

A Validated Liquid Chromatography-Tandem Mass Spectrometry Coupled with Liquid-Liquid Extraction for Indacaterol Quantitation in Human Plasma

Wesam G. Ammari^{1,*}, Zainab Al-Qadhi¹, Mohammad Khalil², Rabab Tayyem², Samir Qammaz¹, Ghaleb Oriquat¹, Iman A. Bashedi³, Henry Chrystyn⁴

¹School of Pharmacy and Medical Sciences, Al-Ahliyya Amman University, Jordan

²ACDIMA Centre for Bioequivalence and Pharmaceutical Studies, Jordan

³School of Pharmacy, Applied Sciences University, Jordan

⁴School of Applied Sciences, University of Huddersfield, United Kingdom

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Abstract Indacaterol is a recent ultra-long-acting inhaled β_2 -agonist bronchodilator. The current work validated an in-house developed high performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) bioassay for indacaterol in human plasma. Ethyl acetate was used in a liquid-liquid extraction (LLE) method to extract indacaterol from plasma samples. A 200 μ L of the mobile phase (water-methanol (30:70, v/v)) was used to reconstitute the indacaterol dry extract. A 5 μ L was then eluted on a C18 column at a mobile phase flow of 1 mL/min. Formoterol was the internal standard (IS). The MS/MS was the detector. Method validation followed the International Guidelines. Indacaterol and IS were detected at a mass to charge ratio of 393.3 and 345.2, respectively. Validated calibration curves were linear over 0.075 - 100 ng/mL. Method specificity was established in 8 different plasma batches. No matrix effect was observed. The intra-batch accuracy (precision) percentages for the lower limit of quantitation (LLOQ), Low, Mid and High quality control levels were 113.6 (10.8), 104.7 (9.1), 99.8 (7.6) and 102.2 (8.5), respectively. The inter-batch accuracy, recovery, short-term and long-term stability results were accepted. Validation has confirmed a specific, accurate and precise HPLC-MS/MS coupled with a simple and fast LLE for indacaterol in human plasma.

Keywords HPLC-MS/MS, Plasma, Validation, Liquid-Liquid Extraction, Indacaterol

1. Introduction

The availability of well-developed and validated bio-analytical methods for the quantitation of drugs in various

human body fluids supports the conduct of research studies of various purposes including pharmacokinetic, bioequivalence and therapeutic monitoring research. Indacaterol is the first ultra-long-acting β_2 -agonist bronchodilator that has been approved by the U.S. Food and Drug Administration (FDA) in July 2011 [1,2]. It is administered as an orally inhaled dry powder through a pre-metered, single-unit-dose capsule-based dry powder inhaler (DPI) [3,4]. Determination of the lung deposition of inhaled therapies can have many applications including studying the pharmacokinetic performance of the drugs themselves, comparing the inhaled products; especially generic against innovator or different inhaler devices, as well as comparing the inhalation techniques and flow rates used by the patients through an inhaler. Consequently, different pharmacokinetic methods have been published in the literature. These methods were based on bio-analytical techniques that determined the inhaled drug concentration in either human plasma [5-7] or urine [8-11] samples.

Recently, our research group have developed and validated a bio-analytical assay that determined indacaterol in human urine [12]. To the best of our knowledge, one work had described the validation of a bioassay for indacaterol in human serum [13]. However, that work used a sophisticated extraction technique; solid-phase extraction (SPE), that was on-line coupled to the high performance liquid chromatography-tandem mass spectrometry detection (HPLC-MS/MS) instrumentation. This on-line SPE set-up may not be available widely in the laboratories for other researchers to reproduce that analytical technique in their research studies. Therefore, we aimed in our present work to validate an in-house developed HPLC-MS/MS analytical method that involved a simple and fast liquid-liquid extraction (LLE) technique for the determination of indacaterol in human plasma. We have followed a consensus of the U.S. FDA [14] and the European Medicines Agency (EMA) [15] Guidelines

on Bio-analytical Method Validation.

