominal values for all levels (20% for the LLOQ) and inaccuracy (RE%) should be within ± 15% of the nominal values for all levels (± 20% for the LLOQ). The calibration curve in the range of 5.00 - 500 ng/mL was fitted by least-square quadratic regression and a weighting factor of 1/X was applied (Table S2). The mean coefficient of determination (R^2) was 0.999 (Table S2).

Selectivity and specificity: The quantification method of indirubin in rat plasma was shown to be selective (CV% = 11.9%, RE % = -0.148%) (Table S3). The peak area of indirubin in the blank rat plasma samples (duplicates, 3 batches) was found to be below 20% (14.8%) of the LLOQ demonstrating that the method was specific (Table S4).

Intra-run repeatability and inter-run reproducibility: Intra-run imprecision (CV%) was in the range of 2.37 % and 6.11% (Table 1), and inaccuracy (RE%) was between -8.22% and 4.51% of the nominal values (Table 1). Inter-run imprecision (CV%) ranged from 1.59% to 7.44%, and inaccuracy (RE%) was between -9.60% and 3.46% (Table 1), demonstrating that the acceptance criteria were met (below 15%).

Carry-over: Mean carry-over (Fig. 2E and F) in blank rat plasma samples was 2.60% (below 20%) for indirubin, and 0.00% for I.S. (below 5%), and thus met requirements of the EMA guideline for industry [13] (Table S5).

Extraction yield: The absolute recovery for indirubin was 78.5% for QCL (5.00 ng/mL), 75.5% for QCM (250 ng/mL), and 87.0% for QCH (400 ng/mL) (Table S6). Hence, recovery was consistent (Table S6). For I.S. an absolute recovery of 105% was found (Table S6).

Dilution test: Dilution of samples up to 100-fold did not affect precision and accuracy of the method, as the imprecision (CV%) was below 15% (10x dilution: 3.04%, 100x dilution: 2.67%), and the inaccuracy (RE %) was within ± 15% (10x dilution: -0.177%, 100x dilution: 8.60%) of the nominal value (Table S7).

Short-term stability: Indirubin samples in rat plasma were subjected to 3 freeze and thaw cycles. Imprecision (CV%) for the 6 replicates at the QCL (15.0 ng/mL) and the QCH (400 ng/mL) was below 15% (2.39% and 4.38%, respectively, data not shown), and inaccuracy (RE%) was within ±

15% (-8.74% and -1.23%, respectively) (Table S8). In addition, indirubin samples were kept for 3 hours at RT. Imprecision (CV%) for the 6 replicates at low concentration (15.0 ng/mL) was 3.96%, and 3.62% for the 6 replicates at high concentration (400 ng/mL) (data not shown). Inaccuracy (RE%) was -8.54% for QCL, and -0.207% for QCH (Table S8). Processed samples of indirubin were stored for 96 hours under autosampler conditions (20°C, protected from light). At both concentration levels (15.0 ng/mL and 400 ng/mL), imprecision (CV%) was below 15% (4.14% and 4.67%, respectively, data not shown), and inaccuracy (RE%) was within ± 15% of the nominal values (-9.29% and 1.61%, respectively) (Table S8). Hence, according to FDA and EMA guidelines indirubin was stable under the above conditions.

Long-term stability: Stability of indirubin in rat plasma could be confirmed for at least 84 days when stored below -65°C (Fig. S1), as the slope of the calculated linear regression was 0.944 (acceptance criteria: 1 ± 0.15).

Stock solution stability: Indirubin SS (dissolved in DMSO) was stored below -65°C for 598 days and kept for 6 hours at RT. Analysis showed that the degradation expressed by the percentage differences (-0.142%) was below 5%, indicating that the SS of indirubin was stable after storage below -65°C for more than 1.5 years (Table S9). The DMSO SS of I.S. stored below -65°C was stable up to 190 days, as already reported by Oufir et al. [16].

3.3. Preliminary PK study

The validated method was applied to the PK study of indirubin in Sprague Dawley rats after single i.v. dose of 2 mg/kg b.w. (N=4). Typical MRM chromatograms of rat plasma samples are given in Fig. S2. The main PK parameters of indirubin calculated by non-compartmental analysis using PKSolver [18] are listed in Table 2, and the mean plasma concentration *versus* time profile is shown in Fig. 3. The initial concentration (C_0) of indirubin was 1052 ng/mL, the clearance was 2.71 L/h/kg, the area under the concentration-time curve (AUC) as calculated with the trapezoidal rule was 737 ng*h/mL, and the half-life ($t_{1/2}$) of indirubin was 35 min (Table 2). Previous PK data reported by Deng et al. obtained from male Wistar rats showed a half-live of 1 hour (i.v., 2.8 mg/kg) [11]. The different $t_{1/2}$ found in male Wistar rats (1 hour) and Sprague Dawley rats (35 min) may be explained by differing cytochrome P450 isozyme expression in the two strains. Furthermore, Koster et al. [19], reported that different strain suppliers, differences in diet, husbandry, and microflora could additionally influence the variability of isozyme expression [20]. Also, different administered doses (male Sprague Dawley rats: i.v., 2 mg/kg *vs.* male Wistar rats: i.v., 2.8 mg/kg) and body weight (male Sprague Dawley: 320 - 350 g *vs.* male Wistar rats: 280 - 300 g) might have impacted the excretion of the compound. Independent of that, our data obtained by a fully validated UPLC-MS/MS assay are significantly more reliable than the data in the previous study which were obtained by HPLC-UV analysis [11].

