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Validation of an on-line solid-phase extraction method coupled to liquid chromatography–tandem mass spectrometry detection for the determination of Indacaterol in human serum

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ABSTRACT

Indacaterol has been recently approved in Europe for the treatment of chronic obstructive pulmonary disease (COPD). In the present study, we have developed and validated a rapid and sensitive on-line solid phase extraction (SPE) method coupled to liquid chromatography–tandem mass spectrometry (LC–MS/MS) detection for the determination of Indacaterol in human serum. The sample preparation involves the serum dilution with a 0.2% acetic acid solution prior to the on-line SPE on a mixed-mode cationic (MCX) polymer based sorbent. The samples were then eluted on a reversed phase column with a mobile phase made of acidified water and methanol and detection was performed by MS using electrospay ionization in positive mode. The analysis time between 2 samples was 7.0 min. Standard curves were linear over the range of 10.0 pg/mL (LLOQ) to 1000 pg/mL with correlation coefficient (r^2) greater than 0.990. The method specificity was demonstrated in six different batches of human serum. Intra-run and inter-run precision and accuracy within ±20% (at the LLOQ) and ±15% (other levels) were achieved during a 3-run validation for quality control samples (QCs). The stability at room temperature (38 h) was determined and reported. In addition, the comparison between an off-line SPE procedure and our method gave equivalent results. The results of the present work demonstrated that our on-line SPE–LC–MS/MS method is rapid, sensitive, specific and could be applied to the quantitative analysis of Indacaterol in human serum samples. Our method effectively eliminated the tedious conditioning and rinsing steps associated with conventional off-line SPE and reduced the analysis time. The on-line SPE approach appears attractive for supporting the analysis of several hundreds of clinical samples.

1. Introduction

Indacaterol (Fig. 1) is a novel, ultra long-acting β_2-agonist [1] recently approved in the European Union for the maintenance treatment of moderate-to-severe COPD. The long duration of action of Indacaterol is due to the addition of a long, lipophilic side-chain that binds to an exosite on adrenergic receptors. By acting on the β_2-adrenergic receptor, Indacaterol induces smooth muscle relaxation, which results in dilation of bronchial passages. Indacaterol is administrated via inhalation using a specific device. Hence the amount of drug found in the systemic circulation is rather low. As a consequence, to support the pharmacokinetic and pharmacodynamic studies of Indacaterol, the development of ultra sensitive bioanalytical methods is required. Currently, LC–MS/MS is accepted as a preferred technique for the quantification of small and large molecules in biological matrices. In the literature, LC–MS/MS has been used and proven to be an effective technique for the validation of various pharmaceutical ingredients in human samples [2–5]. It has been also shown that on-line SPE techniques coupled to LC–MS/MS offer speed, high sensitivity due to the pre-concentration factor, and low extraction cost per sample [3,4,6–10]. With the on-line approach, the sample preparation step is embedded into the chromatographic separation resulting in the reduction of the sample preparation time. In addition, robotic liquid handling workstations can be used for parallel sample processing in the on-line approach. Several on-line sample extraction procedures allowing direct injection of biological fluids or extracts followed by LC–MS/MS analysis have recently been developed. With this approach, the quantification of the compound of interest in biological matrix at low nanogram

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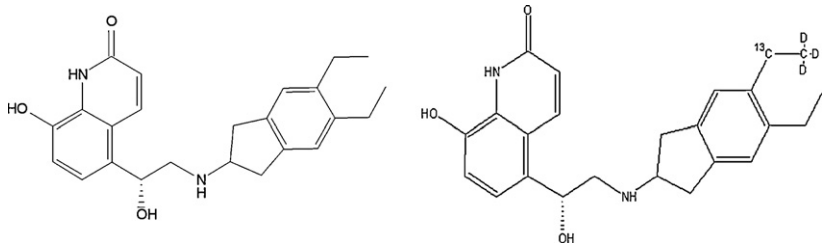

Fig. 1. Chemical structures of Indacaterol (reference standard) and $^{13}CD_3$-Indacaterol (internal standard).

per milliliter level [11–13] was then possible. Even though many SPE sorbents are commercially available, the most commonly used sorbents in the on-line SPE–LC–MS/MS methods are based on the polar and non-polar retention mechanisms [14,15]. However, hydrophobic, non-polar retention mechanism (e.g. C_{18} cartridge) is the least selective because many components are retained from the biological matrices along with the compounds of interest. This retentiveness could be advantageous in cases metabolites and parent drug with very different polarities, have to be simultaneously determined. A Recent study has shown that sorbent that utilizes an ion-exchange interact has been proven to be highly selective for compounds that can be ionized under either acidic or basic conditions [16].

The aim of this study was to develop and validate an ultra-sensitive on-line SPE LC–MS/MS method for the quantification of Indacaterol at a low pg level in the human serum. Mixed-mode cationic polymer-based sorbents was used as the sorbent for Indacaterol on-line extraction prior its separation on a reversed phase column. The validation results presented here demonstrated that our method is well suited for the quantitative analysis of Indacaterol in human serum in order to support the pharmacokinetic studies of the drug during the clinical trials.

