AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Determination of Darifenacin in Human Plasma by a Novel LC–MS/MS method by using Protein Precipitation technique

Boligarla Gopi Kalyan Kumar[1], Vasu Babu Ravi[2], Sesharatnam Padala [3] and N. Y. Sreedhar [1] *

1. Electroanalytical Lab, Department of Chemistry, Sri Venkateswara University, Tirupati–517 502, India

2. PCR Laboratories, Ramanthapur, Hyderabad–500 013, India

3. Nirmala College of Pharmacy, Atmakuru Road, Mangalagiri, Vijayawada–522 503,

ABSTRACT

In this paper the authors proposed a simple, sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay method for the determination of darifenacin in human plasma. This method employed solifenacin as an internal standard (IS). Analyte and the IS were extracted form 200 µL of human plasma using protein precipitation technique. The chromatographic separation was achieved on a C_{18} column by using a mixture of acetonitrile and 5mM ammonium formate in 0.01% formic acid (90:10, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The linearity of the method was established in the concentration range 0.05–20.5 ng/mL with $r^2 \geq 0.99$. The intra–day and inter–day precision (%CV) and accuracy results in three validation batches across five concentration levels were well within the acceptance limits. The validated method was successfully applied to a pharmacokinetic study in humans under fasting condition with 15 mg darifenacin extended release tablet.

Keywords: Darifenacin, Human plasma, Protein precipitation (PP), LC–MS/MS, Pharmacokinetics

*Corresponding Author Email: gopi_kalyan@rediffmail.com

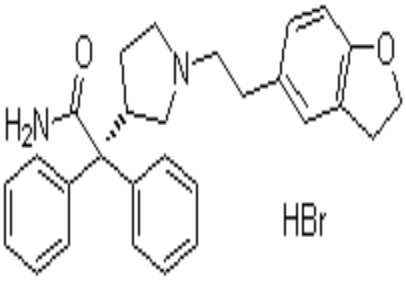
Received 23 December 2013, Accepted 02 January 2014

Please cite this article in press as: Boligarla G. *et al.*, Determination of Darifenacin in Human Plasma by a Novel LC–MS/MS method by using Protein Precipitation technique. American Journal of PharmTech Research 2014.

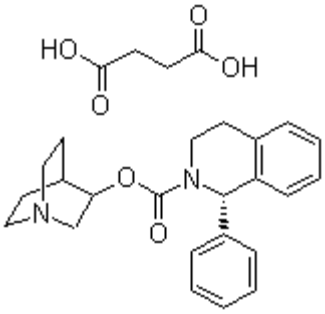
INTRODUCTION

Darifenacin hydrobromide is a selective muscarinic M_3 acetylcholine receptor antagonist used to treat overactive bladder syndrome. These receptors are primarily responsible for bladder muscle contractions. Thereby decreases the urinary urgency. The drug has short elimination half–life after intravenous and immediate release oral dosage forms (3–4 h), but has long half–life (14–16 h) with a prolonged–release formulation. Darifenacin is well absorbed from the gastrointestinal tract and peak plasma concentrations are reached about 7 h after repeated once–daily oral dosage of a prolonged released formulation. The drug has high plasma protein binding affinity (98%) especially α1–acid glycoprotein. The pharmacokinetics of darifenacin is not affected by food [1].

As per the literature, only two [1, 2] LC–MS/MS methods have been reported for the determination of darifenacin in biological samples. Kaye *et al.*, (1996) reported a HPLC/APCI–MS/MS method for the determination of darifenacin in human plasma samples using multi step solid–phase extraction technique (SPE) which involves many complicated method development protocols. This method utilizes relatively high plasma sample volume (>0.5 mL) which may not be favorable for routine drug analysis for a pharmacokinetic/bioequivalence study. Recently, Karavadi and Challa (2012) reported an LC–MS/MS method for the determination of darifenacin in rat plasma samples is highly sensitive with the concentration range of 0.01–20.0 ng/mL. This method employs liquid–liquid (L–L) extract, evaporation, drying and reconstitution steps for sample preparation. Moreover, LLE technique having disadvantages, such as the formation of emulsions and the use of large volumes of toxic organic solvents, complexity in sample preparation (evaporation, drying and reconstitution). In the present paper the authors have been proposed a simple, rapid and sensitive LC–ESI–MS/MS method for the determination of darifenacin in 200 µL of human plasma with a chromatographic run time 2.0 min. This method employs simple protein–precipitation (PP) technique for the sample preparation. We have also employed solefenacin as an internal standard to obtain the better accuracy and precision results. The validated method was successfully applied to determine the darifenacin concentrations for a pharmacokinetic study in humans. Additionally, for the first time method reproducibility was demonstrated through incurred samples reanalysis (ISR).



