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Development and full validation of an UPLC-MS/MS method for the determination of an anti-allergic indolinone derivative in rat plasma, and application to a preliminary pharmacokinetic study

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ABSTRACT

The natural product (*E,Z*)-3-(4-hydroxy-3,5-dimethoxybenzylidene)indolin-2-one (indolinone) was identified some years ago as a nanomolar inhibitor of FcεRI-receptor dependent mast cell degranulation. To further explore the potential of the compound, we established an UPLC-MS/MS assay for dosage in rat plasma. The method was fully validated according to FDA Guidance for industry. Results of this validation and long term stability study demonstrate that the method in lithium heparinized rat plasma is specific, accurate, precise and capable of producing reliable results according to recommendations of international guidelines. The method was validated with a LLOQ of 30.0 ng/mL and an ULOQ of 3000 ng/mL. The response versus concentration data were fitted with a first order polynomial with $1/X^2$ weighting. No matrix effect was observed when using three independent sources of rat plasma. The average extraction recovery was consistent over the investigated range. This validation in rat plasma demonstrated that indolinone was stable for 190 days when stored below −65 °C; for 4 days at 10 °C in the autosampler; for 4 h at RT, and during three successive freeze/thaw cycles at −65 °C. Preliminary pharmacokinetic data were obtained in male Sprague–Dawley rats (2 mg/kg BW i.v.). Blood samples taken from 0 to 12 h after injection were collected, and data analyzed with WinNonlin. A short half-life (4.30 ± 0.14 min) and a relatively high clearance (3.83 ± 1.46 L/h/kg) were found.

1. Introduction

(*E,Z*)-3-(4-hydroxy-3,5-dimethoxybenzylidene)indolin-2-one (indolinone) is an alkaloid occurring in low concentrations in the TCM herbal drug Banlangen (dried roots of *Isatis indigotica* L., family Brassicaceae) [1] and in leaves of European woad (*Isatis tinctoria* L.) [2,3]. Both drugs have a long history of traditional use in the context of inflammatory ailments [4], which could be rationalized in recent years through the discovery and characterization of various pharmacologically highly active constituents such as tryptanthrin [5–8], indirubin [9,10] and indolinone [2]. Indolinone was identified as the constituent responsible for inhibition of mast cell degranulation of lipophilic *I. tinctoria* extracts [4], as it inhibited compound 48/80 induced histamine release from mast cells ref [2]. More recently, indolinone was shown to block immunoglobulin E (IgE) mediated degranulation of sensitized mast cells at nM concentrations (IC_{50} = 54 nM) without direct inhibition of kinases downstream of the FCεRI [11]. While the mode of action is not yet fully understood at the molecular level, the lack of cytotoxicity and drug-like physicochemical properties [12] of indolinone earmarked the compound as an interesting lead for further development.

To further explore the potential of indolinone, a bioanalytical method was needed in view of pharmacokinetic studies with the compound. A HPLC-ESIMS assay for quantification of indolinone in *Isatis* extracts has been previously reported by us [13], but this

Abbreviations: CV, coefficient of variation; UPLC-MS/MS, Ultra performance liquid chromatography with tandem mass spectrometric detection; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; RE, relative error; ca. (circa), about; Cal, calibrator; con., concentration; CV, % coefficient of variation; min, minute; QC, quality control(s); QCH, quality control high; QCL, quality control low; QCM, quality control medium; RE%, relative error of measurement; rcf, relative centrifugal force; SD, standard deviation; v/v, volume per volume; RT, room temperature; SS, stock solution; WS, working solution; I.S., internal standard; BW, body weight; I.V., Intravenous; MRM, Multiple reaction monitoring; SE, standard error; C_0, the concentration at time zero; $t_{1/2}$, half-life of elimination; AUC_{0-last}, area under curve from time zero to 30 min; $AUC_{0-\infty}$, area under the curve with extrapolation to infinity; K_e, elimination rate constant; MRT, mean residence time; V_z, volume of distribution at the terminal phase; CL, clearance.

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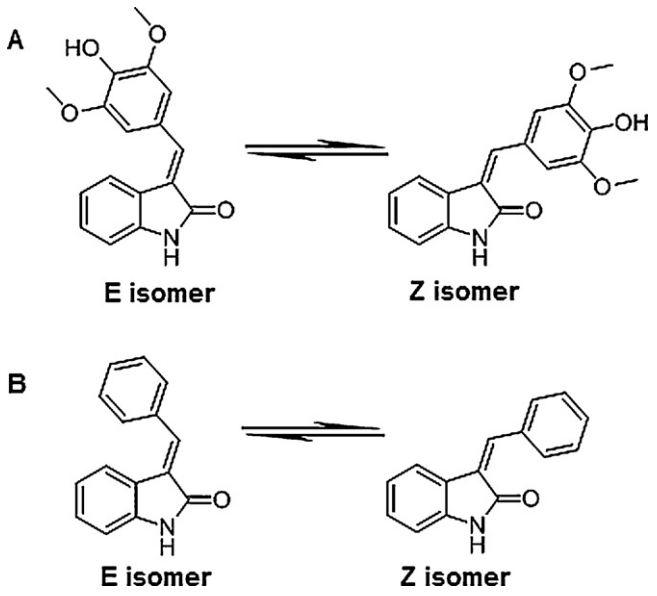

Fig. 1. Chemical structures of (*E,Z*)-3-(4-hydroxy-3,5-dimethoxybenzylidene)indolin-2-one (indolinone) (A) and internal standard, (*E,Z*)-3-(benzylidenyl)-indolin-2-one (B).

method was not applicable to the dosage in biological fluids. We here describe the development and validation, according to FDA guidance for industry [14,15], of an UPLC-MS/MS assay for dosage of indolinone in lithium heparinized rat plasma, and its application in a preliminary pharmacokinetic study in Sprague–Dawley male rats (2 mg/kg BW i.v.).