2. Experimental

2.1. Chemicals and reagents

The reference standard Indacaterol (purity 99.4%), $C_{24}H_{28}N_2O_3{\cdot}C_4H_4O_4$, molecular weight 508.6 (salt) was synthesized by Novartis Pharma AG, Basel, Switzerland as a maleate salt with a salt to base ratio of 1.296. The internal standard was $^{13}CC_{23}D_3H_{25}N_2O_3$ (free base of Indacaterol, purity 80%), molecular weight 396.5, synthesized by Novartis Pharma Inc., East Hanover, USA. All the solvents and reagents were of analytical grade and were used without further purification. Acetonitrile, 100% acetic acid (glacial) and 32% ammonia solution were obtained from Merck (NJ, USA). Formic acid was obtained from Fluka (Buchs, Switzerland). Water was deionized and purified on Milli-Q gradient system from Millipore (MA, USA). The human serum samples were obtained from the internal blood bank.

2.2. Instrumentation

The on-line SPE system consisted of a Prospekt-2 apparatus (Spark Holland, Emmen, Netherlands) composed of an auto-sampler (Endurance), a solvent delivery unit (SDU) and an automatic cartridge exchange (ACE) module. The cartridge used, Oasis MCX, 1× 10 mm was from Waters (Milford, MA, USA). The HPLC system consisted of a Shimadzu LC-10ADVP mobile phase delivery pump (Kyoto, Japan). The HPLC analytical column used was a Capcell Pack ACR C18 (150 mm × 4.6 mm, 5 μm) from Shiseido (Minato-ku, Tokyo, Japan). Mass spectrometric detection was performed on an API 5000 triple quadrupole mass spectrometer from AB/MDS Sciex (Ontario, Canada) equipped with a Turbo V Ionspray source operating in the positive mode. Data acquisition was performed with Analyst 1.4.2 software distributed by AB/MDS Sciex. The curtain gas, ion source gas 1, ion source gas 2 and collision gas (all nitrogen) were set at 40, 25, 40 and 5 instrument units, respectively. The spray voltage was 5500 V, the heater temperature was 750 °C, the interface heater was turned on, and the entrance potential was set to 10 V. Data acquisition was performed with a dwell time of 50 ms for each transition. Specific settings for each compound are shown in Table 1. Under these MS conditions, the predominant analyte precursor ion was the protonated species $[H+H]^+$. The different monitored transitions used were as follows: Indacaterol (m/z) 393.5 → 173; [$^{13}CD_3$]Indacaterol (m/z) 397.5 → 177.

2.3. Preparation of stock and working solutions

Stock solution containing Indacaterol was prepared in a mixture of water/methanol (1/1, v/v) to give a final concentration of 200 μg/mL. Individual working calibration standard (Cs) solutions with concentrations of 0.400, 0.800, 1.60, 4.00, 20.0, 40.0 and 80.0 ng/mL were prepared after serial dilutions of the stock solution in water/methanol (1/1, v/v). The working quality control sample (QCs) solutions with concentrations of 0.300, 1.20, 4.00, 20.0 and 80.0 ng/mL were prepared in the same manner.

2.4. Preparation of Cs and QCs

Two different batches of human serum were used for the preparation of Cs and QCs. The Cs samples were prepared by spiking each Indacaterol individual working Cs solution with blank human serum. This yielded Cs concentrations of 10.0 (LLOQ), 50.0, 75.0, 100, 250, 500 and 1000 pg/mL. The QCs were prepared in the same manner to give final concentrations of 10.0, 30.0, 75.0 and 750 pg/mL.

2.5. Sample preparation

A volume of 150 μL of serum Cs, QCs or study samples was transferred into a 96-well, then 150 μL of a 0.2% acetic acid in water solution containing the IS at 500 pg/mL were added. The 96-well plate was then sealed with a film, shaken for 10 min and centrifuged at 2250 × g for 10 min at 5 °C. After the centrifugation, an aliquot of the supernatant (200 μL) was directly injected onto the on-line SPE–LC/MS/MS system.

Table 1
SRM transitions and ion optics parameters for Indacaterol and that of its internal standard [$^{13}CD_3$]Indacaterol.

Compounds	Declustering potential (V)	Collision energy (eV)	Collision cell exit potential (V)
Indacaterol	71	33	18
[$^{13}CD_3$]Indacaterol	71	33	18

Table 2
Gradient program used for the LC-analysis of Indacaterol.

Time (mm:ss)	Solvent A (%)	Solvent B (%)
0:00	5	95
3:90	5	95
4:00	0	100
5:00	0	100
5:08	5	95
6:00	5	95

2.6. SPE, LC, and MS conditions

The MCX cartridges were conditioned with 2000 μL solution of methanol/water (90/10, v/v) containing 1% acetic acid and subsequently with 2000 μL of water containing 1% acetic acid. Then 200 μL of diluted serum (calibration standards, QCs, and study samples) were loaded onto the cartridge using 2000 μL water containing 1% acetic acid solution and the cartridges were successively washed with 1500 μL water containing 1% acetic acid, 1500 μL metahnol/water (90/10, v/v) containing 1% acetic acid and 2000 μL methanol/acetonitrile (40/60, v/v) solutions. After completion of the sample preparation cycle, the cartridge was switched in-line with the mobile phases to desorb the analytes and transfer them into the LC column (elute position). After 60 s of elution, the cartridge was switched back off-line (load position), replaced by a new one and the sample preparation cycle started again with the next sample. The total time of the SPE cycle was less than the chromatographic run-time. Thus, once synchronized, the time of sample cleanup is virtually nonexistent after processing the first cartridge. The mobile phases water containing 0.1% of a 32% ammonia solution (A) and methanol (B) were delivered at 1000 μL/min as described in Table 2.

