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A VALIDATED BIOANALYTICAL METHOD FOR QUANTIFICATION OF SOLIFENACIN BY LC-MS AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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ABSTRACT

A simple, high throughput, direct-injection high-performance liquid chromatography tandem mass spectrometry method (LC/MS/MS) has been developed and validated for the quantitation of Solifenacin in human plasma using Solifenacin D_5 as internal standard (IS). Analyte and the IS were extracted from the 100 µL of K_2 EDTA human plasma by simple Solid Phase extraction. The on-line extraction was achieved on a Strata-X 33µm polymeric sorbent cartridge (30 mg/1 mL). The extracted analyte was eluted by a mobile phase which contained 5mM ammonium acetate buffer in 0.1% formic acid and methanol (20:80% v/v). The analytical column was a Zorbax SB C_{18} column (4.6mm×50 mm, 3.5 µm). The standard curve, which ranged from 0.20 to 30.1ng/ml, was fitted by a weighted (1/*x*2) quadratic regression model. The Mass detection of Solifenacin involves m/z - 363.20 (parent) and 110.00 (product) and Solifenacin D5 involves m/z - 368.20 (parent) and 110.00 (product) as internal standard in Positive ion mode.Method validation was performed as per FDA guidelines and the results met the acceptance criteria. The intra-day and inter-day precision (%CV) and accuracy results in six validation batches across six concentration levels were well within the acceptance limits. A run time of 1.60 min for each sample made it possible to analyze more number of samples in short time, thus increasing the productivity. The proposed method successfully applied to a pharmacokinetic study of Solifenacin 10 mg tablet in healthy, adult, human male subjects under fed condition.

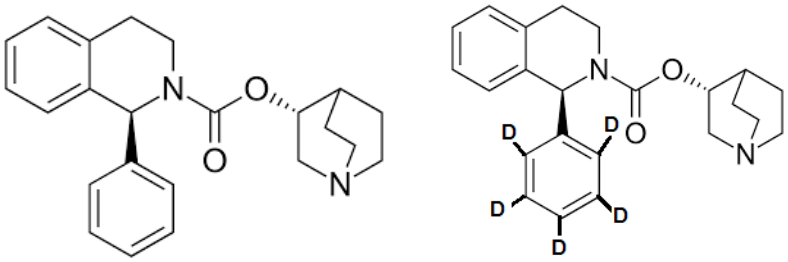
KEYWORDS: Solifenacin, human plasma, Solid Phase extraction, LC-MS/MS.

INTRODUCTION

Solifenacin (Vesicare)[1-2] is a medicine of the antimuscarinic class and was developed for treating contraction of overactive bladder with associated problems such as increased urination frequency and urge incontinence. Solifenacin is used to treat invasive aspergillosis and candidiasis and fungal infections caused by Scedosporium and Fusarium species, which may occur in immunocompromised patients. It is also used for the treatment of oropharyngeal candidiasis (OPC), including OPC refractory to itraconazole and/or fluconazole therapy.[3-5] It is also used to treat invasive infections by Candida, Mucor, and Aspergillus species in severely immunocompromised patients.[4,5] Solifenacin works by disrupting the close packing of acyl chains of phospholipids, impairing the functions of certain membrane-bound enzyme systems such as ATPase and enzymes of the electron transport system, thus inhibiting growth of the fungi. It does this by blocking the synthesis of ergosterol by inhibiting of the enzyme lanosterol 14α-demethylase and accumulation of methylated sterol precursors. Solifenacin is significantly more potent at inhibiting 14-alpha demethylase than itraconazole. Few methods were reported on estimation of Solifenacin by different analytical methods. Störzinger.D et.al.[6,7] reported Development and validation of a high-performance liquid chromatography assay for Solifenacin utilizing solid-phase extraction. Groll AH et.al.[8,9] shown clinical pharmacology and potential for management of fungal infections. Chhun.S et.al.[10-12] reported Simultaneous quantification of voriconazole and Solifenacin in human plasma by high-performance liquid chromatography[13-17] with ultra-violet detection. Among the methods present method involve less retention time and solid-phase extraction which is modern and less reported method. The analytical method should satisfy the scientists in terms of simplicity, sensitivity, runtime, time consumption, sample volume and efficient extraction procedure.[18-20]

MATERIALS AND METHODS

A Reference sample of Solifenacin (100.55%) was obtained from Indoco Remedies Limited (Mumbai, India), while Solifenacin D5 (99.87%) was form VIVEN Life Sciences Pvt. Limited (Mumbai, India). Their chemical structures are shown in Fig. 1. Water used for the LC-MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). HPLC grade Methanol was purchased from J.T. Baker (Phillipsburg, USA), while analytical grade formic acid and ammonium acetate was from Merck Ltd (Mumbai, India). The control K2–human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).