2. Experimental

2.1. UPLC-MS/MS analysis

2.1.1. Chemical and reagents

(*E,Z*)-3-(4-hydroxy-3,5-dimethoxybenzylidene)indolin-2-one (Fig. 1A) was synthesised in the labs of the Division of Pharmaceutical Biology, University of Basel, Switzerland, and the internal standard (I.S.), (*E,Z*)-3-(benzylidenyl)-indolin-2-one (Fig. 1B) was previously synthesised at the Institute of Pharmaceutical Biology, University of Jena, Germany, according to published procedures [2,16]. Purity (>99.9%) of both compounds was checked by HPLC–UV–ESI-MS and ^{1}H and ^{13}C NMR (data not shown). The ratio of E to Z isomers for indolinone and I.S. was assessed by ^{1}H NMR and HPLC as 71:29 and 75:25, respectively. Preparative separation of isomers failed because of slow spontaneous isomerization at RT [2,16]. Acetonitrile, methanol, formic acid, trifluoroacetic acid (TFA), acetone, isopropanol were all ULC/MS grade from BioSolve (Valkenswaard, The Netherlands). HPLC grade water was obtained by an EASYpure II (Barnstead; Dubuque, IA, USA) water purification system. Blank male Sprague–Dawley rat plasma in lithium heparin batches were from BioChemed Services (Winchester, VA, USA).

2.1.2. UPLC-MS/MS instrument and chromatographic conditions

An Acquity UPLC (Waters Corp., Milford, MA, USA) coupled to an Acquity TQD was used. The UPLC system consisted of a binary pump, a cooling autosampler (sample syringe size of 100 μl with an injection loop of 20 μl) set at 10 °C and protected from light, and of a column heater set at 45 °C. A Waters UPLC HSS T3 column (100 mm × 2.1 mm i.d.; 1.8 μm) (Waters Corp., Milford, MA, USA) protected by an Acquity UPLC column in-line filter unit (0.2 μm in-line frit) was used for the separation of both compounds. The following gradient was used: 0–0.5 min, B 2%; 0.5–2 min, B 2–100%; 2–2.5 min, B 100%; 2.5–2.6 min, B 100–2%; 2.6–4 min, B 2%, where mobile phase A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. The flow-rate of the mobile phase was set at 0.5 mL/min.

Seal wash solvent was water–acetonitrile (70:30, v/v), weak and strong wash solvents were water–acetonitrile (50:50, v/v; containing 0.2% TFA) and acetonitrile–isopropanol–acetone (40:30:30, v/v/v; containing 0.2% TFA), respectively. Data were acquired by MassLynx V4.1 software and processed for quantification with QuanLynx V4.1 (Waters Corp., Milford, MA, USA). The MS/MS system was operated with an ESI interface in positive ionization mode (ESI+). Cone and desolvation gas flow rates, obtained from a nitrogen generator N_2 Mistral-4 (Schmidlin Labor + Service AG, Neuheim, Switzerland), were 25 L/h and 1000 L/h, respectively. Argon was used as collision gas at a pressure of ca. 5.0×10^{-3} mbar.

MS parameters were defined with Waters IntelliStart software (automatic tuning and calibration of the Acquity TQD) and manually optimized as follow: capillary voltage of 3.5 kV, source temperature at 150 °C and desolvation temperature at 400 °C. Cone voltage was 47 V, and collision energy was 20 eV for both indolinone and I.S. Quantification was determined using multiple reaction monitoring (MRM) mode for the transitions m/z 298.05 > 265.9 for indolinone, and 222.0 > 194.0 for I.S. The dwell time was automatically set at 0.161 s.

2.1.3. Standards and stock solutions

Standard stock solutions (SS) of indolinone and I.S. were separately prepared in DMSO, aliquoted and stored below −65 °C. Prior to analytical run, standard working solutions (WS) of both compounds were freshly prepared by serial dilution of SS with methanol and stored below −65 °C. The first I.S working solution (WS1) at 100 μg/mL was diluted with methanol to give a second working solution (WS2) at 1000 ng/mL. Prior to spiking of plasma samples, this WS2 for the I.S. was prepared daily for the full validation and assays.

2.1.4. Preparation of calibration and quality control samples

Working solutions were vortex mixed before use and a pool of plasma containing 3000 ng/mL (Cal 7) was prepared by spiking blank rat plasma with appropriate volume of indolinone working solution (WS1) at 100 μg/mL into a clean glass tube. Other calibrators were prepared in bulk, consisting of serial dilutions starting from the Cal 7 with blank rat plasma.

A pool of plasma containing 2400 ng/mL (QCH) was prepared by spiking blank rat plasma with appropriate volume of indolinone working solution (WS2) at 100 μg/mL into a clean glass tube. Pools of other QCs consisted of serial dilutions starting from the QCH with blank rat plasma.

After preparation, pools of calibration standards and QCs were homogenized by vortex mixing and aliquots of 75.0 μl of sample were dispatched in polypropylene tubes and stored below −65 °C until use for analysis. Aliquots were thawed and vortex mixed before use.