Darifenacin hydrobromide



Solifenacin succinate (IS)

Figure 1.: Chemical structures of darifenacin hydrobromide and solifenacin succinate (IS).

MATERIALS AND METHOD

Standards and chemicals

Reference sample darifenacin hydrobromide (>99.60%) was obtained from Hetero Drugs Limited (Hyderabad, India). Solefenacin succinate (>99.85%) was employed as an internal standard and was obtained from Vivan Life Sciences Limited (Mumbai, India). Their chemical structures are shown in Figure. 1. HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, USA). Analytical grade ammonium formate and formic acid were purchased from Merck Ltd (Mumbai, India). Water used for the LC–MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). The control human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).

LC–MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Hypurity Advance column (50 mm × 4.6 mm, 5 µm), 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 10 µL of the processed samples were injected into the column, which was kept at room temperature (20±5°C). An isocratic mobile phase consisting of a mixture of acetonitrile and 5mM ammonium formate in 0.01% formic acid (90:10, v/v) was used to separate the analyte from the endogenous components. The mobile phase was pumped at a flow rate of 1.0 mL/min into the electrospray ionization chamber of the mass spectrometer. MS–MS quantification was achieved in positive ion mode for the analyte and the IS using an MDS Sciex API–4000 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 40, 40, 25, 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 100, 46, 10, 7 V for darifenacin and 80, 43, 10, 5 V for the IS. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 427.30 precursor ion to the *m/z* 147.10 for darifenacin and *m/z* 363.20 precursor ion to the *m/z* 193.20 product ion for the IS. Both the quadrupoles (Q1 and Q3) were set on unit resolution. The analysis data obtained were processed by Analyst Software™ (version 1.4.2).

Preparation of stock and working solutions

Primary stock solutions of darifenacin and the IS were prepared separately in HPLC grade methanol (1 mg/mL). All the working standards were prepared in a mixture of acetonitrile and water (60:40, v/v; diluent).

Calibration samples were prepared by spiking control K_2 EDTA human plasma with the standard solution of the analyte as a bulk, to obtain darifenacin concentration levels of 0.05, 0.10, 0.51, 1.03, 2.06, 4.11, 8.22, 12.3, 16.4 and 20.5 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 0.05 (LLOQ), 0.15 (low; LQC), 3.08 (middle; MQC–1), 10.3 (MQC–2) and 17.4 ng/mL (high; HQC) as a single batch at each concentration. All the samples were stored in the freezer at -70 ± 10^0C until analyses.

Sample processing

All frozen plasma samples were thawed and allowed to equilibrate at room temperature prior to analysis. The samples were vortexed to mix for 10 s prior to spiking. A 200 µL aliquot of human plasma sample was mixed with 20 µL of the internal standard working solution (250 ng/mL of solefenacin). To this, 50 µL of the 5mM ammonium formate buffer solution and 1.0 mL of acetonitrile were added. After vortex–mixing for 60 s and centrifugation at 4000 rpm for 20 min, the supernatant was transferred to another clean test tube and evaporated to dryness at 45 °C under a gentle stream of nitrogen. The residue was reconstituted with 200–µL of the mobile phase and 10 µL were injected into LC–MS/MS system.

Bioanalytical method validation

A thorough and complete method validation of darifenacin in human plasma was carried out as per US FDA guidelines [4]. The parameters determined were selectivity, specificity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability. Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma obtained from six different sources including one lipemic and hemolyzed plasma. 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 K2–EDTA

plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (36 QC samples in total).

The linearity of the method was determined by analysis of three standard calibration curves (CC) containing 10 non–zero concentrations. In addition, each curve contains one blank plasma sample and one blank plasma sample with internal standard (zero sample). Each CC was analyzed individually by least square weighted ($1/x^2$) linear regression. Intra–day accuracy and precision was determined using six replicates of LLOQ QC, LQC, MQC–1, MQC–2, and HQC samples were analyzed along with a calibration curve in a single day. Inter–day accuracy and precision were assessed by analyzing three batches of samples on two consecutive days. The precision (% CV) at each concentration level from the nominal concentration should not be greater than 15%, except for LLOQ QC where it should be 20%. The accuracy (%) must be within ±15% of their nominal value at each QC level except LLOQ QC where it must be within ±20%.