Solifenacin (A) **Solifenacin D_5 (B)**

Fig. 1: Chemical structures of Solifenacin (A) and Solifenacin D5 (B) (IS).

LC–MS/MS instrument and conditions

An HPLC system consisting of a Zorbax SB-C_{18} column 4.6 mmx50 mm x3.5µm; Agilent Technologies, Santa Clara, CA, USA), a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–$20A_3$) was used for the study. Aliquot of 15 µL of the processed samples were injected into the column, which was kept at 35°C. An isocratic mobile phase consisting of a mixture of Methanol and 5mM ammonium acetate in 0.1% formic acid buffer (80:20, v/v) was used to separate the analyte from the endogenous components and delivered at a flow rate of 0.80 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 an MDS Sciex API–4500 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 500 °C. The ion spray voltage was set at 5500 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 30, 35, 40, and 8 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 50, 34, 10, 8 V for Solifenacin and IS. Detection of the ions was carried out in the multiple reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 363.20 parent ion to the *m/z 110.00* for product ion of Solifenacin and *m/z* 368.20 parent ion to the *m/z* 110.00 product ion for the IS were shown in Fig.2. Quadruples Q1 and Q3 were set on unit resolution. The analysis data obtained were processed by Analyst Software™ (version 1.6.3). The MRM technique provided intrinsic selectivity and sensitivity, hence chosen for the study.

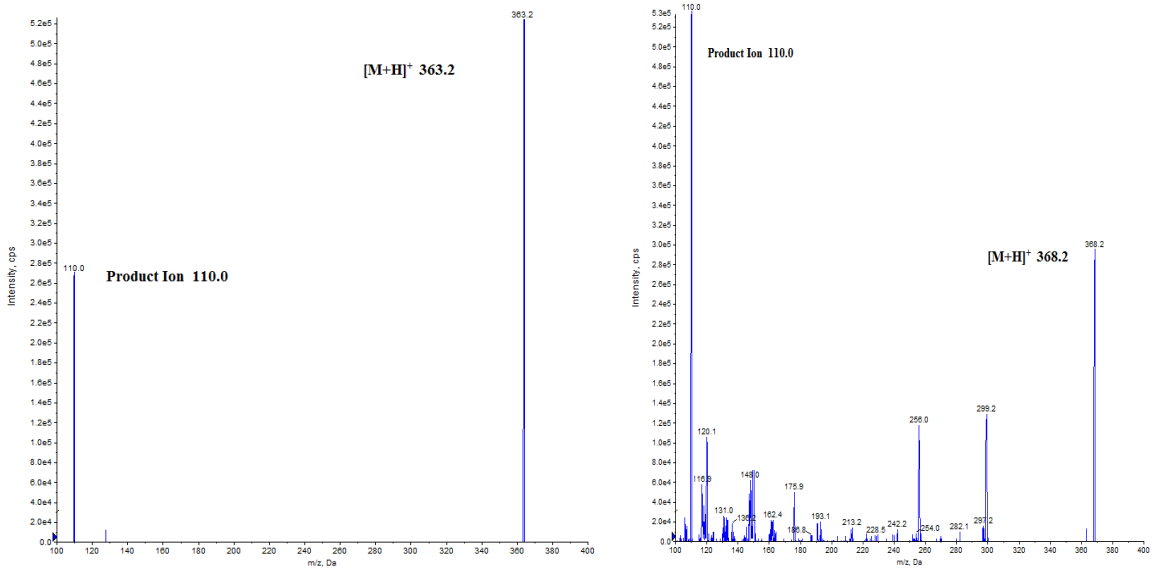


Fig. 2: Product ion mass spectra of $[M+H]^+$ of Solifenacin and Solifenacin D5 (IS).

