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A Novel LC-MS/MS Method for Simultaneous Determination of Ivabradine and its Active Metabolite N-Desmethyl Ivabradine in Human Plasma: Its Pharmacokinetic Application

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Abstract : The method was new simple, rapid, sensitive and simultaneous liquid chromatography/tandem mass spectrometry assay method for the determination of Ivabradine and N-Desmethyl Ivabradine in human plasma using Ivabradine-d_6 and N-Desmethyl Ivabradine-d_6 as internal standards (IS). Analyte and the internal standards were extracted from the human plasma *via* Solid phase extraction (SPE) using Strata ^{TM} X 33µM polymeric sorbent cartridges (1cc/30mg). The chromatographic separation was achieved on a Kromasil 100-5 C_{18}, 100 x 4.6 mm, 5 µm columns by using a gradient programme at a flow rate of 0.60 mL/min with a total runtime of 3.0 min and the elution was monitored by multiple reaction monitoring modes using electropspray ionization. The calibration curve obtained was linear ($r^2 \geq 0.99$) over the concentration range of 0.20–201 ng/mL for Ivabradine and 0.10–15.14 for N-Desmethyl Ivabradine. Method validation was performed as per FDA guidelines and the results met the within the acceptable limits. The proposed method was found to be applicable to pharmacokinetic studies.

Keywords : Ivabradine, N-Desmethyl Ivabradine, Solid Phase Extraction, LC-MS/MS, Validation, Human plasma, Pharmacokinetics.

Introduction

Ivabradine is a novel heart rate lowering agent for the management of coronary artery disease (CAD) and heart failure (HF). Ivabradine inhibits the pacemaker cells of the sino-atrial node without affecting atrioventricular and intraventricular conduction time, ventricular repolarization and myocardial contractility. Thus, this drug gives the possibility to evaluate effect and outcomes of pure HR reduction in patients with CAD and HF [1, 2]. Ivabradine has a half-life of 2 h in plasma [3]. The drug metabolized to its active metabolite N-desmethyl ivabradine has also been shown to decrease heart rate [4, 5]. Hence it is important to analyze ivabradine and its active metabolite N-desmethyl ivabradine for a pharmacokinetic or bioavailability/bioequivalence studies. Very few LC-MS/MS methods have been reported for the determination of Ivabradine in biological

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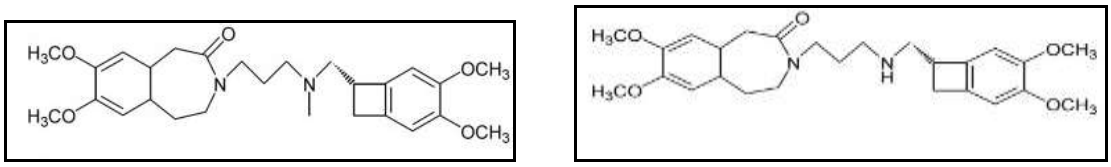
samples. Jiang et al.,[6] reported a method for the determination of Ivabradine alone (without metabolite N-Desmethyl Ivabradine) using solid phase extraction technique (SPE) for sample preparation. Lu et al.,[7] analyzed Ivabradine and N-desmethyl Ivabradine in human plasma and urine samples by using liquid-liquid extraction for sample preparation. The method employs diazepam as internal standard. Both the methods are having run time more than 4 min. which is not suitable for high throughput bioanalysis. Also, these methods are employed non-deuterated compounds as internal standard. Use of deuterated compounds as internal standards helps to obtained better precision and accuracy results and also minimizes matrix effect related errors. The analytical method should be simple, enough sensitive, rapid, consume less sample volume and efficient extraction procedure[8, 9]. In view of above, the authors proposed a simple and rapid LC-MS/MS method for the simultaneous determination of Ivabradine and N-desmethyl Ivabradine in of human plasma using isotope labeled compounds Ivabradine-d_6 and N-desmethylivabradine-d_6 as internal standards respectively. The validated method was successfully applied to a pharmacokinetic study[10] in healthy male south Indian subjects.