Recovery for the analyte and the IS was calculated by comparing the mean peak response of pre–extraction spiked samples (spiked before extraction) to that of post–extraction spiked samples (spiked after extraction) at each QC level. Recovery of the analyte was determined at LQC, MQC–2 and HQC concentration levels, whereas for the IS was determined at working concentration level (250 ng/mL). Six replicates each at a concentration of about 1.65 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 (12 h), processed samples stability including autosampler stability (41 h), wet extract stability (37 h) and reinjection reproducibility (29 h), freeze–thaw stability (4 cycles), long–term stability (30 days) were performed at both the QC levels (LQC and HQC) 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).

Pharmacokinetic study design

A pharmacokinetic study was performed in healthy male subjects (n = 6). The ethics committee approved the protocol and the volunteers provided with informed written consent. Blood samples

were collected following oral administration of darifenacin (15 mg extended release tablet) at pre–dose and 1, 2, 3, 4, 5, 6, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 14, 16, 24, 36, 48 and 72 h, in K_2–EDTA vacutainer 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 °C till their use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Along with the clinical samples, the QC samples at low, middle 1, middle 2 and high concentration levels were also assayed in triplicate. Plasma concentration–time profile of darifenacin was analyzed by non–compartmental method using WinNonlin Version 5.1. An incurred sample re–analysis (ISR) was also conducted by selecting the 12 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% [5, 6].

RESULTS AND DISCUSSION

Mass spectrometry

MS parameters were optimized by infusing the tuning solution (100 ng/mL) into the mass spectrometer using electrospray as the ionization source and operating in the MRM mode. The signal intensities obtained in positive mode were much higher than those in negative ion mode since the analytes and IS have the ability to accept protons. Protonated form of each analyte and the IS, $[M+H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was monitored from *m/z* 427.30 to 147.10 for darifenacin and from *m/z* 363.20 to 193.20 for the IS. As earlier publications have discussed the details of fragmentation patterns of darifenacin [3] and the IS [7], we are not presenting the data pertaining to this. LC–MRM was chosen for the assay development due to its inherent selectivity and sensitivity [8].

Method development

During the method development different parameters includes mobile phase selection, flow rate, column type and injection volume were investigated. Methanol and acetonitrile were tried in different ratio with buffers like ammonium acetate, ammonium formate as well as acid additives like formic acid and acetic acid in varying strength. It was observed that acetonitrile and 5 mM ammonium formate in 0.01% formic acid (90:10, v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. Use of acidic buffer helped in achieving the good peak shape and high spectral response. Additionally, C_8 and C_{18} columns of different make were tested and finally Hypurity advance (50 mm × 4.6 mm, 5 µm) column helped in achieving

the adequate retention time, better separation form endogenous components, symmetric peak shape and satisfactory response. The retention time of analyte and the IS obtained with the above optimized chromatographic conditions were low enough (0.80 and 0.95 min) allowing short run time of 2.0 min.

The earlier authors have employed liquid–liquid extraction (LLE) procedure [3] and solid–phase extraction (SPE) procedure [2] to extract darifenacin from rat plasma and human plasma, respectively. As per the literature, darifenacin has high plasma protein (98%) binding affinity[1].Therefore, protein precipitation (PP) was tested with methanol/acetonitrile under acidic and basic conditions. But although methanol gave good results, the recovery was not consistent at lowest level. Finally, promising results were obtained with acetonitrile, which can produce a clean chromatogram for a blank sample and yields the maximum recovery for the analyte from the plasma. Addition ammonium formate to the plasma samples as an extraction additive helped achieving reproducible and quantitative recoveries for the analyte and the IS.

A good internal standard should mimic the analyte during chromatography, ionization and extraction. At the initial stages of this work, several compounds were investigated to find a suitable IS and finally solifenacin was found to be best for the present purpose.

Selectivity and sensitivity

The selectivity of the method was examined by analyzing blank human plasma extract (Figure. 2A) and a blank plasma extract spiked only with the IS (Figure. 2B). As shown in Figure. 2A, 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. Similarly, Figure. 2B shows the absence of direct interference from the IS to the MRM channel of the analyte. Figure. 2C depicts a representative ion–chromatogram for the LLOQ sample (0.05 ng/mL). Figure. 4 depict a representative chromatograms resulting from the analysis of subject blank plasma sample and 9.50 h subject plasma sample after the administration of a 15 mg oral single dose of darifenacin extended release tablet.