2.1.5. Plasma sample extraction

Aliquots (25 μl) of lithium heparinized rat plasma were subjected to protein precipitation with the addition of exactly 100 μl of WS2 (1000 ng/mL in methanol) of corresponding IS and 1000 μl ice cold acetonitrile (stored at −20 °C). This mixture was briefly vortexed, mixed for 10 min at RT in an Eppendorf Thermomixer (1400 rcf), and centrifuged at 13,200 rcf for 20 min at 10 °C. An exact volume of 1000 μl of supernatant was transferred into an Eppendorf 96-well plate and dried under nitrogen gas flow at 40 °C by using a SPE dry 96 (Argonaut, Foster City, CA, USA). The dried extract was reconstituted with 200 μl of injection solvent (65% solvent A + 35% solvent B), and mixed for 45 min at RT in an Eppendorf

Mixmate (1500 rcf). The supernatant was transferred into a 300 μl insert of a HPLC vial. Injections for UPLC-MS/MS analysis were in full loop mode (20 μl).

2.2. Full bioanalytical method validation

For all validation tests, imprecision are expressed by the CV (%) on results tables, and inaccuracy as the mean percentage of error RE% (bias) with regard to the theoretical (or nominal) values. Following the FDA guidance for industry [14,15], the imprecision (CV %) must not exceed 15% for all levels (20% for the LLOQ as exception), and the inaccuracy (RE%) must be within ±15% of the nominal value for all levels (±20% of the nominal value for the LLOQ as exception).

2.2.1. Chromatography performance

The calibration curve consisted of a blank sample (rat plasma sample processed without I.S.), a zero sample or Cal 0 (rat plasma sample processed with I.S.), and seven calibration samples covering the range from 30 to 3000 ng/mL. For the calibration and the run to be valid, the coefficient of determination (r^2) had to be higher than 0.96, and at least 75% of calibration samples had to remain. Moreover, for the lowest (LLOQ) and the highest (ULOQ) levels, only one replicate out of two could be rejected.

2.2.2. Regression parameters

Two sets of seven calibration samples (calibrators) prepared in bulk from indolinone WS and stored below −65 °C were injected in order of increasing concentrations (from 30 to 3000 ng/mL) at the beginning and the end of each analytical UPLC run, respectively. The first concentration level (Fig. 2C) was defined to the lower limit of quantification (LLOQ) and the last (Fig. 2E) to the upper limit of quantification (LLOQ). The weighting factor was determined during the first series in order to obtain a consistent dispersion of measure errors across the range. This appropriateness of this weighing factor was evaluated by plotting the calibration sample inaccuracy (RE% values) values of each run with concentration values in log scale (data not shown) [17,18]. Two replicates of three levels of quality control (QC) samples prepared in bulk from indolinone WS by serial dilution and stored below −65 °C were injected randomly among the analytical run. The first QC level was defined to the QC low (QCL = 90.0 ng/mL), the second level to the QC medium (QCM = 1500 ng/mL) and the last to the QC high (QCH = 2400 ng/mL).

2.2.3. Carry-over

For each analytical UPLC run, one extracted blank biological matrix (without indolinone and I.S) was injected immediately after the ULOQ sample of both calibration sets (Fig. 2A and B). Peak area in blank sample injected after ULOQ calibrator had to be below 20% of the peak area of the LLOQ calibrator for indolinone, and below 5% for I.S.

2.2.4. Selectivity

Six QC samples of indolinone at the LLOQ (duplicates, three different rat plasma batches) were processed and quantified using a valid calibration curve. The imprecision (CV %) had to be below 20%, and the inaccuracy (RE%) within ±20% of the nominal value. For each plasma batch, no more than one QC sample was allowed to have an inaccuracy outside of the ±20% of the nominal value.

2.2.5. Specificity

Six blank rat plasma (duplicates, three different plasma batches) were processed without addition of indolinone and I.S, and were quantified using a valid calibration curve. For the three rat plasma batches, the peak area measured in the blank plasma sample had not to exceed 20% of the LLOQ peak area for indolinone.