Experimental

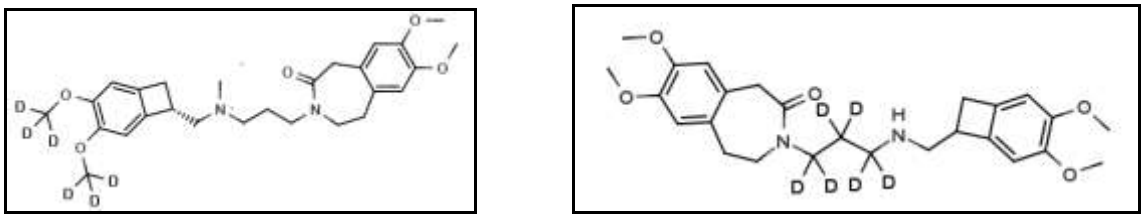
Standards and Chemicals

The reference standards of Ivabradine (99.94%), N-Desmethyl Ivabradine (98.15%), Ivabradine-d_6 (95.20%) and N-Desmethyl Ivabradine-d_6 (99.63%) were obtained from Clearsynth Labs Limited (Mumbai, India), HPLC grade Water was used for the LC–MS/MS was procured from RANKEM Laboratories, India). HPLC grade methanol was purchased from J.T. Baker (Phillipsburg, USA). AR grade Ammonium formate procured from AR grade (MERCK Mumbai India) and SPE Cartridges were purchased from Phenomenex (Hyderabad, India). The control K_2 human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India). Ivabradine and N-Desmethyl Ivabradine structures were shown in Fig.1.Where as Ivabradine-d_6 and N-Desmethyl Ivabradine-d_6 structures were shown in Fig.2.



(A) **(B)**

Fig. 1: Chemical structures of Ivabradine (A) & N-Desmethyl Ivabradine (B)



(A) **(B)**

Fig.2: Chemical structures of Ivabradine-d_6 (A) & N-Desmethyl Ivabradine-d_6 (B)

LC–MS/MS Instrument and Conditions

An HPLC system (Waters-Acquity I class Waters Corporation 34 Maple Street Milford, MA 01757 USA) equipped with a Kromasil 100-5 C_{18}, 100 x 4.6 mm, 5 µm (Make: Akzonobel), A Binary Solvent Manager (BSM), Sample Manager - Flow-Through-Needle (SM - FTN) and Class IVD column heater (CH-A) was used for the study. Aliquot of 7 µL of the processed samples were injected into the column, which was kept at ambient (35±2°C) temperature. A gradient mobile phase composed of a mixture of 5mM ammonium formate and methanol (20:80, v/v) pump A and pump B was used to separate the analyte from the endogenous components and pumped at a flow rate of 0.600 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS–MS detection in positive ion mode for the analyte and the IS using a Waters Xevo TQ-S mass spectrometer (Waters Corporation 34 Maple Street Milford, MA 01757 USA) equipped with a off-axis step wave technology used to ion transfer from source to MS analyzer. Disolvation temperature at 500°C. The capillary voltage was set at 3.4 V. The source temperature 150°C, the nebulizer gas 7.0 bars, Disolvation gas flow 1000L/h. The Cone voltage and collision energy (CE) for

Ivabradine, Ivabradine-d_6, N-Desmethyl Ivabradine, N-Desmethyl Ivabradine-d_6 were set at 8, 4, 16, and 28 V, and 34, 22, 24, 24V respectively. Detection of the ions was carried out in the multiple reaction monitoring mode (MRM), by monitoring the transition pairs of Ivabradine, Ivabradine-d_6, N-Desmethyl Ivabradine, N-Desmethyl Ivabradine-d_6 m/z 469.37, 475.35, 455.40, and 461.31 were selected as precursor ions and m/z 177.15 product ions was selected for all analytes and internal standards. Dwell time was set at 0.079 sec. The analysis data obtained were processed by Mass Lynx® version 4.1 software.

Preparation of Calibration Standards and Quality Control Samples in Human Plasma

Two separate standard stock solutions of Ivabradine and N-Desmethyl Ivabradine were prepared separately in HPLC grade methanol (1 mg/mL) for the preparation of calibration curve standards and quality control samples, respectively. Further dilutions of analytes were prepared in a mixture of methanol and water (80:20, v/v; diluent). A 1mg/mL of Ivabradine-d_6 and N-Desmethyl Ivabradine-d_6 stock solutions were prepared by dissolving the compound in HPLC grade methanol. The working concentration of internal standards (100 ng/mL and 20.00 ng/mL) was prepared from the above stock solutions using the diluent.

The Calibration curve samples were prepared by spiking 950 µL of control K_2 EDTA human plasma with the 50 µL of combined working standard solutions of the Ivabradine and N-Desmethyl Ivabradine as a bulk, to obtain concentration levels of 0.20, 0.40, 1.01, 5.03, 20.13, 40.26, 80.52, 120.78, 161.03, 201.29 ng/mL for Ivabradine and 0.10, 0.20, 0.51, 1.01, 2.03, 4.09, 6.05, 9.08, 12.11, 15.14 ng/mL for N-Desmethyl Ivabradine. Similarly, quality control (QC) samples also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 0.20 (LLOQ QC), 0.61 (LQC), 22.11 (MQC1), 100.50 (MQC2), 140.71 ng/mL (HQC) for Ivabradine and 0.10 (LLOQ QC), 0.30 (LQC), 1.66 (MQC1), 7.51 (MQC2), 10.52 ng/mL (HQC) for *N*-Desmethyl Ivabradine. The aliquots of the calibration and quality control bulk samples were obtained by distributing into micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at –70 ± 10 °C until analyses.