Preparation of plasma standards and quality controls

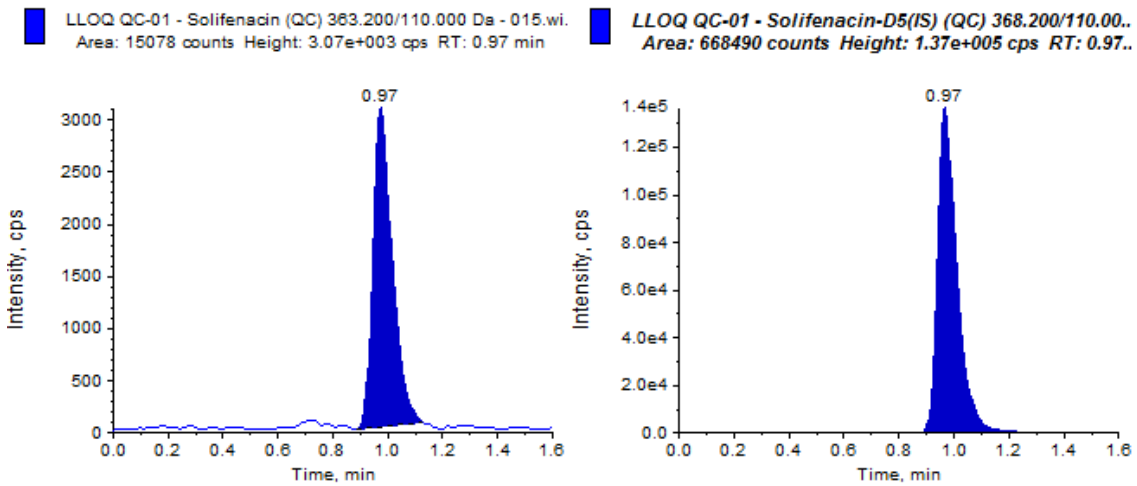
Standard stock solution of Solifenacin and IS (1mg/mL) were prepared in Acetonitrile. Working solutions for calibration and controls were prepared by appropriate dilution in Acetonitrile, ammonia and water (70.0:0.1:29.9, v/v/v; diluent). The IS working solution (100 ng/mL) was prepared by diluting its stock solution with diluent. Stock solutions of Solifenacin and IS were found to be stable for 8 days at 2-8 °C. Calibration samples were prepared by spiking 950 µL of control K_2 EDTA human plasma with the 50 µL working standard solution of the analyte as a bulk, to obtain Solifenacin concentration levels of 0.20, 0.40, 0.75, 1.51, 3.01, 6.02, 12.0, 18.1, 24.1 and 30.1 ng/mL as a single batch at each concentration. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 0.20 (LLOQ QC), 0.54 (LQC), 3.82 (MQC1), 15.3 (MQC2) and 23.0 ng/mL (HQC) as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at -70 ± 10 °C until analyses.

Sample processing

All frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature prior to analysis. Withdraw one set of calibration curve standards, one or more sets of quality control samples and subject plasma samples from the deep freezer and allow to thaw at room temperature. Vortex the thawed samples to ensure complete mixing of the contents. Pipette out 100 µL of the sample into pre-labelled RIA vial tubes. Add 10 µL of internal standards dilution (100ng/mL of Solifenacin D5) except in blank and subject pre dose sample wherein add 10 µL of diluent and vortex. Also process 100 µL of each pre-dose sample with addition of 10 µL of internal standard dilution and vortex. To this add 500 µL of 50 mM Ammonium acetate in 1% Formic Acid will be added and vortex. Load the entire content of the sample onto Strata X^{TM}-33µm polymeric sorbent cartridges (30mg/1mL) that were pre-conditioned with 1.0 mL of HPLC grade methanol, 1.0 mL Milli Q/HPLC grade water followed by 1.0 mL 50mM Ammonium acetate buffer in 1% formic acid. After applying the maximum pressure wash the extraction cartridge with 1mL of 50 mM Ammonium acetate in 1% Formic Acid buffer followed by 2.0 mL of water (each time 1mL). Then elute the samples with 1 mL of mobile phase and the eluted sample were transferred into loading vials and would be loaded into the auto sampler and it was injected into the LC-MS/MS system. The typical chromatograms of Solifenacin were shown in Fig.3.