2.7. Off-line LC–MS/MS method for Indacaterol quantitative analysis in human serum

Serum samples (200 μL) were mixed to 10 μL of IS and 400 μL of 1% phosphoric acid 1% in water solutions. The mixture was vortexed for 10 s and then centrifuged at 14,000 rpm for 10 min. Off-line SPE was performed by passing all supernatant fluids through a MCX cartridge which was preconditioned with 500 μL solution of methanol/water (90/10, v/v) acidified with 1% acetic acid, followed by 500 μL distilled water acidified with 1% formic acid before loading 625 μL of the supernatant fluid at a constant flow rate of 1 mL/min. Then, the cartridge was washed with 300 μL distilled water acidified with 1% formic acid, 300 μL, methanol/water (90/10, v/v) acidified with 1% acetic acid, and 500 μL, methanol/acetonitrile (60/40, v/v). Finally, the cartridge was slowly eluted with 2× 200 μL 2% ammonia solution in methanol/water (90/10, v/v). The eluent was evaporated to dryness in a water bath at 37 °C under a nitrogen stream. The residues were reconstituted in 60 μL methanol/water (50/50, v/v) acidified with 0.5% acetic acid. The plate was shaken for 1 min and an aliquot of 20 μL was then transferred to the LC–MS/MS system for analysis. The samples were analyzed on a reversed-phase HPLC on a Thermo Hypersil Gold C18 1.9 μm (50 mm × 2.1 mm) column at 45 °C using gradient elution (0.1% formic acid in MeOH–H_2O, 5:95 (v/v)–0.1% formic acid in MeOH) at a flow rate of 400 μL/min with at total run-time of 5 min.

2.8. Method validation

2.8.1. Specificity

The specificity of the analytical method was investigated in four replicates by preparing and analyzing the blank samples prepared from six different batches of human serum. The specificity was assessed by comparing the mean apparent analytical response for Indacaterol and $^{13}CD_3$-Indacaterol in the blank samples to the mean analytical response obtained for samples spiked with a concentration of Indacaterol at LLOQ and $^{13}CD_3$-Indacaterol at the working concentration (zero samples). Potential contribution of $^{13}CD_3$-Indacaterol to Indacaterol was assessed by comparing the mean analytical response for Indacaterol in a blank sample spiked with $^{13}CD_3$-Indacaterol at the working concentration to the mean analytical response obtained for samples spiked with a concentration of Indacaterol at LLOQ. Potential contribution of Indacaterol to $^{13}CD_3$-Indacaterol was assessed by comparing the mean analytical response for $^{13}CD_3$-Indacaterol in a blank sample spiked with Indacaterol at the ULOQ to the mean analytical response obtained for samples spiked with $^{13}CD_3$-Indacaterol at the working concentration. The acceptance criterion for Indacaterol was interference <20% of analytical response at LLOQ, and acceptance criteria for $^{13}CD_3$-Indacaterol

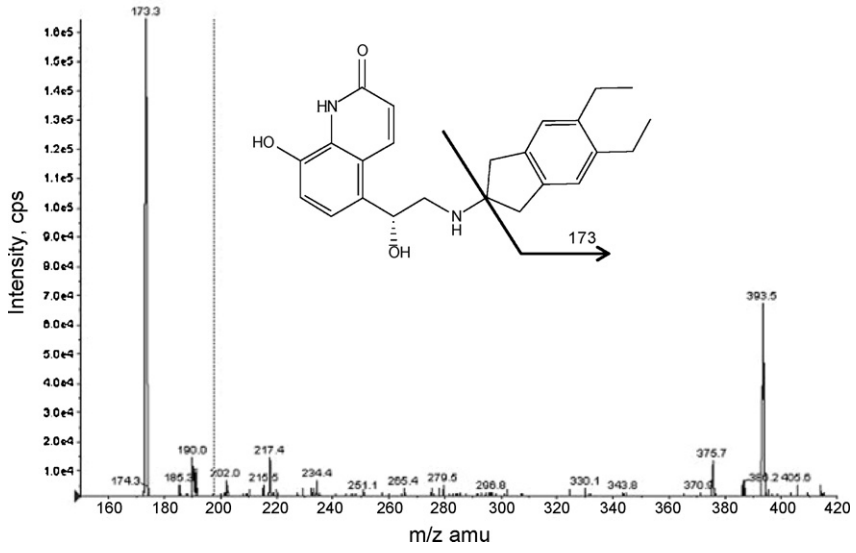

Fig. 2. Representative ion mass spectrum and proposed fragmentation for Indacaterol.