2. Materials and Methods

Standards and Chemical Reagents

Indacaterol maleate, $C_{24}H_{28}N_2O_3 \cdot C_4H_4O_4$, molecular weight 508.6 as a maleate salt (392.49 as a base), was the reference standard (analyte) of the study. Its approximate water solubility, log P, pKa (strongest acidic) and pKa (strongest basic) are 7.9×10^{-3} g/L, 4.0, 8.5 and 9.8, respectively. It was synthesized by Novartis Pharma (Batch No. 0323006) and was obtained through the Jordan Food and Drug Administration (JFDA), Amman, Jordan. The internal standard (IS) was formoterol fumarate dihydrate, $(C_{19}H_{24}N_2O_4)_2 \cdot C_4H_4O_4 \cdot (H_2O)_2$, molecular weight 840.92 as salt (344.405 as base), Astra Zeneca Limited (Batch No. 11401). Its approximate water solubility, log P, pKa (strongest acidic) and pKa (strongest basic) are 4.2×10^{-2} g/L, 1.9, 8.6 and 9.8, respectively. The used chemical solvents and reagents were of an HPLC analytical grade. Methanol and formic acid were obtained from Merck, Germany. Ethyl acetate was obtained from Tidia, USA. Water was deionised and purified using the Milli-Q gradient system from Millipore, MA, USA. The blank human plasma batches were obtained free-of-charge from the Jordan National Blood Bank, Amman, Jordan. These were different batches that were donated by anonymous, healthy adult subjects.

Instrumentation

The HPLC system consisted of Agilent 1200 series HPLC isocratic pump, Agilent 1260 series in-line degasser, Agilent 1200 series auto-sampler, Agilent 1200 series column oven, USA. The HPLC analytical column was a Reprosil 100 C18 (150 x 4.6 mm, 5 μ m), USA. The mass spectrometric detection was performed using Applied BioSystem API 4000 Triple Quadrupole tandem mass spectrometer detector, MDS Sciex, Canada, which was equipped with a turbo V ion spray source in a positive mode. Nitrogen gas (99.99%) was used (Peak nitrogen generator, Scotland). The ion source gas 1, ion source gas 2, collision gas and curtain gas were set at 50, 50, 10 and 25 psi, respectively. The ion spray voltage was set at 5500 V. The entrance and declustering potentials were 10 V and 70 V, respectively. The interface heater was set at 600 °C. The collision energies for indacaterol and IS were 33.5 V and 29 V, respectively. Multiple reactions monitoring (MRM) was the scanning detection mode. The acquisition of data was performed with a dwell time of 200 ms for each transition. The predominant indacaterol precursor ion was the protonated species $[H+H]^+$. The monitored transitions used for indacaterol (m/z) were Q1: 393.3 \rightarrow Q3: 173.2; and for the IS (m/z) were Q1: 345.2 \rightarrow Q3: 149.1. Other used instruments were an analytical balance Mettler Toledo XP2U (Switzerland), a pH meter Mettler Toledo (Switzerland), Centrifuge Eppendorf 5810R (Germany) and Vortex IKA[®] VX1 (Germany). Gilson micropipettes, France, were used.

The processing of the chromatograms, calibration curves

and regression calculations was performed by the Analyst[®] 1.6 software (MDS Sciex, Canada). The management of the acquired data was also done using Watson Thermo Quest Lab Management system (LMS[®]).

Preparation of Indacaterol and IS Stock and Working Solutions

An indacaterol stock solution was prepared by dissolving indacaterol maleate salt in methanol to give a final stock concentration equivalent to 500 μ g/mL of indacaterol free base. A volume of 100 μ L of this stock solution was then diluted with methanol in a 25 mL volumetric flask to give a 2 μ g/mL indacaterol working solution. In the same manner, the IS stock and working solutions were prepared to give final concentrations of 500 μ g/mL and 2 μ g/mL of formoterol free base, respectively.

Preparation of Standard Calibration Curve Points

Nine serial dilutions to the indacaterol working solution in a diluent solvent of water/methanol (50/50 v/v) mixture were performed to give indacaterol concentrations of 0.75, 1.0, 2.0, 10.0, 50.0, 100.0, 400.0, 800.0 and 1000 ng/mL. Then, a volume of 500 μ L of each of these solutions was used to spike a blank human plasma to give the following nine standard calibration curve points with final concentrations of 0.075, 0.10, 0.20, 1.0, 5.0, 10.0, 40.0, 80.0 and 100 ng/mL.

Preparation of Quality Control (QC) Points

Three serial dilutions to the indacaterol working solution in a diluent mixture of water/methanol (50/50 v/v) were performed to give indacaterol concentrations of 2.25, 500 and 850 ng/mL. Then, a volume of 500 μ L of each of these solutions was used to spike a blank human plasma to give the following three QC points with final concentrations of 0.225, 50.0 and 85.0 ng/mL.

Additionally, a 500 μ L of the indacaterol working solution was diluted up to 5 mL with blank plasma to give a final concentration of 200 ng/mL which was used specifically as a QC solution for the dilution integrity validation test.