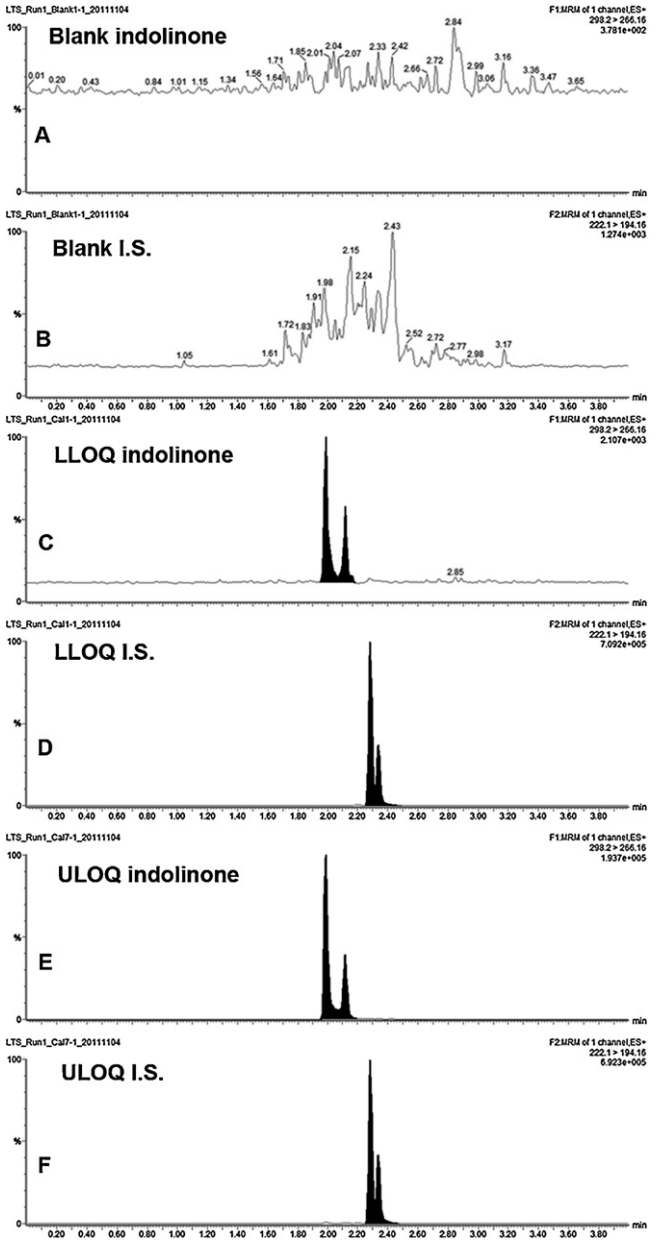

Fig. 2. Typical MRM chromatograms of blank rat plasma monitored for indolinone (A) and for I.S. (B), of blank rat plasma spiked at 30.0 ng/mL (LLOQ) of indolinone (C), and 1000 ng/mL of I.S. (D), of blank rat plasma spiked at 3000 ng/mL (ULOQ) of indolinone (E) and 1000 ng/mL of I.S. (F.)

2.2.6. Within and between series repeatability

Six replicates of QC samples at five concentration levels (30.0–90.0–1500–2400–3000 ng/mL) were processed and injected, bracketed between two sets of calibrators the same day. The imprecision had not to exceed 15% (20% at the LLOQ) and the inaccuracy had to be within ±15% (±20% at the LLOQ) of the nominal value. The reproducibility was assessed on the same QC determinations on at least 3 series during 3 days. The same acceptability criteria as within-series repeatability were used for the imprecision. At the end of the 3 series, the overall means, standard deviations and CV were calculated at each level of QC. The between-series variability was expressed by calculating the between-series CV at each concentration level. The between-series CV took into account the

variability of 6 replicates of each series. The inaccuracy over 3 series was expressed as RE% calculated with regard to the nominal values and had not to exceed ±15%, except for the LLOQ where it had to be limited to ±20%.

2.2.7. Extraction yield

The extraction yield of indolinone was determined using six replicates spiked with indolinone at three concentration levels (90.0–1500–2400 ng/mL) before extraction, and spiked with I.S. after extraction, compared to six blank biological matrix samples spiked after extraction with indolinone at three concentrations and I.S. The extraction yield of I.S. was determined using six replicates spiked with I.S. before extraction and with indolinone at medium concentration (1500 ng/mL) after extraction, compared to six blank biological matrix spiked after extraction with indolinone at medium concentration (1500 ng/mL) and I.S.

2.2.8. Dilution test

In order to assess the reliability of the method at concentration levels outside the calibration range (30–3000 ng/mL), twelve replicates of QC samples at 15,000 ng/mL were prepared. Six were diluted at 1/10 with blank rat plasma and six at 1/100 with blank rat plasma (following this procedure a first dilution at 1/10 then this first dilution was diluted at 1/10 resulting to a final dilution at 1/100).The mean concentration and the imprecision (CV%) were calculated for each dilution factor, and the inaccuracy (RE%) was calculated for each sample and dilution factor.

The imprecision had not to exceed 15%, and the inaccuracy had to be within ±15% of the nominal value.

2.2.9. Short-term stabilities

2.2.9.1. Three freeze and thaw cycles below −65 °C. Six replicates at two concentrations (90.0–2400 ng/mL) were subjected to three overnight freeze/thaw cycles (freezing temperature: below −65 °C) and were quantified. At each concentration level, the imprecision had not to exceed 15%, and the inaccuracy had to be within ±15% of the nominal value.

2.2.9.2. Biological sample stability on benchtop at RT. Six replicates at two concentrations (90.0–2400 ng/mL) were stored for ca. 4 h at room temperature and were quantified. At each concentration level, the imprecision had not to exceed 15%, and the inaccuracy had to be within ±15% of the nominal value.

2.2.9.3. Processed sample stability in the autosampler at 10 °C. Six replicates of QC samples at two concentration levels (90.0–2400 ng/mL) were processed and quantified. After 96 h of storage in the autosampler conditions (10 °C and protected from light), the run was re-injected and re-analysed with freshly prepared two sets of calibrators and QCs. The imprecision had not to exceed 15%, and the inaccuracy had to be within ±15% of the nominal value.

2.2.10. Long-term stability below −65 °C

Three replicates at three concentrations (90.0–1500–2400 ng/mL) stored below −65 °C for at least 1 month, 3 and 6 months were processed. They were quantified using fresh calibration samples and the stability samples were bracketed by freshly prepared quality control samples, in duplicate at three concentrations (90.0–1500–2400 ng/mL). A linear regression, forced through zero, was calculated by plotting the results of $T = X$ month(s) in function of t_0. The stability of the samples was confirmed if the slope obtained was within 1 ± 0.15.

2.2.11. Stock solutions stability test

The stability of indolinone and I.S. stock solutions was assessed under this storage conditions: 190 days of storage below −65 °C and about 6 h at RT. This test was performed by comparison of results from a solution kept in these storage conditions, and the results from a freshly prepared solution. For this purpose, a working solution of indolinone at 10.0 μg/mL and a working solution of I.S. at 10.0 μg/mL in the injection solvent (65% solvent A + 35% solvent B) were prepared from each corresponding stock solution and injected 6 times in the UPLC/MS-MS system. The eventual degradation should not exceed 5% for both compounds [15].