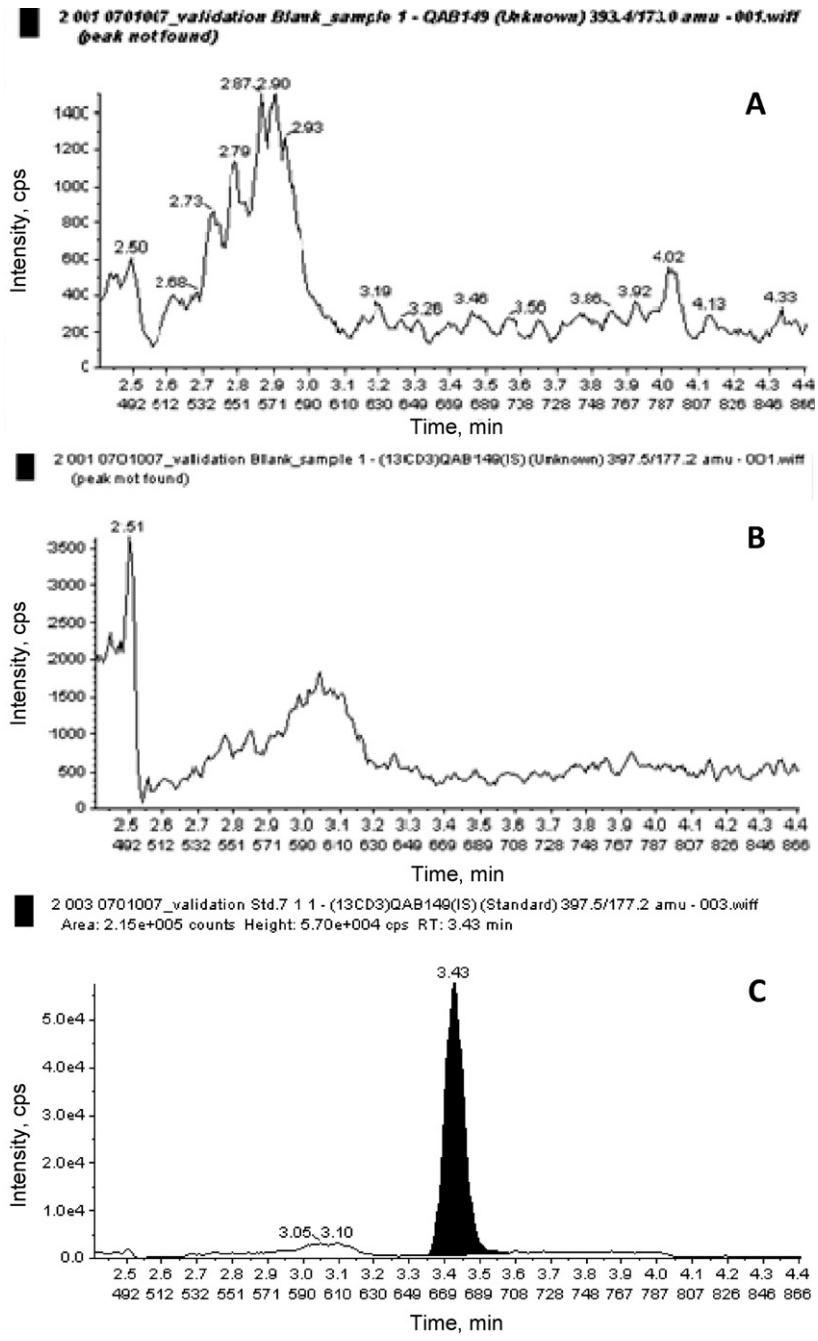

Fig. 3. Representative SRM chromatograms of extracted blank serum sample (A) without Indacaterol (m/z 393.5 → 173.3) and (B) $^{13}CD_3$-Indacaterol (m/z 397.5 → 177.3) or (C) extracted blank serum sample spiked with the IS (zero control sample) at a concentration of 0.05 ng/mL (m/z 397.5 → 177.3).

was interference ≤5% of analytical response at working concentration.

2.8.2. Carry over

The carryover was the observed response of the analyte(s) and internal standard(s) in an injected blank sample following the injection of a ULOQ sample. The extent of any carryover was assessed in one validation run by injecting a series of three blank samples directly after the injection of the ULOQ sample.

The carryover level should <20% of the response observed for the analyte(s) LLOQ and<5% of the response observed for the internal standard(s) at the working concentration.

2.8.3. Matrix effect

The matrix interference (or matrix effect), was assessed by post-column analyte infusion at two concentrations (low and high-QCs) as previously described [17].

2.8.4. Calibration curves and LLOQ

The linearity of the method was evaluated from calibration curves of six calibration points prepared in duplicate and run on three different days. The calibration curve was established using the standards at concentrations 10.0, 50.0, 75.0, 100, 250, 500 and 1000 pg/mL. Calibration curve ($y = ax + b$), represented by the plots of the peak-area ratios (y) of the response for Indacaterol to the internal standard vs the concentration (x) of the calibration