Sample Processing

A 100 µL aliquot of human plasma sample was mixed with 10 µL of the internal standard combined working solution (100 ng/mL Ivabradine-d_6, 20.00 ng/mL of N-desmethyl Ivabradine-d_6). To this, 300 µL of 5mM ammonium formate was added after vortex mixing for 10 s. The sample mixture was loaded onto a Starata™ -X 33µM polymeric sorbent cartridges (1cc/30mg) extraction cartridge that was pre-conditioned with 1.0 mL of methanol followed by 1.0 mL water. The extraction cartridge was washed with 2.0 mL of water (1.0mL of each time). Ivabradine Ivabradine, N-desmethyl Ivabradine, Ivabradine-d_6 and N-desmethyl Ivabradine-d_6 were eluted with 1.0 mL of eluent. Aliquot of 7 µL of the extract was injected into the LC-MS/MS system.

Method Validation Parameters

The proposed method was validated as per recent US FDA guidelines. The parameters determined were selectivity, matrix effect, sensitivity, linearity, precision and accuracy, recovery, dilution integrity and different stability studies.

Pharmacokinetic Study

A pharmacokinetic fed study was performed in healthy male subjects (n = 6). The Ethics Committee (Samkshema Independent Ethics Committee, Hyderabad, India) approved the protocol and the volunteers provided with written informed consent. All the subjects were fasted for at least 10 h before the drug administration. A high-calorie, high-fat breakfast comprising of 800-1000 k.cal was given to the subject before drug administration. Blood samples were collected following oral administration of 7.5 mg Ivabradine tablet at pre-dose and 0.17, 0.33, 0.50, 0.67, 0.83, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 36.00 and 48.00 hours post-dose and collected in K_2 EDTA vacutainer (5 mL) collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 4000 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70 ± 10 °C till their use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. The pharmacokinetic parameters of Ivabradine and N-desmethyl Ivabradine were calculated by Non-compartmental model was used for the pK analysis and the pharmacokinetic values were calculated using linear trapezoidal with linear interpolation.

Results and Discussion

Method Development

LC-MS/MS based methods are preferable because of high sensitive, selective, rapid and commonly used in bioanalytical laboratories[11,12]. The main objective of the present work was to develop and validate an LC–MS/MS method for the simultaneous determination of Ivabradine and N-Desmethyl Ivabradine in human plasma with high sensitivity to measure the concentration of Ivabradine and N-Desmethyl Ivabradine for the pharmacokinetic/bioequivalence studies. Mass spectrometric parameters were optimized by infusing 5µL/min analytes and internal standards at a concentration of 50 ng/mL infusion to the electropspray ionization chamber by using intellistart technology to mass spectrometer giving automatically possible mass to charge ratios. We checked both positive and negative mode. Found that more intense signals in positive mode than the negative mode. The most sensitive mass transitions were observed from m/z 469.37 to 177.15 for Ivabradine, m/z 475.35 to 177.15 for Ivabradine-d_6, m/z 455.40 to 177.15 for N-desmethyl Ivabradine and m/z 461.31 to 177.15 for *N*-desmethyl Ivabradine-d_6 with very less dwell time at 0.079 sec. The MRM technique provides inherent selectivity and sensitivity for the analytes, hence employed for the current study. The method development includes buffer selection, mobile phase selection, column type, flow rate, and injection volume. Various combinations of methanol/acetonitrile with acidic buffers (ammonium acetate/ammonium formate–acetic acid/formic acid) in different ratios were tested. It was observed that 5mMAmmonium formate and methanol (20:80, v/v) as the mobile phase with gradient flow was used to give best sensitivity, efficiency and peak shape. Kromasil 100-5 C_{18}, 100 x 4.6 mm, 5 µm column gave good peak shape and response even at lowest concentration levels for the analytes and the both the internal standards. The mobile phase was operated at a flow rate of 0.60 mL/min. The retention time of analytes and the internal standards were low enough allowing a run time of 3.0 min. As a purpose to develop a simple, fast, less exclusive and robust extraction procedure SPE was tested. SPE was carried out using different types of extraction cartridges like waters HLB, Orochem C_{18}, Agilent Plexa, coppure, Phenomenex Strata ^{TM}-X, 33µm 1cc/30mg. Among all the different makes Phenomenex Starata^{TM}-X, 33µm 1cc/30mg cartridges was found to be optimal, which can produce a clean chromatogram for a blank sample and yields the highest recovery for the analyte from the plasma. Addition of 5mM ammonium formate buffer to the plasma samples as an extraction additive helped achieving reproducible and quantitative recoveries for the analytes and the internal standards. The stable labeled isotopes of internal standards are used for bioanalytical assay to increase assay precision and limit variable recovery between analytes and the internal standards.