2.3. Preliminary PK study

Male Sprague–Dawley rats with jugular vein catheters (weighing between 320 and 350 g) were purchased from Charles River (Wilmington, MA, USA). The animals were single-housed in plastic cages and received a standard chow and water *ad libitum* during the experiments. All the animals were maintained on a 12 h/12 h light/dark cycle. Non-fasted animals were used for the study. All animal experiments 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. Indolinone was dissolved in DMSO [19] and administered intravenously in a concentration of 2 mg/kg. Blood samples (300 μl) were collected from the sublingual vein into Vaccuette® heparinized tubes 0 (prior to dosing), 2, 5, 10, 20, 30 min, 1, 2, 3, 4, 6, 8 and 12 h. Before each blood collection, the animals were briefly anesthetized with isoflurane (2%). The loss of blood volume was replaced with 1 ml normal saline (i.p. injection) after each blood sampling. Blood samples were centrifuged at 4000 rcf for 15 min at 4 °C. Plasma samples were then transferred into 1.5 ml tubes and stored below −65 °C until analysis.

2.4. Data analysis

Mean plasma concentrations of indolinone after intravenous administration versus time curve were generated in Graphpad Prim (version 5.01, San Diego, CA, USA). The pharmacokinetic (PK) parameters were determined by non-compartmental analysis using WinNonlin software (version 5.2.1, Pharsight Corporation, St. Louis, MO, USA). Non-compartmental PK: The PK parameters determined were the concentration at time zero (C_0), the terminal elimination half-life ($t_{1/2}$), AUC $_{0-\infty}$: area under the curve extrapolated to infinity; 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 30 min after administration.

3. Results and discussion

3.1. Chromatography performance

Considering the slow inter-conversion (Fig. 1) at room temperature [2,16] of (*E,Z*)-3-(4-hydroxy-3,5-dimethoxybenzylidene)indolin-2-one (indolinone), we decided to integrate both E and Z isomers peak areas for the quantification of this compound in rat plasma, as shown in Figs. 2 and 3. Since indolinone is a rare natural product, and a suitably labelled compound was not available as internal standard (I.S.), we selected a structurally closely compound, (*E,Z*)-3-(benzylidenyl)-indolin-2-one, and performed an identical integration of both E and Z isomers peaks. The response versus concentration data, in the range 30.0–3000 ng/mL for indolinone, was fitted by least-squares quadratic regression with $1/X^2$ weighting factor. According to

Table 1
Calibrators and calibration curve parameters.

UPLC Run	Nominal level (ng/mL)							Regression parameters			
	30.0	100	250	500	1000	2000	3000	*A*	*B*	*C*	R^2
1	29.4	110	230	503	990	2080	3085	−0.00000000458	0.000139	0.000188	0.9974
	31.3	101	249	483	939	2017	2922				
2	30.0	104	226	506	960	2028	3035	−0.00000000224	0.000112	0.000256	0.9975
	32.1	103	244	506	965	2020	3009				
3	30.1	96.8	236	467	995	2094	3048	−0.00000000352	0.000104	0.000177	0.9978
	31.5	108	253	493	1013	1933	2970				
4	31.2	113	244	500	984	2006	3072	−0.00000000589	0.000101	0.000100	0.9963
	28.6	105	232	478	957	1906	3158				
Mean	30.5	105	239	492	975	2010	3037	−0.00000000406	0.000114	0.000180	0.9972
S.D.	1.19	5.19	9.74	14.5	24.2	64.6	72.5	0.00000000185	–	–	–
CV%	3.90	4.94	4.07	2.95	2.48	3.21	2.39				
RE %	1.75	4.99	−4.33	−1.65	−2.46	0.521	1.24				

Response: A × Conc.^{2} + B × Conc. + C, Quadratic regression, $1/X^2$ weighting

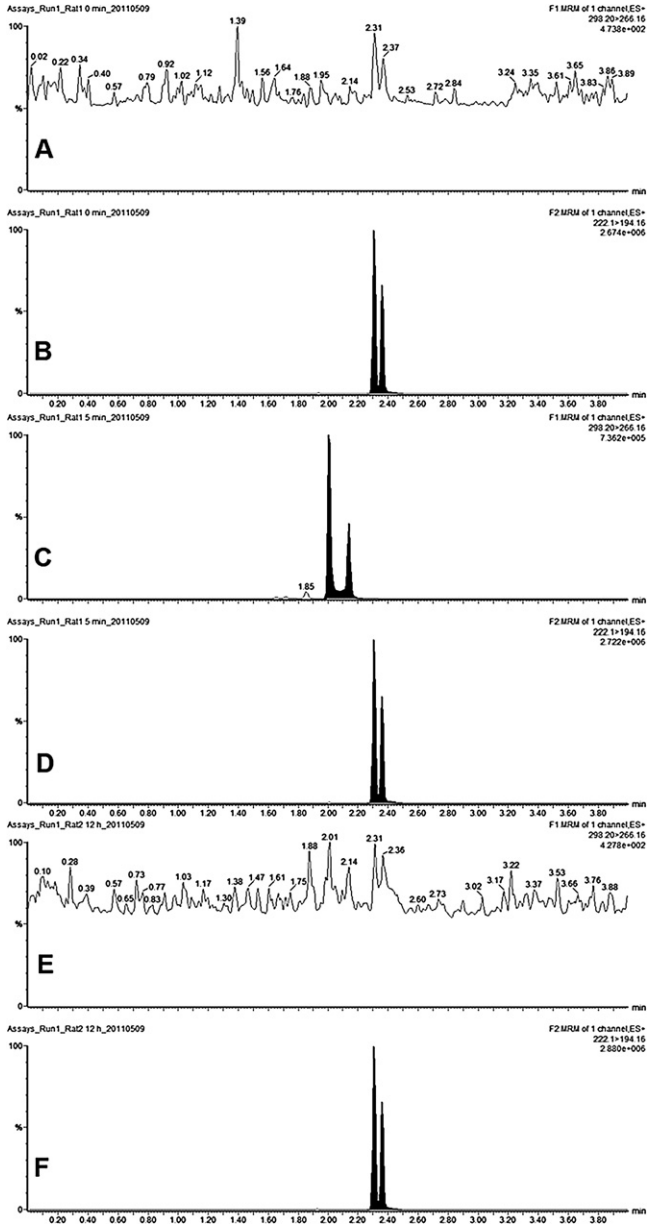

Fig. 3. Typical MRM chromatograms of rat plasma sample at t_0 monitored for indolinone (A), for I.S. (B), of rat plasma sample at 5 min after i.v. injection monitored for indolinone (C), and for I.S. (D), and of rat plasma sample at 12 h after i.v. injection monitored for indolinone (E), and for I.S. (F).