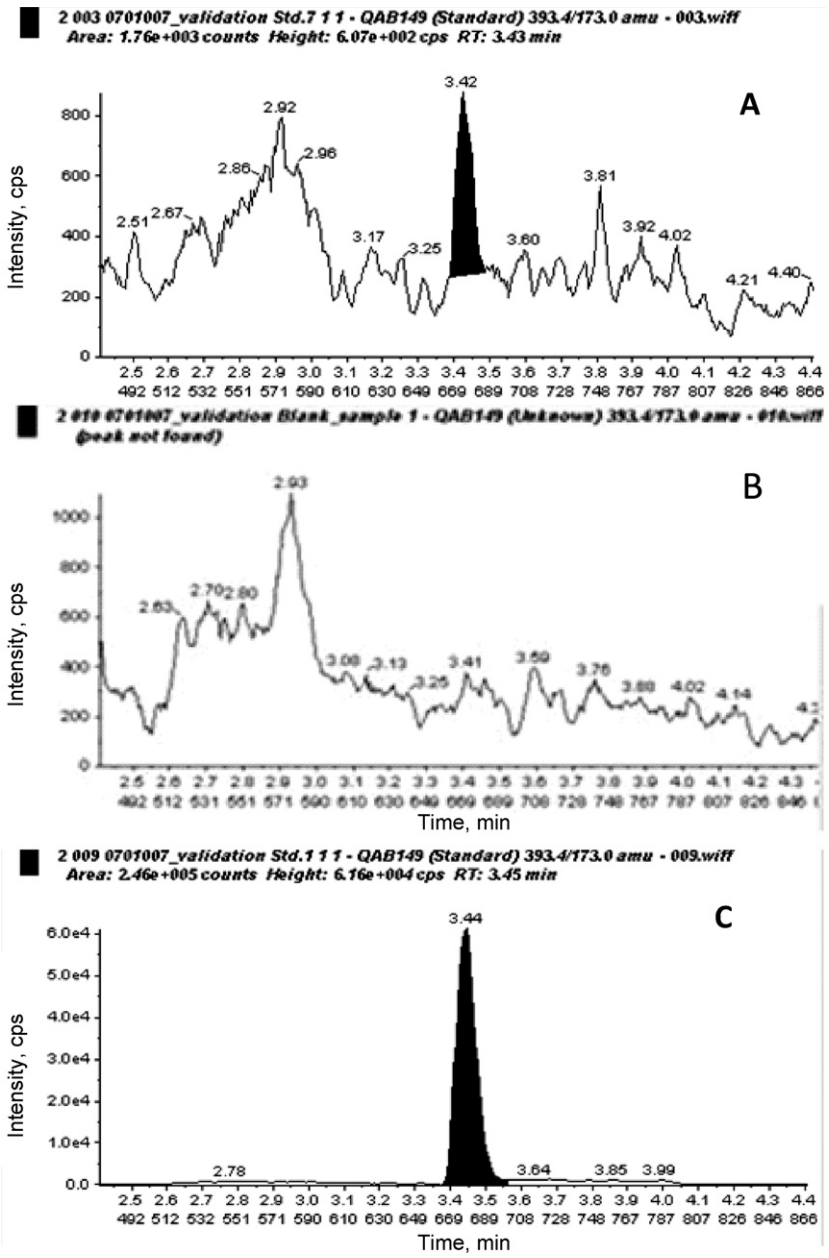

Fig. 4. Representative SRM chromatograms of extracted serum sample spiked with (A) 10 pg/mL low limit of quantification (LLOQ), (B) 1000 pg/mL (ULOQ) of Indacaterol (m/z 393.5 → 173.3) and (C) blank matrix injected right after the ULOQ Indacaterol (m/z 393.5 → 173.3).

standards, were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model. The acceptance criteria were deviation ±15% (±20% at LLOQ) for 75% of the calibration standards from nominal values (with a minimum of six different levels).

2.8.5. Accuracy and precision

Intra-day and inter-day accuracies and precisions were evaluated by analysis of the QC samples at a minimum of four concentrations 10.0 pg/mL (LLOQ), 30.0 pg/mL (within 3 times LLOQ), 75.0 pg/mL and 750 pg/mL analyzed on each day. The deviation (bias) from the nominal value was used to evaluate the accuracy. The intra-day and inter-day (overall) accuracy as well as precision were calculated as the mean bias and precision of all individual concentrations of QC samples analyzed during a single validation day and three validation days, respectively. The acceptance criteria were mean bias within ±15% (±20% at LLOQ) of the nominal values and precision of <15% (<20% at LLOQ).

3. Results and discussion

3.1. Mass spectra analysis

On the positive ion electrospray MS/MS product-ion spectra of Indacaterol, the most abundant product ion was observed at m/z 173.3 (Fig. 2), This resulted from the cleavage as shown in Fig. 2. Similar cleavage pattern was observed with the IS (data not shown).

3.2. Specificity

In the LC–MS/MS chromatograms of six lots of blank serum no peaks were observed at the retention times (around 3.3 min) of Indacaterol and that of IS. This indicated that our method is highly

selective. An example of SRM chromatograms of one extracted blank human serum is depicted in Fig. 3A (Indacaterol channel) and in Fig. 3B (IS channel). There was no interference between the IS and Indacaterol peak at the IS concentration used in the present work. Representative SRM chromatogram of blank serum sample spiked with the IS (zero sample) at the concentration used in this study is depicted in Fig. 3C. This demonstrated that our on-line SPE–LC–MS/MS assay is highly specific for the determination of Indacaterol in human serum.

3.3. Sensitivity

The limit of quantification (LOQ) of the method was 10.0 pg/mL for Indacaterol when using 200 μL diluted human serum. Representative SRM chromatogram of blank serum sample spiked with Indacaterol at a concentration of 10.0 pg/mL (LOQ) is depicted in Fig. 4A. As can be seen, the signal of Indacaterol at the LOQ was above the noise level.