3. Conclusions

A highly selective, rapid and sensitive UPLC-MS/MS assay for quantification of indirubin in lithium heparinized rat plasma was developed and validated according to current regulatory guidelines [12,13]. The calibration curve in the range of 5.00 – 500 ng/ml was quadratic, with a weighting factor of 1/X and $R^2 > 0.999$. The validated method was applied to a pilot PK study in male Sprague Dawley rats after intravenous administration (2mg/kg b.w.), where a relatively high clearance of 2.71 L/h/kg and a $t_{1/2}$ of 35 min were found. The assay will be subsequently used in a full PK study addressing oral bioavailability in Sprague Dawley rats. Moreover, *in vitro* metabolism of indirubin will be studied in human hepatocytes and liver microsomes.

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FIGURES

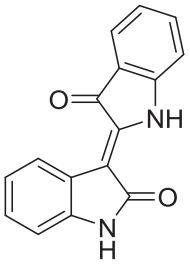
Fig. 1: Chemical structures of (Z)-[2,3'-biindolinylidene]-2',3-dione (indirubin) (A), and internal standard (*E*,*Z*)-3-(benzylidenyl)-indolin-2-one (B).

Fig. 2: Typical MRM chromatograms of rat plasma spiked with 5.00 ng/mL (LLOQ) of indirubin (**A**), with 1000 ng/mL of I.S. (**B**), with 500 ng/mL (ULOQ) of indirubin (**C**), with 1000 ng/mL of I.S. (**D**), of blank rat plasma injected directly after the ULOQ and monitored for indirubin (**E**), and I.S. (**F**).

Fig. 3: Mean plasma concentration *versus* time profile of indirubin in male Sprague Dawley rats (N = 4) after i.v. administration (2 mg/kg b.w.).

Fig. 1

A



B

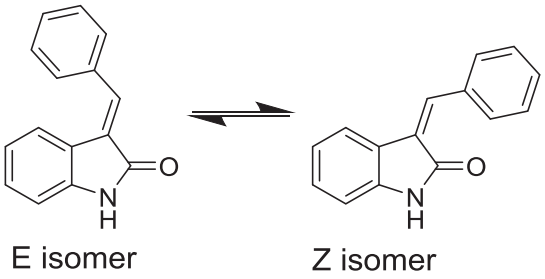


Fig. 2

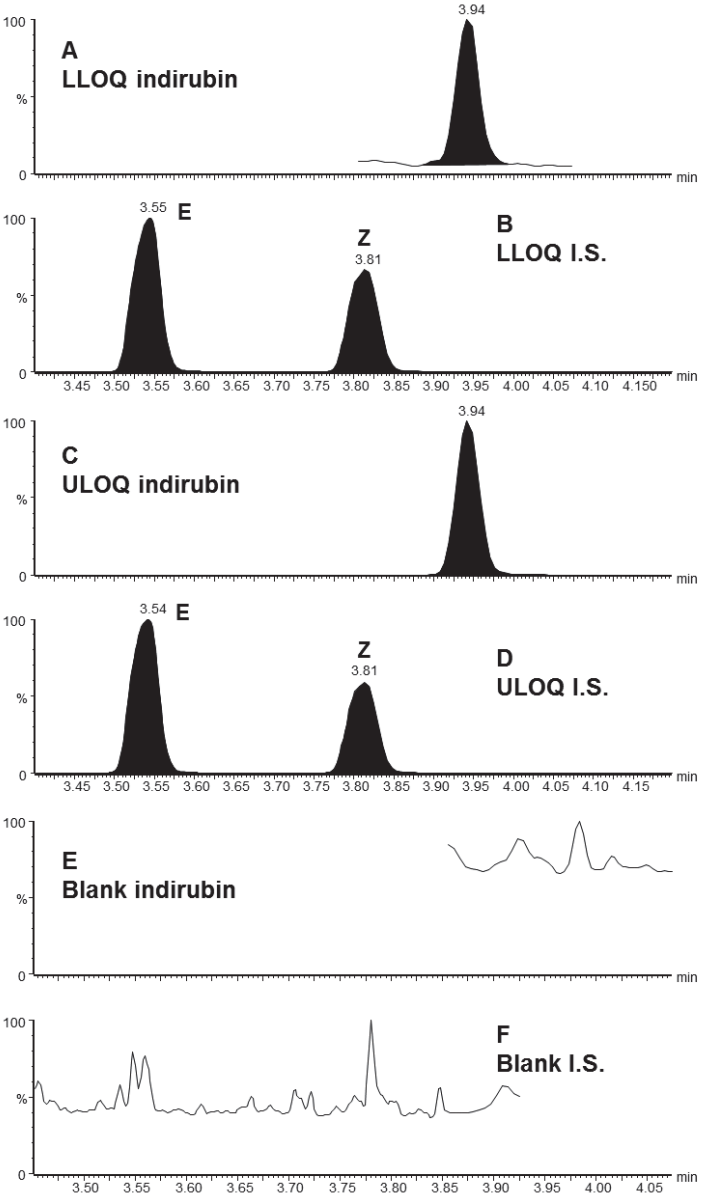
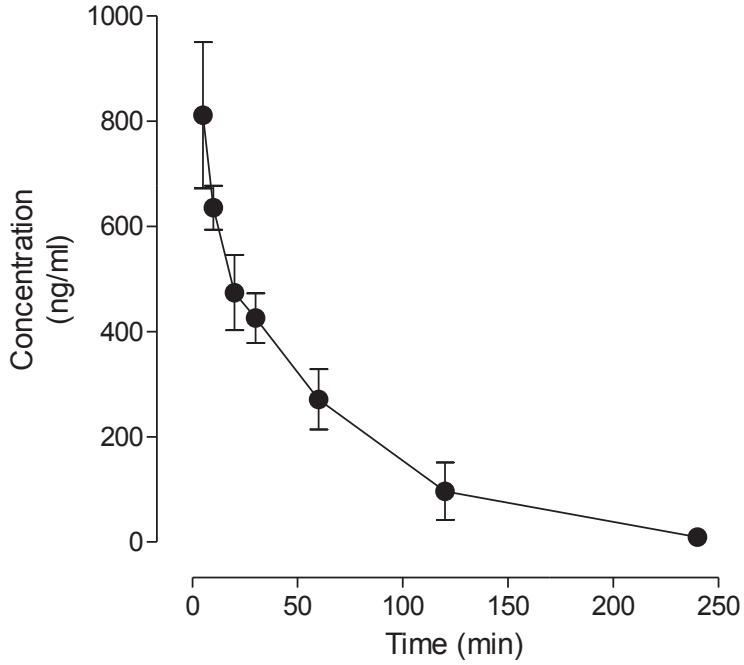