Table 1, the average coefficient of determination (R^2) was equal to 0.9972, and 100% of calibrators were valid during this validation fulfilling the acceptance criteria of the FDA guidance for industry (R^2 higher than 0.96 and at least 75% of calibrators should be valid) [15].

3.2. Assessment of carry-over

In the 30.0–3000 ng/mL range, the impact of the carry-over on blank rat samples (Fig. 2A and B) following the highest calibration sample (Fig. 2E and F) was assessed for both indolinone and I.S. The average carry-over was 0.00% (below 20%) for indolinone and 0.145% (below 5%) for the I.S., indicating that the carry-over has no impact on the results (Table 2).

3.3. Selectivity for indolinone

According to International Union of Pure and Applied Chemistry (IUPAC) recommendations [20], selectivity refers to the extent to which a method can determine particular analytes in mixtures or matrices without interferences from other components. The selectivity of the method with regard to endogenous compounds was assessed by analysis of six QC samples of indolinone (duplicates, 3 different plasma batches) at the LLOQ. The selectivity imprecision (CV%) was 7.24%, and the overall inaccuracy (RE%) was 8.67% (Table 3). Since the imprecision was below 20%, and the inaccuracy was within ±20% at the LLOQ [15], this analytical method for the quantification of indolinone in rat plasma was shown to be selective.

3.4. Specificity for indolinone

Specificity is the ability of a method to unequivocally assess the analyte in the presence of other components that may be present (e.g. impurities, degradation products and matrix components) [21]. Six blank plasma samples (duplicates, 3 different plasma batches) were processed and assessed by peak area measurement. The analyte peak area in the blank samples was compared to the average peak area of the calibration samples at the LLOQ. The peak areas measured in the blank plasma sample were equal to 0.00% (data not shown) and, hence, less than 20.0% of the LLOQ average peak area [11], demonstrating that this analytical method is specific for indolinone.

3.5. Within and between-series repeatability for indolinone

In the range 30.0–3000 ng/mL, within and between-series imprecision and inaccuracy were evaluated at 5 concentration

Table 2
Carry-over assessment for indolinone as analyte, and for (*E,Z*)-3-(benzylidenyl) indolin-2-one as I.S.

UPLC Run	Replicates	Peak response (counts)				Carry-over (%)		Mean Carry-over (%)	
		Blank sample		LLOQ		Analyte	I.S.	Analyte	I.S.
		Analyte	I.S.	Analyte	I.S.				
1	1	0.00	0.00	325	75,878	0.00	0.00	0.00	0.559
	2	0.00	780	318	69,776	0.00	1.12		
2	1	0.00	0.00	329	90,688	0.00	0.00	0.00	0.0216
	2	0.00	37.0	331	85,647	0.00	0.04		
3	1	0.00	0.00	283	85,892	0.00	0.00	0.00	0.00
	2	0.00	0.00	272	78,836	0.00	0.00		
4	1	0.00	0.00	253	77,695	0.00	0.00	0.00	0.00
	2	0.00	0.00	214	71,429	0.00	0.00		
					Mean	0.00	0.145		

levels (30.0–90.0–1500–2400–3000 ng/mL) by repeated determination ($n = 6$) of pooled QC samples in the same run. The within-series imprecision expressed as intra-run CV % did not exceed 8.90% at the LLOQ, and 4.34% at other concentration levels. Inaccuracy expressed as intra-run RE% was between −1.99% and 3.17% (Table 4). The between-series imprecision expressed as inter-run CV % was below 6.11%, and the inaccuracy expressed as inter-run RE% was between −1.08% and 2.16% (Table 4). For both within- and between-series, imprecision was below 15% (20% at the LLOQ), and inaccuracy was within ±15% (±20% at the LLOQ) for other levels [15]. Hence, this bioanalytical method proved to be precise and accurate.

3.6. Extraction yield

Three sets of three lithium heparinized rat plasma stored QC samples, at low, medium and high concentration levels (90.0–1500–2400 ng/mL) were processed without addition of I.S. The extracts samples were spiked with I.S. at a concentration matching the final concentration of the processed samples (444 ng/mL). Eighteen blank lithium heparinized rat plasma samples were processed without addition of I.S. The extracts samples were spiked with concentrations of indolinone and I.S. matching the final concentration of the processed samples. The responses obtained in these study conditions represented the 100% recovery reference. Six blank lithium heparinized rat plasma samples were processed with addition of internal standard. The extracts were spiked with indolinone at a final concentration matching a processed sample at medium level (1500 ng/mL). The extraction yield was calculated by dividing the mean peak area ratio of the extracted samples by the corresponding mean peak area ratio of the spiked extract. The absolute extraction recovery was between 100 and 104% for indolinone and 121% for I.S. (Table 5). Thus, the absolute recovery of indolinone was consistent and reproducible across the entire range (30–3000 ng/mL).