Sample Preparation and Extraction Method

The sample preparation and LLE procedure for indacaterol and IS from the human plasma is presented in Table 1. In addition to the 9 non-zero calibration curve points, a blank sample and a zero sample were also included in the calibration curve. The blank sample was a plasma sample that was prepared and extracted without being spiked with indacaterol and IS. The zero sample was a plasma sample that was prepared and extracted without being spiked with indacaterol. The procedure in Table 1 was followed to prepare and extract the blank samples, however without performing steps 3 and 4. Instead, the blank samples were spiked with 50 μ L of methanol (step 6) to compensate for the 50 μ L volume of the IS. The same procedure was followed to prepare and extract the zero samples, however without performing step 3.

Table 1. Sample Preparation and LLE Procedure of Indacaterol and IS from Human Plasma.

1.	Pipette 1 mL of blank human plasma into a 10 mL test tube.
2.	Take the blank sample apart.
3.	Spike each sample (non-blank and non-zero) with a certain volume of indacaterol working standard solutions, as appropriate, to get a concentration equivalent to LLOQ, Low, Mid or High QC level according to the validation test being performed.
4.	Spike each sample with 50 μ L IS working solution.
5.	Add 400 μ L of 25% formic acid.
6.	Spike the blank sample with 50 μ L of methanol to compensate for the 50 μ L of IS solution used for spiking the other samples.
7.	Add 4 mL of ethyl acetate (the extraction solvent) and vortex for 60 seconds.
8.	Centrifuge for 5 minutes at a speed of 4000 rpm at 5 $^{\circ}$ C.
9.	Freeze the sample and decant the upper organic layer into a new test tube.
10.	Evaporate the organic layer under a nitrogen stream at 40 $^{\circ}$ C.
11.	Reconstitute the dry residue with 200 μ L of the mobile phase.
12.	Inject 5 μ L directly into the LC-MS/MS system using the auto-sampler kept at 5 $^{\circ}$ C.

HPLC-MS/MS Analytical Method

A volume of 5 μ L of the extracted samples was injected, via the auto-sampler (kept at 5 $^{\circ}$ C), into the HPLC-MS/MS system for analysis. The samples were analysed on a reversed-stationary phase using a Reprosil 100 C18 (150 x 4.6 mm, 5 μ m) column at a temperature of 40 $^{\circ}$ C. The mobile phase consisted of a mixture of deionized water and methanol (30:70 (v/v)) acidified with 300 μ L of formic acid per litre of the mixture. An isocratic elution was used at a flow rate of 1 mL/min with a total analytical run-time of 3 minutes. The retention times (t_R) of indacaterol and IS were 2.5 and 1.5 minutes, respectively.

3. Method Validation

A consensus of the U.S. FDA[14] and the European Medicines Agency [15] (EMA) Bioanalytical Method Validation Guidelines was followed.

Calibration Curve Linearity and Lower Limit of Quantitation (LLOQ)

Initially, the lower limit of detection (LOD), the lowest concentration of indacaterol that the bioanalytical procedure can reliably differentiate from the background noise, was determined. This LOD was differentiated from the LLOQ which was the lowest amount of indacaterol in a sample that could be quantitatively determined with acceptable precision and accuracy. Then to validate the method's calibration curve, six different calibration curves were analysed on six different days to check for linearity and accuracy. The peak-area ratios (peak area of indacaterol to peak area of IS) on y-axis vs the calibration curve points concentrations on x-axis were plotted and fitted to the equation; $y = bx + a$, where (a) represents the y-axis intercept and (b) represents the slope. The $1/x$ weighting was used for linearity fitting. The acceptance criteria included not more than $\pm 20\%$ deviation at LLOQ ($\pm 15\%$ at other points) from nominal concentrations. At least 75% of non-zero

standards should meet the above criteria including the LLOQ and the upper LOQ (ULOQ) with an overall correlation coefficient (r) ≥ 0.95 .

The sample dilution integrity was assessed where six two-fold the ULOQ samples were prepared and then diluted four times with a blank human plasma to get a mid-range calibration curve concentration. These diluted samples were then extracted and analysed. An accuracy of $\pm 15\%$ and a precision $< 15\%$ were the test acceptance criteria.

Intra-Batch and Inter-Batch Accuracy and Precision

The intra-batch accuracy and precision were investigated by analysing six replicate samples at four different indacaterol concentrations (ng/mL); 0.075 (LLOQ), 0.225 (Low QC), 50.0 (Mid QC) and 85.0 (High QC) which were prepared from the same plasma batch and analysed on the same day. The inter-batch accuracy and precision were evaluated by analysing six replicate samples at each of the previous concentration levels prepared from 3 different plasma batches and analysed on 3 different days. The accuracy at each concentration level was the ratio of the actual to nominal concentrations. The deviation of the mean from the true value was used as an accuracy measure. The precision was the ratio of standard deviation to mean (%CV). The acceptance criteria were mean accuracy within $\pm 20\%$ at the LLOQ level ($\pm 15\%$ at other QC levels) and precision of $< 20\%$ at the LLOQ ($< 15\%$ at other QC levels).