A
LLOQ indirubin
3.94
B
LLOQ I.S.
3.55 E
Z
3.81
C
ULOQ indirubin
3.94
D
ULOQ I.S.
3.54 E
Z
3.81
E
Blank indirubin
F
Blank I.S.
100
%
0
min
3.45 3.50 3.55 3.60 3.65 3.70 3.75 3.80 3.85 3.90 3.95 4.00 4.05 4.10 4.150
3.45 3.50 3.55 3.60 3.65 3.70 3.75 3.80 3.85 3.90 3.95 4.00 4.05 4.10 4.15
3.50 3.55 3.60 3.65 3.70 3.75 3.80 3.85 3.90 3.95 4.00 4.05

Fig. 3


1000
800
600
400
200
0
Concentration (ng/ml)
0
50
100
150
200
250
Time (min)

TABLES

Table 1: Intra-run (N=6) and inter-run (N=18) imprecision (CV%) and inaccuracy (RE%) of QC samples, based on 3 series of 6 replicates for each level.

	LLOQ	QCL	QCM	QCH	ULOQ
Nominal level (ng/mL)	**5.00**	**15.0**	**250**	**400**	**500**
Intra-run Mean	4.97	13.8	238	418	490
Intra-run S.D.	0.304	0.414	5.65	14.3	14.3
Intra-run CV%	**6.11**	**3.01**	**2.37**	**3.42**	**2.92**
Intra-run RE%	**-0.564**	**-8.22**	**-4.73**	**4.51**	**-1.95**
Inter-run Mean	5.17	13.6	233	412	498
Inter-run S.D.	0.385	0.512	3.70	10.6	13.5
Inter-run CV%	**7.44**	**3.77**	**1.59**	**2.57**	**2.71**
Inter-run RE%	**3.46**	**-9.60**	**-6.66**	**2.96**	**-0.487**

Table 2: PK parameters after a single intravenous dose of 2 mg/kg bw indirubin in rats (N = 4). Data were calculated by PKSolver using non-compartmental analysis.

Parameter	Mean	SD
C_0 (ng/mL)	1052	329
$t_{1/2}$ (min)	35.0	4.20
T_{max} (min)	5.00	0.00
C_{max} (ng/mL)	811	140
$AUC_{0\text{-last}}$ (ng*h/mL)	737	190
$AUC_{0-\infty}$ (ng*h/mL)	763	177
MRT (min)	50.2	8.53
Vz (L/kg)	2.25	0.296
CL (L/h/kg)	2.71	0.520

SD: standard deviation; C_0: the concentration at time zero; $t_{1/2}$: half-life of elimination; AUC_{0-last}: area under curve from time zero to 240 min; $AUC_{0-\infty}$: area under the curve with extrapolation to infinity; MRT: mean residence time; V_z: volume of distribution at the terminal phase; CL: clearance.

*Graphical Abstract