3.7. Processed sample stability in the autosampler conditions

Six replicates of QC samples of indolinone, at 2 concentration levels (90.0–2400 ng/mL), were processed and injected bracketed between two sets of calibration standards. After 4 days of storage at 10 °C and protected from light, the run was re-analysed. At both concentration levels, the imprecision (CV %) did not exceed 3.75%. The inaccuracy (RE%) was between −0.111% and 12.0% (Table 6), demonstrating that indolinone in lithium heparinized rat plasma was stable up to 4 days in the autosampler conditions (10 °C, protected from light).

Table 3
Selectivity test at the LLOQ for indolinone, based on three different plasma batches.

Nominal level (ng/mL)	30.0
Mean	33.1
S.D.	2.39
CV%	7.24
RE%	8.67

3.8. Dilution test

Twelve pooled lithium heparinized rat plasma QC samples at 15,000 ng/mL were diluted (six at 1/10 and six at 1/100), then processed and injected bracketed between two sets of calibration standards. The resulting concentrations were multiplied by the dilution factor. For both dilution factors, the imprecision (CV %) was below 1.49% and the inaccuracy (RE%) was between −5.33% and −1.77% (Table 7). Thus, the dilution had no effect on the precision and accuracy of the results.

3.9. Freeze/thaw stability

Six replicates lithium heparinized rat plasma samples at two concentration levels (90.0–2400 ng/mL) were subjected to three freeze/thaw cycles (thawed for 4 h on benchtop at room temperature and freeze for at least 20 h). These samples were then processed and quantified with a duplicate set of calibration samples that were not submitted to the freeze/thaw cycles. For both concentration levels, the imprecision (CV %) was below 3.88%, and the inaccuracy (RE%) was between −0.0299% and 0.204% (Table 6). Hence, that indolinone in lithium heparinized rat plasma proved to be stable after three freeze/thaw cycles below −65 °C.

3.10. Biological samples stability on benchtop at room temperature

Six replicates of lithium heparinized rat plasma QC samples at two concentrations (90.0–2400 ng/mL) were thawed, kept at room temperature for ca. 4 h, and then processed. The samples were quantified with duplicate set of calibration samples that were processed immediately after thawing. At both concentration levels, the imprecision (CV %) was below 3.98%, and the inaccuracy (RE%) between −6.22% and −0.870% (Table 6). This demonstrated that indolinone in lithium heparinized rat plasma was stable after standing for ca. 4 h at RT prior to processing.

3.11. Biological samples long−term stability below −65 °C

Three replicates of lithium heparinized rat plasma samples freshly prepared at three concentrations (90.0–1500–2400 ng/mL) were analysed at time 0 (t_0). Three other replicates at the same concentrations were stored below −65 °C during 33, 119 (data not shown) and 190 days, and processed. They were quantified using a

Table 4
Within- and between-series imprecision (expressed as CV %), and inaccuracy (expressed as RE%) of QC samples, based on 3 series of 6 replicates for each level.

Nominal level (ng/mL)	30.0 (LLOQ)	90.0 (QCL)	1500 (QCM)	2400 (QCH)	3000 (ULOQ)
Intra-run Mean	30.0	90.2	1474	2476	2940
Intra-run S.D.	2.67	3.18	64.1	41.9	117
Intra-run CV %	8.90	3.52	4.34	1.69	3.98
Intra-run RE %	0.00	0.241	−1.72	3.17	−1.99
Inter-run Mean	29.8	89.8	1484	2452	2974
Inter-run S.D.	1.82	3.21	41.7	40.8	105
Inter-run CV %	6.11	3.57	2.81	1.67	3.53
Inter-run RE%	−0.741	−0.185	−1.08	2.16	−0.871

Table 5
Absolute extraction yield of indolinone and I.S. (*E*,*Z*)-3-(benzylidenyl)-indolin-2-one ($n = 6$).

QC nominal level (ng/mL)	90.0	1500	2400
Absolute recovery (%)	100	100	104
CV%	8.06	7.50	5.97
SD	8.06	7.52	6.22

I.S. level (ng/mL)	444
Absolute recovery (%)	121
CV%	11.2
SD	13.4

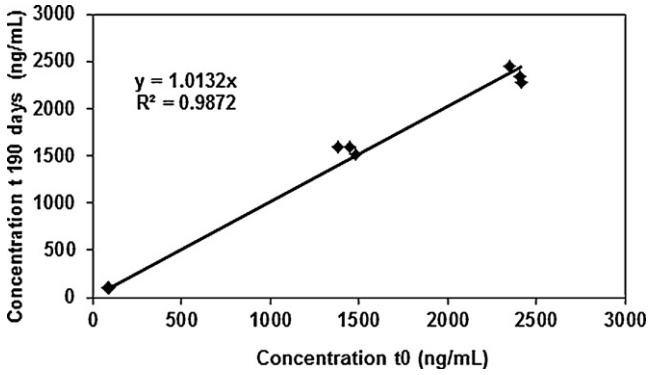

Fig. 4. Long-term stability of indolinone in rat plasma stored for 190 days below −65 °C.

fresh set of calibration samples. The stability samples were bracketed by freshly prepared quality control samples, as duplicates at three concentrations (low, medium and high). These QCs were prepared with an independent stock solution. The difference calculated between the mean of QC at t_0 and the mean of QC stored 190 days below −65 °C ($n = 3$) was between −1.48 and 10.1% (Table 6). As shown in Fig. 4, a linear regression, forced through zero, was calculated plotting the results of stored samples in function of t_0 measurement. As the slope (1.0132) was equal to 1 ± 0.15, the stability of the samples stored in these conditions was confirmed.

Table 6
Short-term and long-term stabilities during storage in various conditions (expressed as RE%) ($n = 6$).