Specificity

The specificity of the bioanalytical method was evaluated by screening six different batches of human plasma to ensure that the analytical response of the LLOQ prepared samples was at least five times the response of the blank samples at the t_R of indacaterol, and to ensure that the analytical response in each corresponding blank sample at the t_R of the IS was less than 5% of that of the IS spiked sample (zero sample). Moreover, the specificity was studied in two additional batches of lipemic and hemolyzed plasma samples.

Matrix Effect

Plasma matrix interference was investigated by comparing the analytical response (peak areas) of six low QC-equivalent standard working solutions (SET 1) with that of low QC samples prepared and extracted from six different plasma batches (SET 2). Two additional batches of lipemic and hemolyzed plasma batches were also assessed. The matrix factors (MF) were calculated for both indacaterol and IS for each plasma batch by dividing each peak area of the post-extracted spiked samples in SET 2 by the corresponding mean peak areas of SET 1 and then multiplied by 100. The IS-normalized MF was then calculated by dividing the MF of indacaterol by the MF of IS for each plasma batch. To have no matrix effect, the %CV of the IS-normalized MF should be <15%.

Carryover

The carryover was assessed by injecting a blank sample immediately after the end of the third inter-batch accuracy and precision validation test (to cover the maximum number of samples of the batch). To have no carryover effect, the peak areas of the blank sample at the t_R of indacaterol and IS should be <20% of the peak area observed for indacaterol at the LLOQ level and <5% of the response observed for the IS.

Recovery

The recovery of indacaterol was examined at the Low QC, Mid QC and High QC levels. The recovery of the IS was evaluated at a concentration of 200 ng/mL. Using one plasma batch, two sets of samples (6 replicates each) were prepared at each of the three QC levels (named SET 1 and SET 2). For SET 1, the six samples of blank plasma were extracted first according to the extraction method and were then spiked with the appropriate indacaterol standard solution to get the required QC level.

For SET 2, the six samples of blank plasma were spiked first with the appropriate indacaterol standard solution corresponding to the required QC level and were then extracted as per the extraction procedure. SET 1 and SET 2 were then analysed. The peak areas of the IS were also evaluated in both sets at the Mid concentration level. At each QC level, the recovery % was calculated by dividing the peak area of each run of SET 2 by the mean of the peak areas of SET 1 multiplied by 100. The recovery was accepted if the precision (%CV) at each QC level was $\leq 15\%$.

Stability

Sufficient volumes of six replicate samples of the Low QC and High QC levels were prepared and immediately analysed to evaluate the initial stability. Then, the remaining volumes were frozen immediately at -70°C to be analysed later to evaluate the short-term, long-term and freeze-thaw stabilities of the samples, as follows. The stability acceptance criteria were an accuracy between 85-115% ($\pm 15\%$) and a precision (%CV) <15%.

Short-Term and Long-Term Stabilities

The short-term stability samples were frozen at -70°C for about two hours (until completely frozen) and were then left at room temperature (RT) for around 24 hours. After that these samples were extracted and analysed along with freshly prepared calibration curve points and two fresh replicate samples at each QC level. The long-term stability was investigated in a similar manner; however the samples were frozen at -70°C for 60 days before being thawed unassisted at RT, extracted and analysed.

To check for the calibration curve stability, sufficient volumes of the calibration curve points were prepared and analysed immediately for the initial stability evaluation. Additional calibration curve points were prepared and stored at -70°C for 60 days. After which they were analysed to test their long-term stability.

Freeze-Thaw Stability

The stability of the spiked plasma samples was investigated after three freeze-thaw cycles. Each cycle included freezing the samples at -70°C for 24 hours followed by unassisted thawing at RT. At the end of the third freeze-thaw cycle, the plasma samples were extracted and then analysed simultaneously with freshly prepared calibration curve points and two fresh replicate samples at each QC level.

Auto-Sampler Stability

Six replicate samples of the Low, Mid and High QC levels were prepared, extracted and analysed initially with a freshly prepared standard calibration curve on the same batch (run). The whole batch was re-analysed after being stored in the auto-sampler at 5°C for 24 hours. The auto-sampler stability was evaluated using two approaches; the post-preparative stability (PPS) approach where the re-injected QC samples were back-calculated using the initial calibration curve. Whilst, the second approach was the processed samples integrity (PSI) where the re-injected QC samples were back-calculated using the re-injected calibration curve.

Dry-Extract Stability

Six replicate samples of the Low, Mid and High QC levels were prepared and extracted. However, before the reconstitution step, the samples were stored as dry residues at -70°C for 24 hours. The samples were then reconstituted with 200 μL of the mobile phase and analysed simultaneously with a freshly prepared calibration curve.