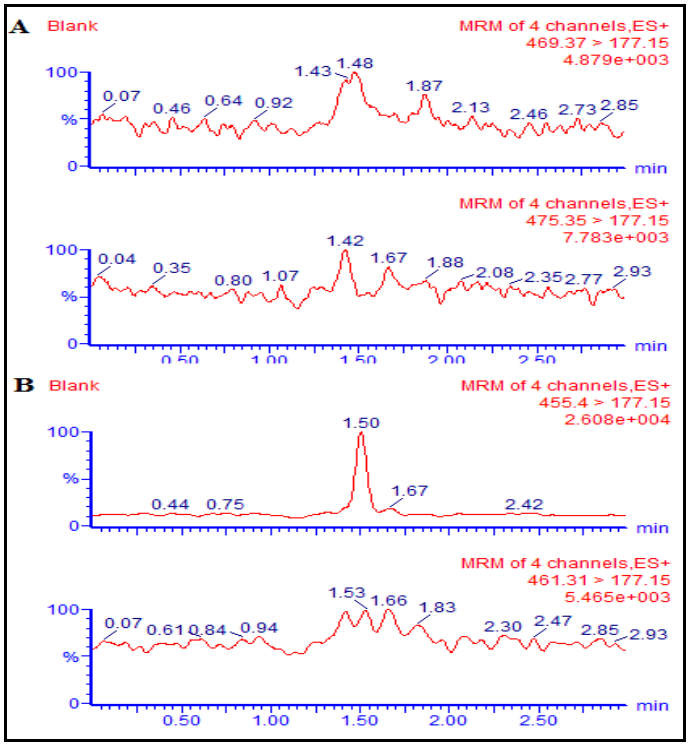


Fig.3: A Representative Chromatogram of a Blank Plasma Sample of (A) Ivabradine and (B) N-Desmethyl Ivabradine.

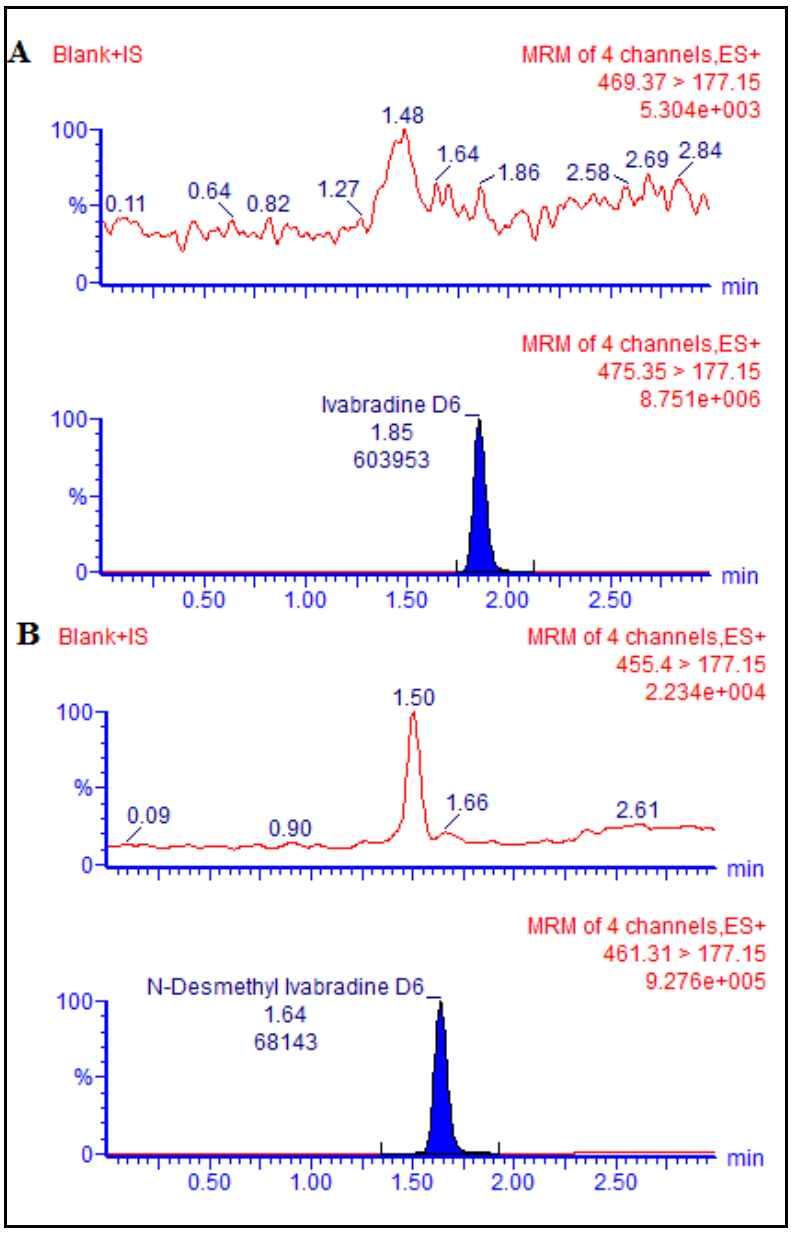


Fig.4: A Representative Chromatogram of a Blank Plasma with internal standard Sample of (A) Ivabradine –d_6 and (B) N-Desmethyl Ivabradine-d_6.