Nominal levels (ng/mL)	90.0	2400
Three successive freeze/thaw cycles below −65 °C	0.204	−0.0299
Stored plasma at RT for 4 h	−0.870	−6.22
Processed plasma at 10 °C for 4 days	−0.111	12.0
Stored plasma below −65 °C for 6 months	10.1	−1.48

Table 7
Dilution test.

Nominal level (ng/mL)	15,000	
Dilution factor	10×	100×
Mean	14,734	14,200
S.D.	127	211
CV%	0.863	1.49
RE%	−1.77	−5.33

3.12. Stock solutions stability test

The results in Tables 8 and 9 demonstrated that stock solutions of indolinone and I.S. stored below −65 °C for 190 days and kept for ca. 6 h at RT were stable, since the degradation expressed by the difference percentage (−1.11% and −1.46% for indolinone and I.S. respectively) was below 5%.

3.13. PK study

The validated method was then applied to a preliminary pharmacokinetic study of indolinone in rats after a single intravenous dose of 2 mg/kg. Table 10 summarizes the main pharmacokinetic parameters of indolinone calculated by non-compartmental analysis using WinNonlin software. The indolinone concentration–time profile obtained after i.v. administration is shown in Fig. 5. The initial concentration (C_0) was 5205 ng/mL, and the half-life time ($t_{1/2}$) was 4.3 min. The area under the concentration-time curve (AUC), calculated based on the trapezoidal rule, was 560 ng h/mL, and the clearance was 3.83 L/h/kg. This preliminary study with a dose of 2 mg/kg demonstrated that the validated bioanalytical method

Table 8
Long-term stability for indolinone stock solution (190 days storage at below −65 °C, and 6 h at RT).

Stock solutions tested	Replicates	Peak area ratios
$t = 190$ days	1	0.104
Stored SS indolinone + Freshly	2	0.107
prepared SS I.S.	3	0.106
	4	0.103
	5	0.107
	6	0.106
Mean		0.106
S.D.		0.00164
CV %		1.56
$t = 0$	1	0.102
Freshly prepared SS indolinone + Freshly	2	0.108
prepared SS I.S.	3	0.101
	4	0.105
	5	0.104
	6	0.106
Mean		0.104
S.D.		0.00258
CV %		2.47
Difference %		−1.11

Table 9
Long-term stability for I.S. stock solution (190 days storage at below −65 °C, and 6 h at RT).

Stock solutions tested	Replicates	Peak area ratios
t = 190 days	1	9.61
Stored SS I.S.	2	9.75
+	3	9.81
Freshly prepared SS indolinone	4	9.99
	5	9.54
	6	9.69
Mean		9.73
S.D.		0.159
CV %		1.64
t = 0	1	9.80
Freshly prepared SS I.S. + Freshly	2	9.26
prepared SS indolinone	3	9.90
	4	9.52
	5	9.62
	6	9.43
Mean		9.59
S.D.		0.237
CV %		2.47
Difference %		−1.46

Table 10
Pharmacokinetic parameters after a single intravenous dose of 2 mg/kg indolinone in rats (n = 4). Data were calculated using non-compartmental analysis.

Parameters	Mean	SE
C_0 (ng/ml)	5205	2232
$t_{1/2}$ (min)	4.30	0.14
K_e (1/h)	9.53	0.21
AUC_{0-last} (ng*h/ml)	561	166
$AUC_{0-\infty}$ (ng*h/ml)	568	162
MRT (min)	5.16	0.59
V_z (L/kg)	0.39	0.15
CL (L/h/kg)	3.83	1.46

SE: standard error; C_0: the concentration at time zero; $t_{1/2}$: half-life of elimination; AUC_{0-last}: area under curve from time zero to 30 min; K_e: elimination rate constant; $AUC_{0-\infty}$: area under the curve with extrapolation to infinity; MRT: mean residence time; V_z: volume of distribution at the terminal phase; CL: clearance.

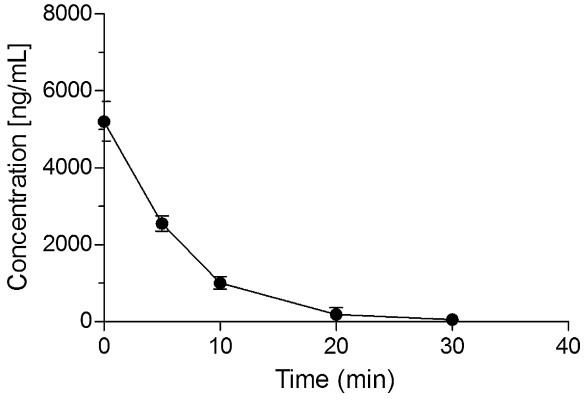

Fig. 5. Mean plasma concentration time profile of indolinone in male Sprague–Dawley rats (n = 4) following i.v. administration (2 mg/kg bw).

was suitable to assess in a specific, selective, precise, accurate and reliable way indolinone levels in rat plasma up to 30 min included. Next PK studies with this validated method will be carried out with doses > 2 mg/kg.

4. Conclusions

The standard calibration curve of indolinone generated between 30.0 and 3000 ng/mL was quadratic and the limit of quantification was 30.0 ng/mL. The rat plasma samples containing indolinone could be diluted up to 100 fold without affecting the precision and accuracy. Indolinone was found to be stable in rat plasma samples kept for 4 h on the bench at RT, after three successive freeze/thaw cycles, and in processed plasma samples stored at ca. 10 °C for 4 days. Indolinone and I.S. stock solutions were found to be stable after 190 days of storage below −65 °C. The carry-over had no impact on the results. The results of this validation study demonstrate that the method is specific, selective, precise, accurate, and capable of producing reliable results. Hence, the method developed here is reliable for the quantification of indolinone in rat plasma samples for routine analysis and will be used for a full PK study with the compound.

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