Stock and Working Solutions Stability

The stability of the stock and working standard solutions of indacaterol and IS after being stored at RT for six hours and after being stored at -70°C for eight days was investigated and confirmed in a previous work of ours [12].

4. Results

Calibration Curve

Table 2 presents the mean accuracy, the precision and the linearity function parameters of the six validated calibration curves along with their regression coefficients (r). The calibration curves were linear over the range of 0.075 – 100 ng/mL. The lower limit of detection (LOD) of indacaterol in plasma was 0.050 ng/mL. However, the LLOQ was identifiable and reproducible at 0.075 ng/mL with a mean accuracy of 99.56% and a precision of 13.0%. Moreover, the sample dilution integrity results met the acceptance criteria. The accuracy and precision were 94.3% and 9.3%, respectively.

Accuracy and Precision

The intra-batch accuracy and precision results for the LLOQ, Low, Mid and High QC levels met the acceptance criteria. The accuracy (precision) percentages were 113.6 (10.8), 104.7 (9.1), 99.8 (7.6) and 102.2 (8.5) for these levels, respectively. Figures 1 – 4 are examples on representative chromatograms for the LLOQ, Low, Mid and High QC levels, respectively. Table 3 presents the inter-batch accuracy and precision results for three human plasma batches which met the acceptance criteria.

Table 2. Accuracy, precision and linearity functions of the calibration curves

Concentration ng/mL	Nominal	0.075	0.10	0.20	1.0	5.0	10.0	40.0	80.0	100.0
	Mean (SD) Back-calculated (n=6 curves)		0.075 (0.010)	0.105 (0.006)	0.194 (0.014)	0.936 (0.069)	5.085 (0.306)	9.970 (0.721)	42.390 (1.260)	80.832 (2.968)
Mean accuracy (%)		99.56	105.33	96.83	93.60	101.71	99.70	105.98	101.04	96.77
Mean precision (%)		13.00	5.72	7.43	7.43	6.02	7.23	2.97	3.67	4.28
Parameters of the calibration curves functions; $y = bx + a$										
	Curve 1	Curve 2	Curve 3	Curve 4	Curve 5	Curve 6				
a*	9.50×10^{-05}	1.83×10^{-04}	2.85×10^{-04}	8.40×10^{-05}	3.25×10^{-04}	-2.00×10^{-05}				
b**	7.89×10^{-03}	8.12×10^{-03}	7.60×10^{-03}	7.50×10^{-03}	9.19×10^{-03}	3.52×10^{-03}				
r***	0.9986	0.9953	0.9999	0.9993	0.9993	0.9998				

* a: Calibration curve y-axis intercept. **b: Calibration curve slope. *** r: Calibration curve regression factor.

Table 3. The inter-batch accuracy and precision results for indacaterol in plasma.

	LLOQ ^a (0.075 ng/mL)	Low QC ^b (0.225 ng/mL)	Mid QC ^b (50 ng/mL)	High QC ^b (85 ng/mL)
Mean ^c	0.081	0.229	49.941	86.782
SD	0.012	0.023	2.484	6.259
Precision (CV%)	14.37	10.16	4.97	7.21
Accuracy (%)	107.85	101.80	99.88	102.10

a: Lower limit of quantitation. b: Quality control level. c: Mean indacaterol concentrations, ng/mL (n=3 plasma batches).

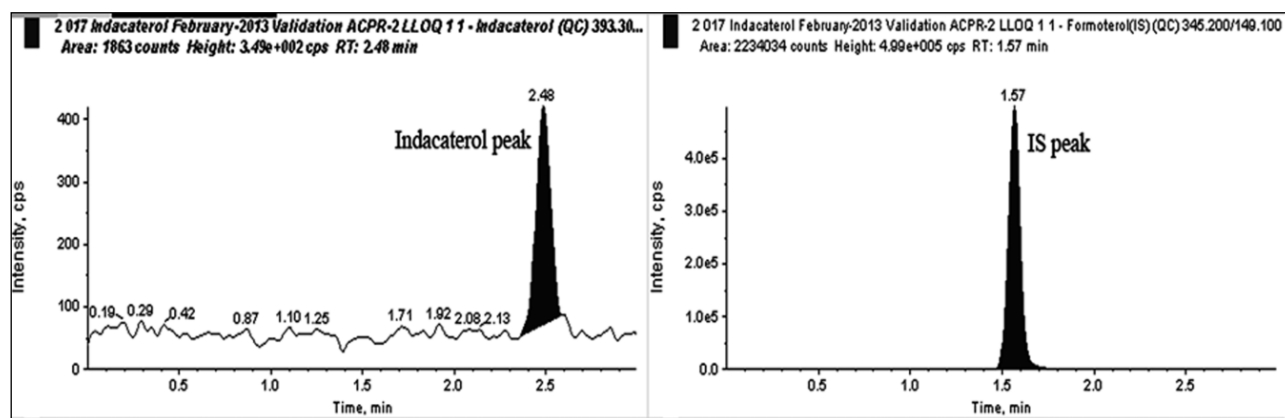


Figure 1. Representative chromatogram at LLOQ – Indacaterol (left) and IS (right).