3.4. Carryover

In the current assay, acetonitrile–MeOH–H_2O, 70:20:10 (v/v/v) followed by 0.1% acid formic acid in MeOH–H_2O, 50:50 (v/v) solutions were used to wash syringe and injection port multiple times before and after each injection. Under these washing conditions, no peak was observed at the retention times of Indacaterol (Fig. 4B) in the SRM chromatogram of a blank extracted sample analyzed right after the injection of Indacaterol at the ULOQ concentration (1000 pg/mL) (Fig. 4C), indicating the absence of carryover.

3.5. Matrix effect

Matrix effect was assessed by post-analyte infusion in order to have from the obtained chromatograms, a qualitative suppression or enhancement. The blank solvent used to diluted Indacaterol was infused (chromatogram shown in Fig. 5A) to have an idea of the background signal. When applying extraction method (SPE), no perturbations in the response of the low and high QCs were seen as evident by post-column analyte infusion experiments (Fig. 5B and C). Thus under our experimental conditions, there was neither enhancement nor suppression of the MS signal of Indacaterol at its retention time (Fig. 5D). This was also true for the internal standard (data not shown).

3.6. Calibration

The concentration of Indacaterol was calculated by reference to standard calibration curve using parameters a and b of the calibration function $y = ax + b$ and coefficient of determination r^2 on each day of the validation. Daily variations of calibration regression coefficient (r^2) ranged from 0.990 to 0.993 and the linear regression fit equations were on average $y = 1.236x + 0.001283$. The calibration curves did not exhibit any non-linearity within the chosen range. As the results showed good accuracy and precision and the calibration curves were linear over the concentration range 10.0–1000 pg/mL, we concluded that the curve produced by this method could be used to reliably determine the serum concentrations in a consistent fashion. The acceptance criteria for the mean bias were met: $-6.6\% \leq$ mean bias $\leq 4.3\%$. The LLOQ was defined as the lowest concentration on the calibration curve for which an acceptable accuracy of ±20% and a precision below 20% were obtained.

3.7. Accuracy and precision

The inter-run bias percentage ranged between 2.0 and 2.9% and the inter-run %CV ranged from 6.9 to 15.0%. The largest mean inter-batch accuracy for Indacaterol was for the LLOQ (10.0 pg/mL, acceptance limit ≤20%). The mean intra- and inter-run bias results are presented in Table 3. Method accuracy, intra- and inter-batch precision for spiked human serum was established. The average accuracy expressed as %bias from the nominal concentration and precision expressed as %CV were calculated. Four validation batches considered for inter- and intra-batch precision and accuracy. The FDA's guidance for industry on bioanalytical method validation and a consensus recommend that the criterion for precision and accuracy for acceptance at each concentration level is within 15%, except for LLOQ, where it is not more than 20%. According to these criteria, our analytical method is reliable for the quantitative analysis of Indacaterol in human serum.

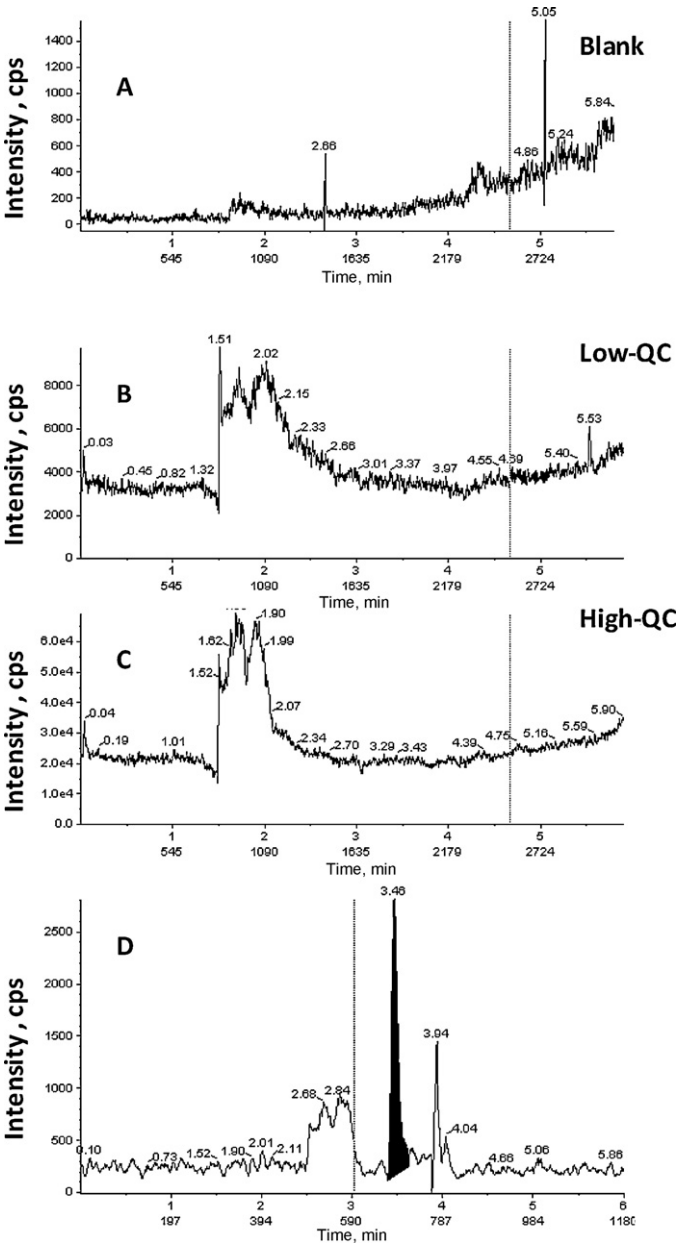

Fig. 5. Post column infusion LC–MS/MS chromatograms of extracted blank human serum extracts from SPE. (A) Post-infusion of blank (solvent used for the dilution of Indacaterol), (B) post-infusion of low-QC, (C) post-infusion of high QC and (D) LC–MS/MS chromatogram of Indacaterol.