Method development

The objective of the present work was to develop and fully validate an LC–MS/MS method for the determination of Solifenacin in human plasma with high sensitivity to monitor the concentration of Solifenacin for pharmacokinetic/bioequivalence studies. To develop a sensitive and selective bioanalytical method requires the judicious selection of chromatography column, mobile phase and organic solvent. These parameters should be carefully monitored to produce the required resolution from endogenous components which in turn affect sensitivity and reproducibility of the analytical method by ion suppression.



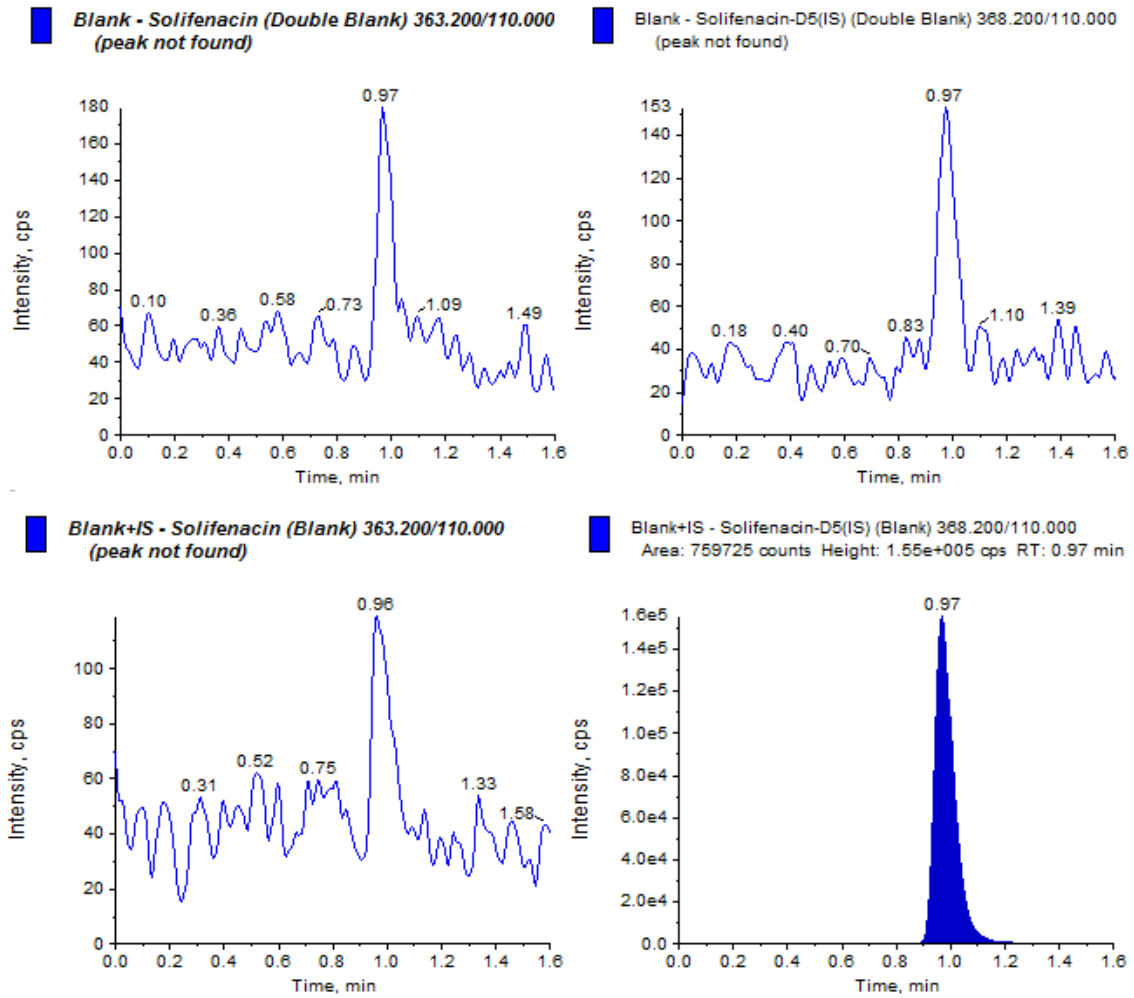


Fig. 3: Typical MRM chromatograms of Solifenacin (left panel) and IS (right panel) in human blank plasma, and human plasma spiked with IS, a LLOQ sample along with IS.