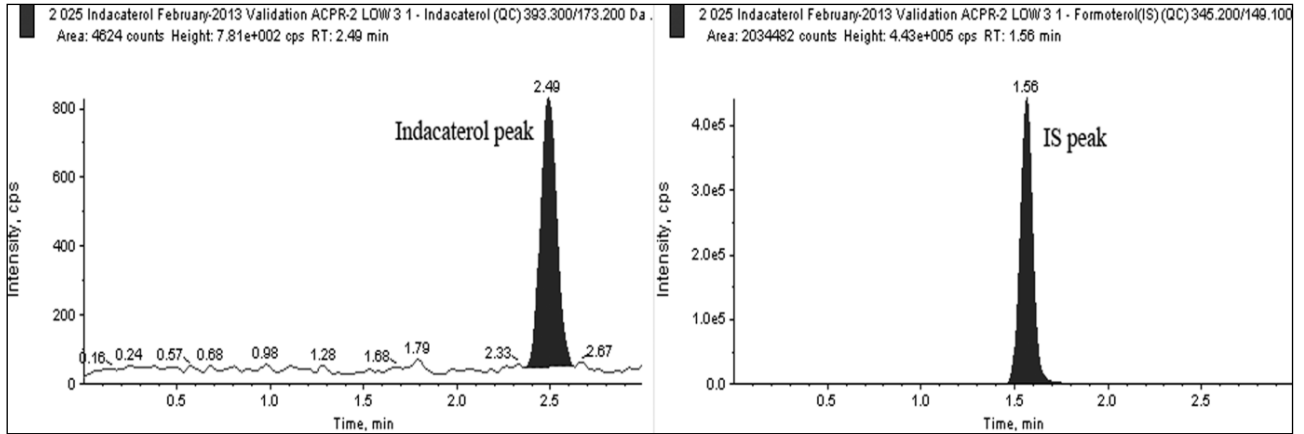


Figure 2. Representative chromatogram at Low QC – Indacaterol (left) and IS (right).

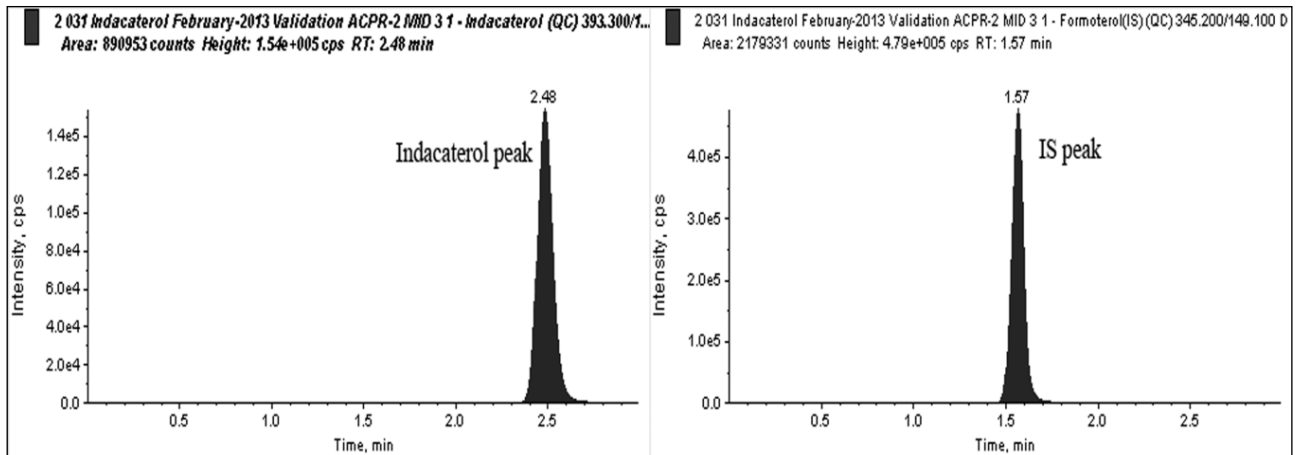


Figure 3. Representative chromatogram at Mid QC – Indacaterol (left) and IS (right).