Table 3
Concentration of QCs results in human serum and accuracy and precision of the method in spiked human serum for Indacaterol.

Run date	Indacaterol nominal concentration in ng/mL							
	0.0100	%Bias	0.0300	%Bias	0.0750	%Bias	0.750	%Bias
28-Apr-2008	0.00895	−10.5	0.0282	−6.0	0.0740	−1.3	0.800	6.7
	0.00909	−9.1	0.0338	12.7	0.0805	7.3	0.767	2.3
	0.00846	−15.4	0.0329	9.7	0.0747	−0.4	0.864[b]	15.2
	0.0119	19.0	0.0271	−9.7	0.0736	−1.9	0.826	10.1
	0.00887	−11.3	0.0341	13.7	0.0776	3.5	0.740	−1.3
	0.0116	16.0	0.0314	4.7	0.0771	2.8	0.798	6.4
Intra-run mean	0.00981		0.0313		0.0763		0.799	
Intra-run %CV	15.5		9.5		3.5		5.4	
Intra-run %bias	−1.9		4.3		1.7		6.5	
n	6		6		6		6	
29-Apr-2008	0.0119	19.0	0.0307	2.3	0.064	−14.7	0.759	1.2
	0.0278[c]		0.0324	8.0	0.0883[b]	17.7	0.785	4.7
	0.00943	−5.7	0.0281	−6.3	0.0819	9.2	0.736	−1.9
	0.0107	7.0	0.0274	−8.7	0.0759	1.2	0.817	8.9
	0.0106	6.0	0.0331	10.3	0.0794	5.9	0.693	−7.6
	0.0125[a]	25.0	0.0312	4.0	0.0776	3.5	0.681	−9.2
Intra-run mean	0.0110		0.0305		0.0779		0.745	
Intra-run %CV	10.9		7.5		10.3		7.1	
Intra-run %bias	10.0		1.7		3.9		−0.7	
n	5		6		6		6	
30-Apr-2008	0.0117	17.0	0.0296	−1.3	0.0718	−4.3	0.706	−5.9
	0.00932	−6.8	0.0267	−11.0	0.0830	10.7	0.826	10.1
	0.0132[c]		0.0265	−11.7	0.0704	−6.1	0.823	9.7
	0.0116	16.0	0.0368[b]	22.7	0.0752	0.3	0.729	−2.8
	0.00807	−19.3	0.0300	0.0	0.0817	8.9	0.817	8.9
	0.00824	−17.6	0.0345	15.0	0.0832	10.9	0.730	−2.7
Intra-run mean	0.00979		0.0307		0.0776		0.772	
Intra-run %CV	18.1		13.6		7.5		7.2	
Intra-run %bias	−2.1		2.3		3.5		2.9	
n	5		6		6		6	
Mean conc. found	0.0102		0.0308		0.0772		0.762	
Inter-run %CV	15.0		9.9		7.3		6.9	
Inter-run %bias	2.0		2.7		2.9		2.9	
n	16		18		18		18	

[a] Bias ≥±20%.
[b] Bias ≥±15%.
[c] Deactivated due to a contamination issue during the preparation of the QC samples.

3.8. Stability and dilution

Stability of a drug in a biological matrix is dependant on the chemical nature of the drug, the matrix effect, the container system and storage conditions with time and temperature and stress conditions. The auto-sampler stability evaluated at 5 °C over 38 h on spiked QCs at 30.0 and 750 pg/mL were successfully validated for Indacaterol with good accuracy and precision data (Table 4). The other stabilities were determined within the frame of a previous validated off-line SPE–LC–MS/MS method.

The dilution test was determined using a 100-fold QCs dilution with blank serum for Indacaterol prior its extraction and assayed in 3 replicates along with Cs and QCs in a validation run. As can be seen in Table 5, the measured concentrations of Indacaterol in these QCs were comparable to the nominal values, with a mean accuracy of 103%. This demonstrated that samples with higher concentration can be diluted with blank serum to obtain acceptable data.

Table 4
Room temperature stability of Indacaterol (*n* = 3) for 38 h.

	Sample 1	Sample 2	Sample 3	Mean	%CV
QC3					
Expected concentration (ng/mL)	0.0300	0.0300	0.0300	0.0300	
Measured concentration (ng/mL) without storage	0.0282	0.0338	0.0329	0.0316	9.5
Measured concentration (ng/mL) after storage	0.0269	0.0390	0.0265	0.0308	23.1
Recovery (%) vs expected				102.7%	
Recovery (%) vs without storage				97.4%	
QC1					
Expected concentration (ng/mL)	0.750	0.750	0.750	0.750	
Measured concentration (ng/mL) without storage (run 3)	0.800	0.767	0.826	0.798	3.7
Measured concentration (ng/mL) after storage	0.738	0.709	0.713	0.720	2.2
Recovery (%) vs expected				96.0%	
Recovery (%) vs without storage				90.3%	

Table 5
Precision and accuracy of dilution QC samples ($n = 3$) for Indacaterol.