Sensitivity

The lowest limits of reliable quantification for the analytes were set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 2.78% and 98.5% for Ivabradine, 3.29% and 99.0% for N-Desmethyl Ivabradine.

Matrix Effect

No significant matrix effect was observed in all the six batches of human plasma for the analytes at LQC and HQC concentrations. The precision and accuracy for Ivabradine at LQC concentration were found to be 2.20% and 101.2% and at HQC level they were 1.59% and 98.7%, similarly, the precision and accuracy for N-Desmethyl Ivabradine at LQC concentration were found to be 1.68% and 98.6%, and at HQC level they were 4.24% and 103.7%. The results were summarized in Tab.1.

Tab. 1: Matrix effect of Ivabradine and N-Desmethyl Ivabradine in human plasma (*n* = 6)

Analyte		**Ivabradine**		**N-Desmethyl Ivabradine**	
		LQC (0.61 ng/mL)	**HQC (140.71 ng/mL)**	**LQC (0.30 ng/mL)**	**HQC (10.58 ng/mL)**
Plasma lot	**QC ID**	**mean Concentration found (ng/mL)**	**mean Concentration found (ng/mL)**	**mean Concentration found (ng/mL)**	**mean Concentration found (ng/mL)**
Lot 1	1,2,3	0.59	135.56	0.29	10.30
Lot 2	1,2,3	0.62	138.85	0.30	11.06
Lot 3	1,2,3	0.62	141.59	0.30	10.66
Lot 4	1,2,3	0.62	139.53	0.31	11.50
Lot 5	1,2,3	0.63	140.73	0.30	10.82
Lot 6	1,2,3	0.61	137.38	0.29	11.44
Mean		0.61	138.94	0.30	10.96
S.D		0.01	2.21	0.01	0.46
%C.V		2.20	1.59	1.68	4.24
% Nominal		101.2	98.7	98.6	103.7
N		6	6	6	6
S.D-Standard Deviation; C.V- Coefficient of Variation					

Linearity, Sensitivity, Precision and Accuracy

The ten-point calibration curve was found to be linear over the concentration range of 0.20-201 ng/mL for Ivabradine and 0.10–15.14 ng/mL for N-Desmethyl Ivabradine. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration-detector response relationship for both the analytes in human plasma. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.99. The results for intra-day and inter-day precision and accuracy in plasma quality control samples are summarized in Tab.2. All the results of intra-day and inter day precision deviation values were all within 15% of the relative standard deviation (RSD) at low, middle and high quality control level, whereas within 20% at LLOQ QCs level was shown in Fig.5. Also the intra-day and inter-day accuracy deviation values were all within 100 ± 15% of the actual values at low, middle, and high quality control level, whereas within 100± 20% at LLOQ QCs level. The results revealed good precision and accuracy.

Tab.2: Precision and accuracy data for Ivabradine and N-Desmethyl Ivabradine in human plasma (*n* = 6)

	Intra-day precision and accuracy (*n*=12; 6 from each batch)				**Inter-day precision and accuracy (*n*=30; 6 from each batch)**		
Analyte	**Concentration added (ng/mL)**	**Concentration found (mean ± SD; ng/mL)**	**Precision (%)**	**Accuracy (%)**	**Concentration found (mean ± SD; ng/mL)**	**Precision (%)**	**Accuracy (%)**
Ivabradine	0.20	0.23± 0.01	3.98	110.4	0.23± 0.01	4.13	110.5
	0.61	0.63± 0.02	3.40	103.8	0.62± 0.02	2.83	102.6
	22.11	23.14± 0.96	4.16	104.7	22.92± 0.71	3.08	103.6
	100.50	109.02± 5.95	5.46	108.5	109.67± 4.02	3.66	109.1
	140.71	146.99± 6.51	4.43	104.5	149.01± 5.06	3.40	105.9
N-Desmethyl Ivabradine	0.10	0.10± 0.00	4.51	96.8	0.10± 0.01	5.74	95.6
	0.30	0.30± 0.01	3.55	98.1	0.29± 0.01	4.70	95.6
	1.66	1.60± 0.09	5.36	96.0	1.67± 0.10	5.81	100.4
	7.51	7.49± 0.18	2.43	99.7	7.57± 0.14	1.85	100.8
	10.52	10.43± 0.26	2.51	99.2	10.61± 0.32	2.97	100.9