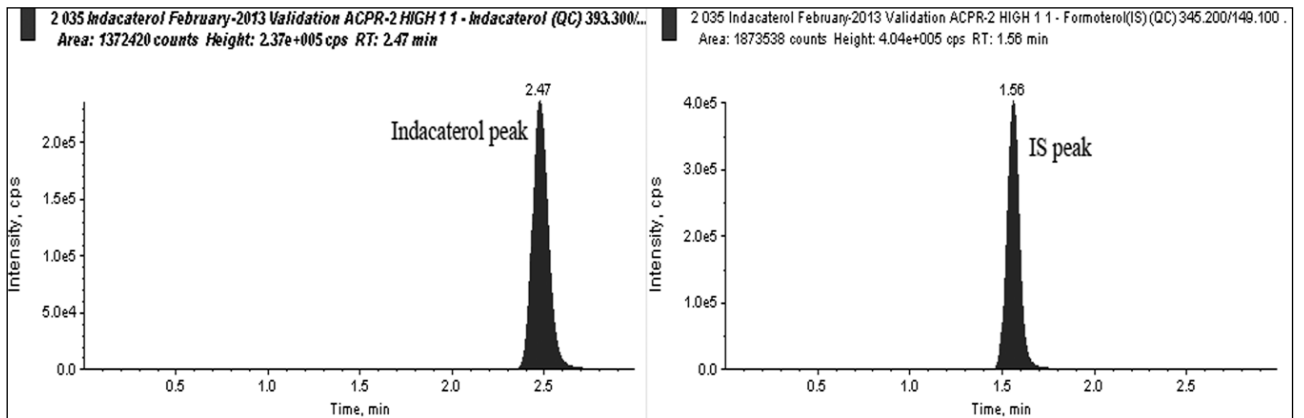


Figure 4. Representative chromatogram at High QC – Indacaterol (left) and IS (right)

Specificity

No interfering peaks were observed at the t_R of both indacaterol (around 2.50 minutes) and IS (around 1.50 minutes) in the chromatograms of the eight analysed plasma batches. Therefore, the ratios of the peak areas of indacaterol and IS in each LLOQ sample of the eight validated plasma batches to their corresponding peak areas (not observed) in each blank sample were zero (acceptance <5%). This confirms that our method is specific for both indacaterol and IS quantification in human plasma.

Matrix Effect

The results of the matrix effect are shown in Table 4. Plasma had no interfering effect on the determination of both indacaterol and the IS in the current analytical method.

Carryover

The carryover results showed that the ratio of the peak area at the t_R of indacaterol in the blank sample to the peak area of indacaterol in the spiked LLOQ sample was zero (acceptance <20%). Similarly, no peak was observed for the IS in the blank sample, therefore the ratio was also zero for the IS carryover assessment (acceptance <5%).

Recovery

The mean recovery (precision) percentages of indacaterol from plasma at the Low, Mid and High QC levels were 55.41 (14.99), 54.32 (14.96) and 54.70 (6.35), respectively. The mean recovery (precision) percentage of the IS was 48.92

(13.22). The recovery of indacaterol and IS from plasma was consistent, precise and reproducible.

Short-Term, Long-Term and Freeze-Thaw Stabilities

Table 5 presents the results of the freshly-prepared (initial), short-term, long-term and freeze-thaw stability of indacaterol in human plasma. The long-term stability results of the calibration curve points showed an accuracy range between 91.66 and 104.55%. The calibration curve had an equation; $y = 0.0035x - 7.80 \times 10^{-5}$, and r value of 0.9996. The stability evaluation met the acceptance criteria.

Auto-Sampler Stability

The PPS results at the Low, Mid and High QC levels showed mean accuracy (precision) percentages of 109.82 (9.93), 106.46 (1.90) and 107.04 (1.69), respectively. The PSI mean accuracy (precision) percentages were 102.31 (8.96), 100.17 (1.83) and 100.56 (1.69) at the corresponding QC levels, respectively. These results confirm that the indacaterol samples were stable in the auto-sampler at 5 °C for 24 hours.

Dry-Extract Stability

The mean accuracy (precision) percentages for the indacaterol dry extract at the Low, Mid and High QC levels were 107.78 (5.80), 112.26 (8.03) and 107.45 (7.42), respectively. These results indicated that the dry extract of indacaterol was stable at -70 °C for 24 hours before being reconstituted for analysis.

Table 4. The matrix effect results for indacaterol and IS determination in plasma.