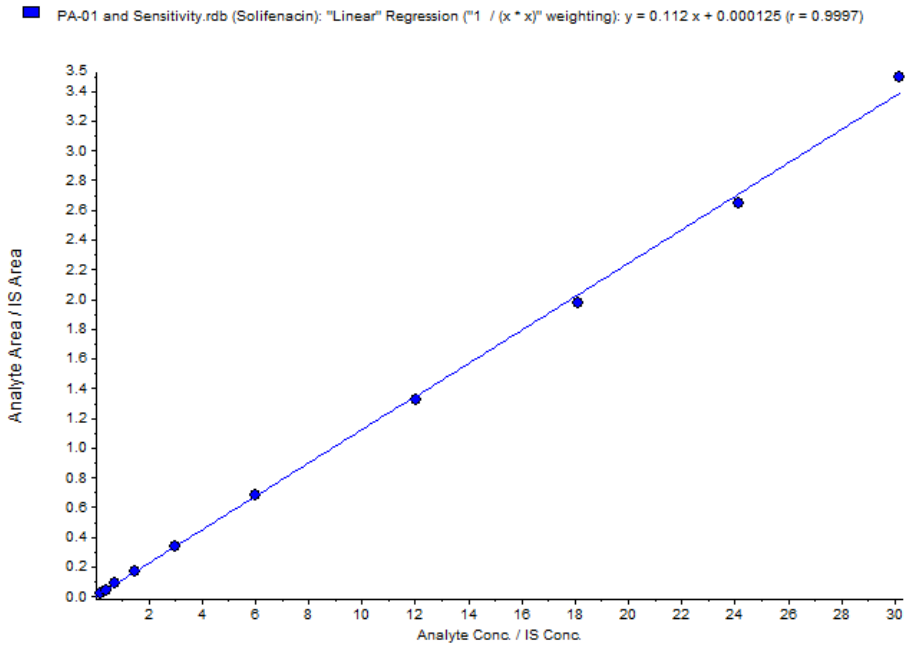


Fig. 4: Typical calibration curve of Solifenacin.

Once Chromatographic column, mobile phase pH and organic solvent are set then flow rate, column temperature and buffer type and concentration can be manipulated for optimal response. An ideal internal standard should mimic the analyte in as many ways as possible. It should have a similar structure, same physicochemical properties or can be a labeled compound. For LC-MS/MS analysis, use of stable isotope-labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible and to increase assay precision and limit variable recovery between analyte and the IS.

Method validation

A thorough and complete method validation of Solifenacin in human plasma was carried out as per USFDA guidelines. The parameters determined were carryover test, selectivity, specificity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity, run size evaluation and stability. Carry over experiment was performed to verify any carryover of analyte and IS which may reflect in subsequent runs. The design of the study comprised of the following sequence of injections i.e. blank plasma sample → six samples of LLOQ → blank plasma sample → ULOQ sample → blank plasma samples to check for any interference due to carry over. Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma obtained from six different sources. Sensitivity was determined by analyzing six replicates of plasma samples spiked with the lowest level of the calibration curve concentrations. Matrix effect was checked with six different lots of K_2 EDTA plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (18 QC samples in total). The linearity of the method was determined by analysis of standard plots associated with a ten–point (non–zero standards) standard calibration curve. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences. To determine intra-day accuracy and precision, a calibration curve and six replicates of LLOQ QC, LQC, MQC–1, MQC–2, and HQC were analyzed on the same day. Inter–day accuracy and precision were assessed by analyzing three batches of samples on two consecutive days. Recoveries of analyte and IS were determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. Recovery of Solifenacin was determined at a concentration of 0.54 (LQC), 15.3 (MQC2) and 23.0(HQC) ng/mL whereas for IS was determined at concentration of 100 ng/mL. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. Six replicates each at a concentration of about 1.60 times of the uppermost calibration standard were diluted two and four -fold with blank plasma. The diluted samples were processed and analyzed. Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8°C) was performed by comparing the area response of the analyte (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (11 h), processed samples stability (autosampler stability for 73 h, wet extract stability for 41 h and reinjection stability for 40 h.), freeze-thaw stability (4 cycles), long-term stability (49 days) were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% SD) and precision (≤15% RSD).

RESULTS AND DISCUSSION

Selectivity and chromatography

The selectivity of the method was examined by analyzing blank human plasma extract and an extract spiked only with the IS. As shown in Fig.3, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte and the IS.