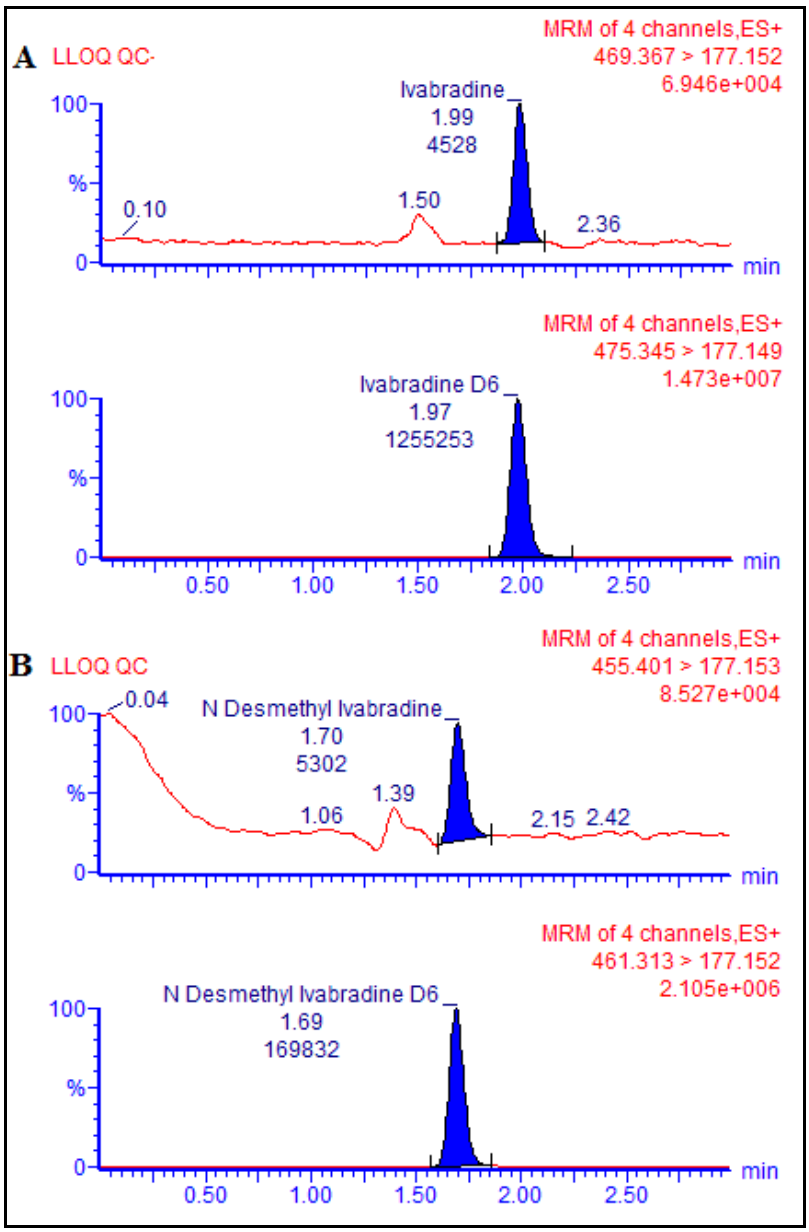


Fig. 5: A Representative Chromatogram of a LLOQ QC with internal standard Sample of (A) Ivabradine and (B) N-Desmethyl Ivabradine.

Recovery and Dilution Integrity

A simple solid- phase extraction with Strata^{TM}-X, 33µm, polymeric sorbent cartridges (1cc/30 mg) was proved to be robust and provided cleanest samples. The recoveries of analytes and internal standards were good and reproducible. The mean overall recovery of Ivabradine and N-Desmethyl Ivabradine was 90.43±4.22% with the %CV range of 1.71 - 4.17% and 90.22±3.43% with the %CV range of 1.20–3.92%.The recoveries of Ivabradine-d6 and N-Desmethyl Ivabradine-d6 was 79.59% with the %CV range of 3.84 - 5.47% and 86.46% with the %CV range of 2.50 - 4.69%. The upper concentration limit of Ivabradine and N-Desmethyl Ivabradine can be extended to 170.86 and 12.52 ng/mL by using two- and four-fold dilution with screened human blank plasma. The mean back–calculated concentrations for two- and four-fold dilution samples were within 85–115% of their nominal values and the coefficients of variations (%CV) were in the range of 1.73- 6.51% and 1.26% to 1.49% respectively.

Stability Studies

In various stability experiments carried out namely bench top stability (16 h), autosampler stability (75 h), repeated freeze-thaw cycles (5 cycles), reinjection stability (25 h), wet extract stability (72 h at 2-8 °C) and

long–term stability at –70 °C for 50 days the mean % nominal values of the analytes were found to be within ±15% of the predicted concentrations for the analytes at their LQC and HQC levels shown in Tab.3. Therefore, the results were found to be within the acceptable limits during the entire validation.