	Sample 1	Sample 2	Sample 3	Mean	%CV
Dilution factor 100					
Expected concentration (ng/mL)	80.0	80.0	80.0	80.0	
Measured concentration (ng/mL)	87.7	84.5	86.1	86.1	1.9
Accuracy (%)	110%	106%	108%	108%	
Dilution factor 1000					
Expected concentration (ng/mL)	80.0	80.0	80.0	80.0	
Measured concentration (ng/mL)	81.7	80.9	84.5	82.4	2.3
Accuracy (%)	102%	101%	106%	103%	

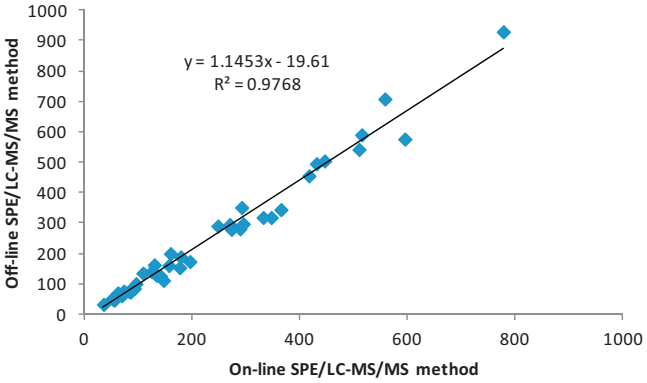

Fig. 6. Comparison of Indacaterol concentration (ng/mL) by the respective on-line and off-line SPE LC–MS/MS methods.

3.9. Method comparison

A previous off-line SPE–LC–MS/MS (see Section 2 for method description) was used to measure a et ($n = 41$) of clinical samples. The results were compared with those obtained by the on-line SPE–LC/MS/MS method. Shown in Fig. 6 are the comparative concentration data for Indacaterol obtained with the two methods. It can be noticed that excellent correlation between the results obtained with the on-line and the off-line SPE–LC–MS/MS methods as indicated by a coefficient of correlation higher than 0.97. Moreover, by evaluating our data with a statistical program making use of the robust-statistics concept of Rousseeuw and Croux [18], our method is comparable to the reference method as, there was no bias at 5% significance level. Also the slope and the intercept do include 1 and 0, respectively.

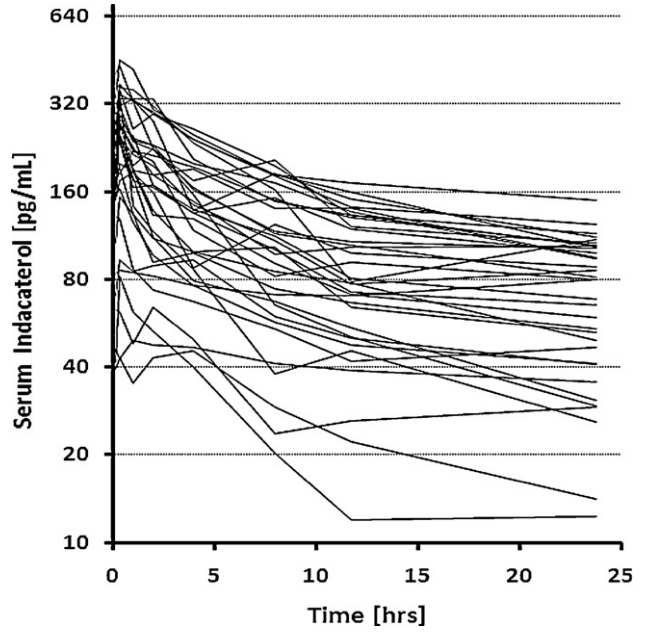

Fig. 7. Representative sample of steady-state concentration–time profiles after administration of a 150 μg dose in a COPD patient population in a pivotal clinical trial.

3.10. Application to clinical studies

The present method was used to support several clinical studies with Indacaterol. All blood samples were taken by either direct venipuncture or via an indwelling cannula inserted in a forearm vein. Blood samples (4 mL) were collected at each of the following time points into polypropylene tubes spray coated with silica (for serum preparation). For one of the clinical study (for which the data are shown in Fig. 7), blood were collected on day 28/29 at the following time points. Pre-dose, 10 min, 20 min, 1 h, 2 h, 4 h, 8 h, 11.75 h, and 23.75 h post-dose. Shown in Fig. 7 are the concentration plot profiles vs time for patients receiving 150 μg of Indacaterol. With our present method it was possible to quantify Indacaterol in serum samples up to 23.75 h post-dose.

4. Conclusions

We have developed a sensitive, selective, reproducible, robust and fully automated on-line SPE LC–MS/MS for quantification of Indacaterol in human serum. The on-line solid phase extraction method tested in this study was suitable for the routine analysis of Indacaterol in human serum over the range from 10.0 to 1000 pg/mL with a limit of quantification of 10.0 pg/mL using a sample volume of 150 μL. The method was also specific and sufficiently selective for Indacaterol in human serum, even in the presence of other components and possible impurities of the chemicals used. The method met the US-FDA requirements for specificity, sensitivity, precision, accuracy, and stability and was suitable to support pharmacokinetic studies of Indacaterol in human serum.

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