Matrix effect

Matrix effect assessment was done with the aim to check the effect of different lots of plasma on the back calculated value of QC's nominal concentration. The results found were well within the acceptable limits. No significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high quality control concentrations. Also, the extraction method was rugged enough and gave accurate and consistent results when applied to real subject samples.

Linearity, precision and accuracy

The ten point calibration curve was found to be linear over the concentration range of 0.20 – 30.1 ng/mL for Solifenacin. 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. 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.1. Typical calibration curve of Solifenacin as shown in Fig.4.

Table 1: Precision and accuracy data for Solifenacin.

Quality control	Run	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
Intra–day variations (Six replicates at each concentration)				
LLOQ	1	0.20±0.00	2.05	96.1
	2	0.18±0.01	3.01	87.2
	3	0.21±0.00	2.20	106
	4	0.19±0.01	7.99	92.2
	5	0.22±0.01	4.67	110
LQC	1	0.51±0.01	1.94	95.5
	2	0.51±0.01	1.00	94.9
	3	0.52±0.01	1.83	97.2
	4	0.51±0.01	1.99	94.8
	5	0.52±0.01	1.30	97.7
MQC1	1	3.36±0.07	2.04	88.0
	2	3.55±0.03	0.80	92.9
	3	3.56±0.04	1.08	93.1
	4	3.63±0.03	0.86	94.8
	5	3.74±0.03	0.86	97.8
MQC2	1	13.6±0.30	2.19	89.1
	2	14.8±0.18	1.21	96.8
	3	14.7±0.11	0.78	96.2
	4	14.8±0.26	1.78	96.6
	5	15.8±0.15	0.94	103
HQC	1	22.7±2.05	9.01	99.0
	2	21.4±0.98	4.59	93.1
	3	21.9±1.30	5.96	95.2
	4	21.6±1.12	5.19	93.9
	5	23.5±0.39	1.67	102
Inter–day variations (Eighteen replicates at each concentration)				
LLOQ		0.20±0.02	9.79	98.3
LQC		0.51±0.01	2.00	96.0
MQC1		3.57±0.13	3.65	93.3
MQC2		14.7±0.72	4.87	96.4
HQC		22.2±1.43	6.45	96.7
Spiked concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 0.20, 0.54, 3.82, 15.3 and 23.0 ng/mL, respectively.				

Stability studies and dilution integrity

In the different stability experiments carried out viz. bench top stability (11 h), autosampler stability (73 h), wet extract stability (41 h), repeated freeze–thaw cycles (4cycles), reinjection stability (40 h) and long term stability at –70 °C for 49 days the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels shown Tab.2. Thus, the results were found to be within the acceptable limits during the entire validation. The MRM chromatograms of Solifenacin were shown in Fig.3.

Table 2: Stability data for Solifenacin in plasma (*n*=6).

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Process[a]	0.54	0.57±0.02	3.46	107
	23.0	23.3±0.32	1.37	102
Process[b]	0.54	0.50±0.01	1.62	93.6
	23.0	23.1±0.32	1.37	100
Bench top[c]	0.54	0.58±0.01	2.20	109
	23.0	23.4±0.22	0.94	102
FT[d]	0.54	0.61±0.04	5.89	113
	23.0	23.3±0.34	1.48	101

Reinjection[e]	0.54	0.52±0.00	0.74	101
	23.0	23.5±0.19	0.80	103
Long–term[f]	0.54	0.55±0.01	1.97	100
	23.0	23.7±0.44	1.85	99.7

[a] after 73 h in autosampler at 15°C; [b] after 41 h at 20°C; [c] after 11 h at room temperature; [d] after 4 freeze and thaw cycles; [e] after 40 h of Reinjection; [f] at –70°C for 49 days