	HPLC-MS/MS peak area for indacaterol		MF ^a (Indacaterol)	HPLC-MS/MS peak area for IS ^b		MF ^a (IS)	IS ^b - normalized MF ^a
	SET 1	SET 2		SET 1	SET 2		
Mean (n=8)	7.057 x10 ³	6.954 x10 ³	98.539	3.347 x10 ⁶	3.846 x10 ⁶	114.926	0.857
SD	3.581 x10 ²	4.438 x10 ²	6.289	8.736 x10 ⁴	1.462 x10 ⁵	4.367	0.040
CV%	5.07	6.38	6.382	2.61	3.80	3.800	4.611

a: Matrix factor. b: Internal standard.

Table 5. The initial, short-term, long-term and freeze-thaw stability results of indacaterol in plasma

	Initial stability of indacaterol in plasma		Short-term stability at RT ^b for 24 hours		Long-term stability at -70°C for 60 days		Freeze-thaw stability	
	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC
Mean ^a	0.236	86.907	0.247	96.901	0.235	86.366	0.241	95.255
SD	0.022	7.392	0.029	3.200	0.024	2.803	0.033	9.092
Precision (CV%)	9.15	8.51	11.62	3.30	10.30	3.25	13.61	9.54
Accuracy (%)	104.74	102.24	109.56	114.00	104.44	101.61	107.04	112.06

a: Mean indacaterol concentrations, ng/mL (n=6). b: Room temperature.

5. Discussion

In the current work, a simple liquid-liquid extraction method was developed to extract indacaterol from a 1 mL sample of human plasma. The extraction solvent was a 4 mL of ethyl acetate (acidified with 400 μ L of 25% formic acid). The indacaterol dry extract was then reconstituted with a 200 μ L of the mobile phase which was simply an acidified mixture of water and methanol (30:70, v/v). After that, only 5 μ L injection volume was needed for the HPLC analysis that involved eluting indacaterol and formoterol IS on a reversed C18 stationary phase with an isocratic mobile phase pumped at a flow rate of 1 mL/min. The detection system involved the tandem mass spectrometer which employed turbo-ion spray ionization in the positive ion mode along with a multiple reactions monitoring (MRM). Accordingly, indacaterol was monitored at a molecular ion m/z ratio of 393.3 and an MS/MS daughter at m/z ratio of 173.2. Whilst, the IS was monitored at molecular ion m/z ratio of 345.2 and an MS/MS daughter at m/z ratio of 149.1. The total analytical run-time was 3 minutes.

This developed method was then validated according to the international guidelines on bio-analytical method validation [14,15]. The results of the various validation measures have shown that our method was specific, accurate, precise, stable and reproducible. The linearity for indacaterol determination in plasma was confirmed over a concentration range between 0.075 and 100 ng/mL with a LLOQ of 0.075 ng/mL.

One previous published work has developed and validated an HPLC-MS/MS bio-analytical method for indacaterol in human serum [13]. However, that method was fully automated with an on-line sophisticated solid-phase extraction (SPE) technique which might not be widely available in the analytical laboratories. Additionally, this solid phase extraction platform for indacaterol from serum involved two solvent-preconditioning steps for the cartridges that consumed 20 minutes, in addition to other several extraction steps with several chemical solvents needed. A labelled $^{13}\text{C}_3$ -indacaterol as an internal standard was employed in that method. Unlike the fast analytical run-time and the simple isocratic mobile phase flow of the current work, Emotte and co-workers (2012) used a gradient mobile phase flow with a total analytical run-time of 6 minutes.

However, the present work was limited by the higher indacaterol LLOQ and accordingly the quantitation range compared with the previously published one which ranged between 10 and 1000 pg/mL [13]. In this reference, indacaterol is clinically approved in 3 once-daily strengths; 75 mcg (in USA), 150 and 300 mcg (in Europe and many other countries) [2]. Additionally, the developmental single-dose and multiple-dose clinical pharmacokinetic studies safely used much higher indacaterol doses of up to 3000 mcg [16]. Therefore, the ability to clinically use this wide range of indacaterol strengths safely would help overcome this study limitation, and thus allow the researchers to use both our current plasma and previous urinary [12] analytical methods in

their future pharmacokinetic, bioequivalence and clinical studies of indacaterol.

6. Conclusions

The validation of an in-house developed simple and fast LLE coupled with an HPLC-MS/MS technique has confirmed an accurate, precise and specific determination of indacaterol in human plasma. This bioanalytical technique is robust and reproducible to support future indacaterol-related research studies.

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Author Disclosure Statement

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Henry Chrystyn has no shares in any pharmaceutical companies. He has received sponsorship to carry out studies, together with some consultant agreements and honoraria for presentations, from several pharmaceutical companies that market inhaled products. These include AbdiIbrahim, Almirall, AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKline, Innovata Biomed, Meda, Mundipharma, Omron, Sandoz, Teva, Truddell, and UCB. Research sponsorship has also been received from grant awarding bodies (EPSRC and MRC).

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