Tab. 3: Stability data for Ivabradine and N-Desmethyl Ivabradine in human plasma (*n*=6)

Analyte	Stability test	Concentration added (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Ivabradine	Process [a]	0.61	0.58 ± 0.01	2.53	95.6
		140.71	141.30± 1.27	0.90	100.4
	Wet extract [b]	0.61	0.59± 0.01	2.00	97.8
		140.71	140.89± 1.25	0.89	100.1
	Bench top [c]	0.61	0.56± 0.01	2.02	92.8
		140.71	139.97± 1.26	0.90	99.5
	FT [d]	0.61	0.59± 0.01	1.39	98.1
		140.71	140.01 ± 0.91	0.65	99.5
	Reinjection [e]	0.61	0.65 ± 0.02	3.73	103.1
		140.71	149.01± 3.29	2.21	104.1
	Short–term [f]	0.61	0.60 ± 0.01	1.36	95.6
		140.71	140.09± 1.44	1.03	97.9
	Long–term [g]	0.61	0.61 ± 0.02	2.73	97.3
		140.71	143.14± 0.97	0.67	100.0
N-Desmethyl Ivabradine	Process [a]	0.30	0.32 ± 0.00	1.11	105.3
		10.52	10.91± 0.12	1.14	103.7
	Wet extract [b]	0.30	0.32± 0.01	1.64	104.8
		10.52	10.74± 0.08	0.75	102.1
	Bench top [c]	0.30	0.31± 0.01	3.84	103.2
		10.52	10.64± 0.21	1.99	101.1
	FT [d]	0.30	0.31± 0.01	1.84	103.8
		10.52	10.76 ± 0.07	0.65	102.3
	Reinjection [e]	0.30	0.31 ± 0.00	1.62	105.0
		10.52	10.67± 0.21	1.96	103.0
	Short–term [f]	0.30	0.31 ± 0.01	1.81	106.2
		10.52	10.84± 0.07	0.62	104.7
	Long–term [g]	0.30	0.31 ± 0.01	2.69	105.9
		10.52	10.18± 0.14	1.37	98.3

[a] after 75 h in autosampler at 10°C; [b] after 72 h at room temperature; [c] after 16 h at room temperature; [d] after 5 freeze and thaw cycles; [e] after 25 h of Reinjection; [f] at –20°C for 5 days; [g] at –70°C for 50 days

Pharmacokinetic Study Results and Incurred Sample Reanalysis (ISR)

The proposed method was successfully used to quantify Ivabradine and N-Desmethyl Ivabradine plasma concentrations for a pharmacokinetic study in healthy adult male subjects (*n*=6) under fed condition. Fig.6 depicts the mean plasma concentration *vs* time profile of Ivabradine and N-Desmethyl Ivabradine in healthy human subjects. The maximum concentration (C_{max}) of Ivabradine and N-Desmethyl Ivabradine in plasma (45.32±7.05 ng/mL, 4.10±0.58 ng/mL) was attained at (t_{max}) (1.22±0.34 h, 1.39±0.49 h). The area under the plasma concentration–time curve from time zero to last measurable time point (AUC_{0-t}) was (219.18±43.15, 46.08±7.60 ng*h/mL), the area under the plasma concentration time curve from time zero to infinity time point (AUC_{0-inf}) (219.80±43.33, 49.05±8.13 ng*h/mL) and The terminal half–life ($t^{½}$) was found to be (0.12±0.01 h, 0.06±0.01 h) for Ivabradine and N-Desmethyl Ivabradine, respectively. The results were summarized in Tab. 4.

Tab. 4: Pharmacokinetic parameters of Ivabradine and N-Desmethyl Ivabradine in human plasma (*n*=6)

Parameter	Ivabradine	N-Desmethyl Ivabradine
t_{max} (h)	1.22±0.34	1.39±0.49
C_{max} (ng/mL)	45.32±7.05	4.10±0.58
AUC_{0-t} (ng h/mL)	219.18±43.15	46.08±7.60
AUC_{0-inf} (ng h/mL)	219.80±43.33	49.05±8.13
$t_{1/2}$ (h)	5.86±0.47	11.93±1.63
Kel (h^{-1})	0.12±0.01	0.06±0.01