A single dose pharmacokinetic study was performed in healthy South Indian male subjects (n = 6). The Ethics Committee (Samkshema Independent Ethics Committee, Hyderabad, India) approved the protocol and the volunteers provided with written informed consent. The subjects were fasted 12 h before administration of the drug formulation. Six healthy South Indian male subjects with an age group of 20–40 years and body–mass index (BMI) of ≥18.5 kg/m^2 and ≤24.9 kg/m^2, with body weight not less than 50 kg were chosen for the study. They were randomly assigned to one group and took a single oral dose of Solifenacin succinate 10 mg tablets. Blood samples were collected at pre-dose (0.00), 0.50, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00, 12.00, 18.00, 24.00, 36.00, 48.00 and 72.00 h, in K_2 EDTA vacutainer (3 mL) collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 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 main pharmacokinetic parameters of Solifenacin were calculated by non–compartmental model using WinNonlin Version 5.2. An incurred sample re–analysis was also conducted by selecting the 6 subject samples (2 samples from each subject) near C_{max} and the elimination phase. The percent change in the value should not be more than ±20% Tab.5. The validated method was successfully applied for a pharmacokinetic study of Solifenacin in 6 healthy South Indian adult male subjects divided in to one group who received a single oral dose of Solifenacin 10 mg tablet under fed condition. With the reported lowest LLOQ (0.20 ng/mL) Solifenacin was not quantifiable beyond 72 h post-dosing. The mean plasma concentration–time profile of Solifenacin was presented in Fig. 6 and the corresponding pharmacokinetic parameters were listed in Tab.3.

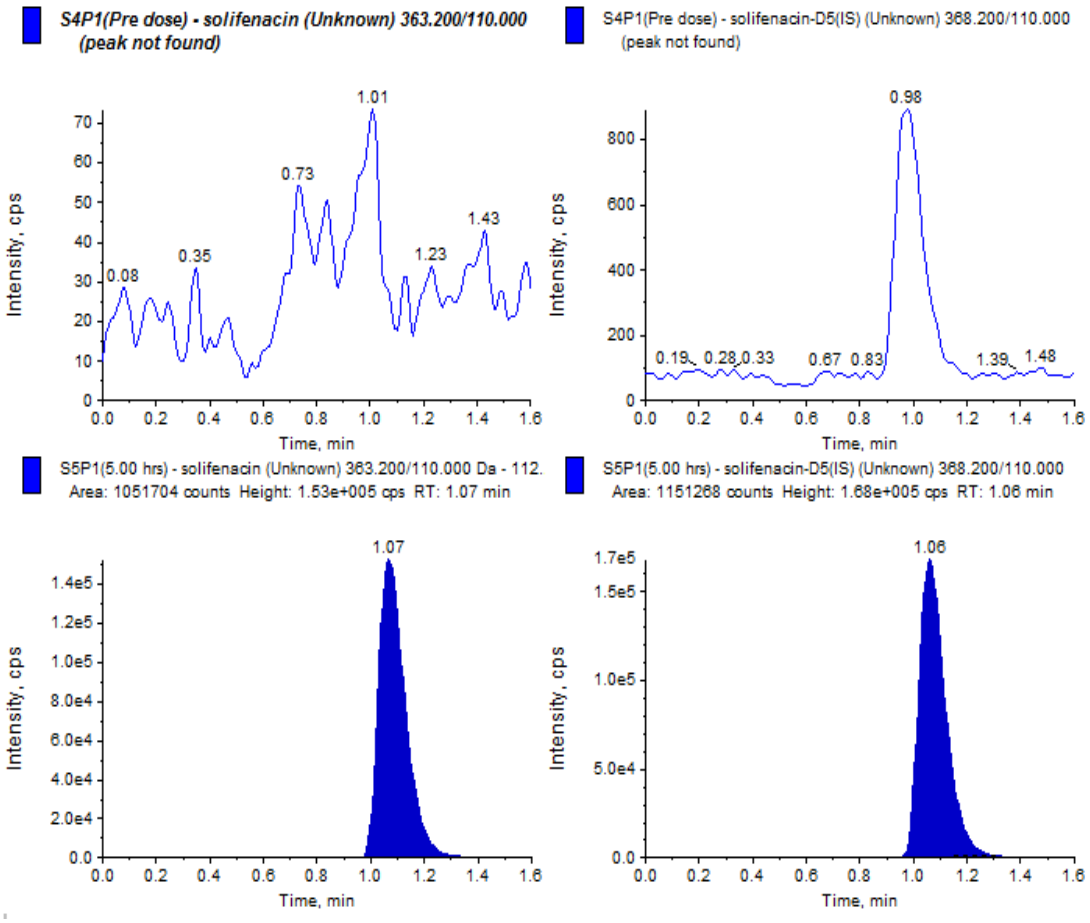


Fig. 5: MRM chromatograms resulting from the analysis of subject pre dose sample (A) and 5.00 hr subject plasma sample (B), after the administration of a Solifenacin 10 mg tablet.

Tab.3: Pharmacokinetic parameters of Solifenacin succinate 10 tablets formulation in healthy, adult, human male subjects under fed condition (n=6, Mean ± SD).

PK Parameter	Mean ± SD
t_{max} (h)	6.00±1.26
C_{max} (ng/mL)	19.7±3.23
AUC_{0-t} (ng h/mL)	970±253
AUC_{0-inf} (ng h/mL)	2727±2176
$t_{1/2}$ (h)	96.0±61.5
Kel (h^{-1})	0.01±0.00

Table 4: Incurred samples re-analysis data of Solifenacin.

S. No.	Sample ID	Solifenacin 10 mg tablet		
		Initial conc. (ng/mL)	Re–assay conc. (ng/mL)	Difference[a] (%)
1	S1P1(10.00 hrs)	24.8	23.8	4.43
2	S1P1(72.00 hrs)	17.8	18.6	4.66
3	S2P1(8.00 hrs)	20.3	22.3	9.53
4	S2P1(72.00 hrs)	9.95	10.7	7.00
5	S3P1(6.00 hrs)	18.1	18.0	0.90
6	S3P1(72.00 hrs)	7.37	7.23	1.85
7	S4P1(10.00 hrs)	16.5	16.4	0.57
8	S4P1(72.00 hrs)	7.85	7.61	3.01
9	S5P1(8.00 hrs)	21.0	20.0	4.89
10	S5P1(72.00 hrs)	13.8	13.5	1.71
11	S6P1(7.00 hrs)	15.7	15.6	0.55
12	S6P1(72.00 hrs)	5.78	5.88	1.70

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

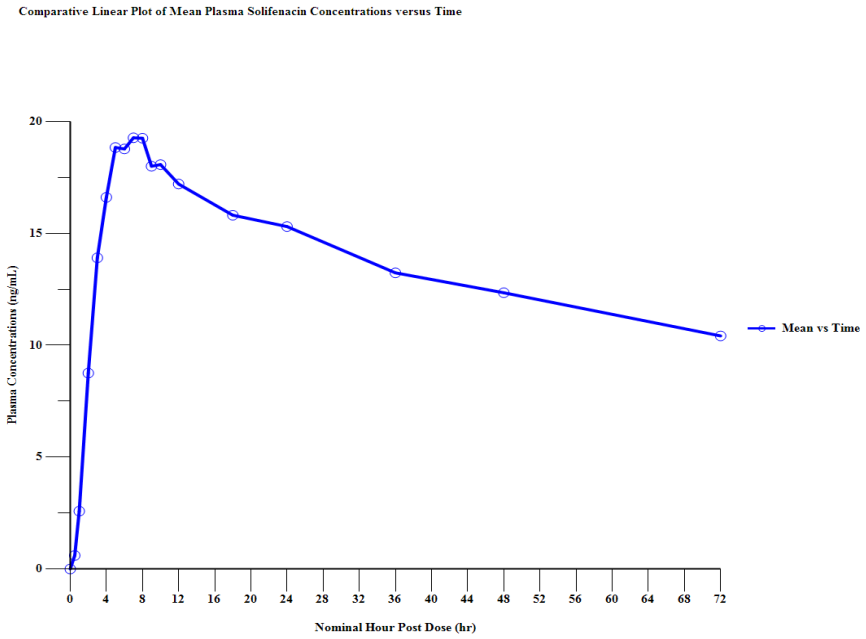


Fig. 6: Mean plasma concentration-time profile of Solifenacin in human plasma following A single oral dose of Solifenacin succinate 10 mg tablets to healthy volunteers (n=6).

CONCLUSION

The present research work involves successful method development and validation of a simple, sensitive and rapid LC-MS/MS method for the determination of Solifenacin in human plasma samples according to commonly acceptable FDA guidelines. Moreover, the total analysis time (extraction and chromatography) is the shortest. The advantage of this method is that a relatively more number of samples can be analyzed in short time, thus increasing the output. The Solid Phase

extraction minimizes the chances of errors, saves considerable time and simplifies the sample preparation procedure. The method showed suitability for pharmacokinetic studies in humans. 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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