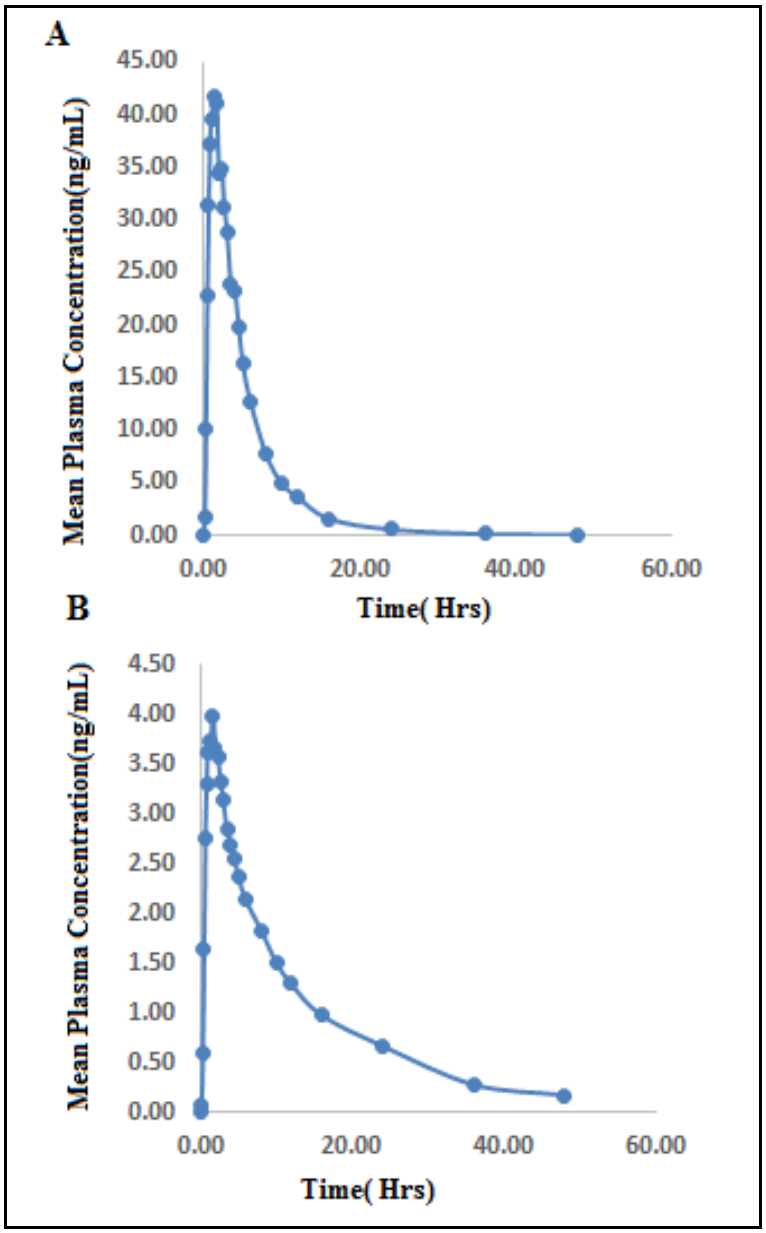


Fig.6: Mean plasma concentration-time profile of Ivabradine (A), and N-Desmethyl Ivabradine (B) in human plasma following oral administration of 7.5 mg of Ivabradine tablet to healthy volunteers (n= 6).

Incurred sample reanalysis

Incurred sample reanalysis was evaluated by selection of two plasma samples from each subject one sample is near to Cmax value and one sample from elimination phase and re–assayed in a separate batch run.

By applying the present method, it showed the % variability for all the experienced samples were less than 20% which was shown in Tab.5.

Tab. 5: ISR Results of Ivabradine and N-Desmethyl Ivabradine in human plasma (*n*=6)

Analyte	Subject No	Time Point (Hrs)	Original Concentration (ng/mL)	ISR Concentration (ng/mL)	Mean Concentration (ng/mL)	%variability [a]
Ivabradine	1	1.33	34.77	30.25	32.51	13.88
	1	16.00	1.20	1.35	1.28	11.76
	2	1.33	45.41	44.03	44.72	3.09
	2	16.00	1.50	1.37	1.43	9.62
	3	1.33	34.97	33.48	34.22	4.37
	3	16.00	1.61	1.51	1.56	5.90
	4	1.00	34.75	33.48	34.12	3.73
	4	16.00	2.46	2.47	2.47	0.57
	5	1.33	52.46	50.20	51.33	4.41
	5	16.00	1.80	1.75	1.77	3.16
	6	0.67	46.67	45.69	46.18	2.10
	6	16.00	1.08	1.05	1.07	3.47
N-Desmethyl Ivabradine	1	1.67	3.68	3.54	3.61	3.82
	1	24.00	0.61	0.59	0.60	3.19
	2	2.33	3.89	3.83	3.86	1.61
	2	36.00	0.36	0.36	0.36	1.94
	3	1.00	3.34	3.26	3.30	2.36
	3	24.00	0.49	0.43	0.46	13.13
	4	2.33	3.05	3.16	3.10	3.38
	4	24.00	0.78	0.75	0.77	3.00
	5	1.33	4.66	5.03	4.84	7.56
	5	36.00	0.31	0.30	0.31	0.66
	6	1.67	4.69	4.72	4.70	0.45
	6	24.00	0.75	0.75	0.75	0.94

[a] Expressed as [(initial conc.−re–assay conc.)/average] ×100%.

Conclusion

The proposed LC–MS/MS method is simple, rapid, specific and sensitive for simultaneous determination of Ivabradine and N-Desmethyl Ivabradine in human plasma and is fully validated according to commonly acceptable FDA guidelines. The current method has shown acceptable precision and adequate sensitivity for the quantification of Ivabradine and N-Desmethyl Ivabradine in human plasma samples obtained for pharmacokinetic studies. The simple SPE method with Strata^{TM}-X, 33µm, polymeric sorbent cartridges (1cc/30mg) gave consistent and reproducible recoveries for the analytes and the internal standards from plasma. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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