The lowest limit of reliable quantification for the analyte was set at the concentration of the LLOQ (0.05 ng/mL). The signal–to–noise ratio (S/N) was measure at this concentration and found to be ≥10. The precision and accuracy at LLOQ concentration were found to be 6.46% and 108%, respectively.

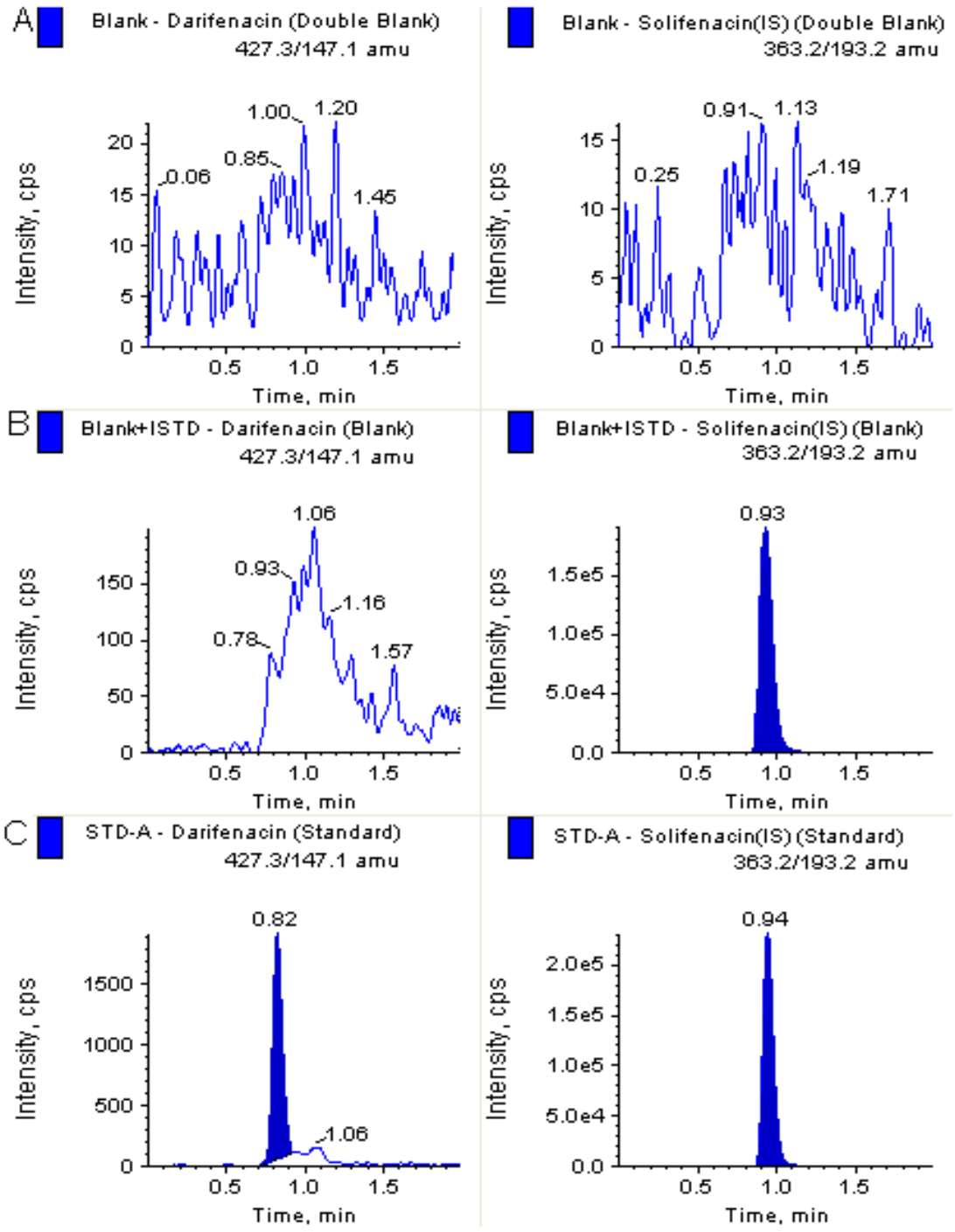


Figure 2: Typical MRM chromatograms of darifenacin (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).

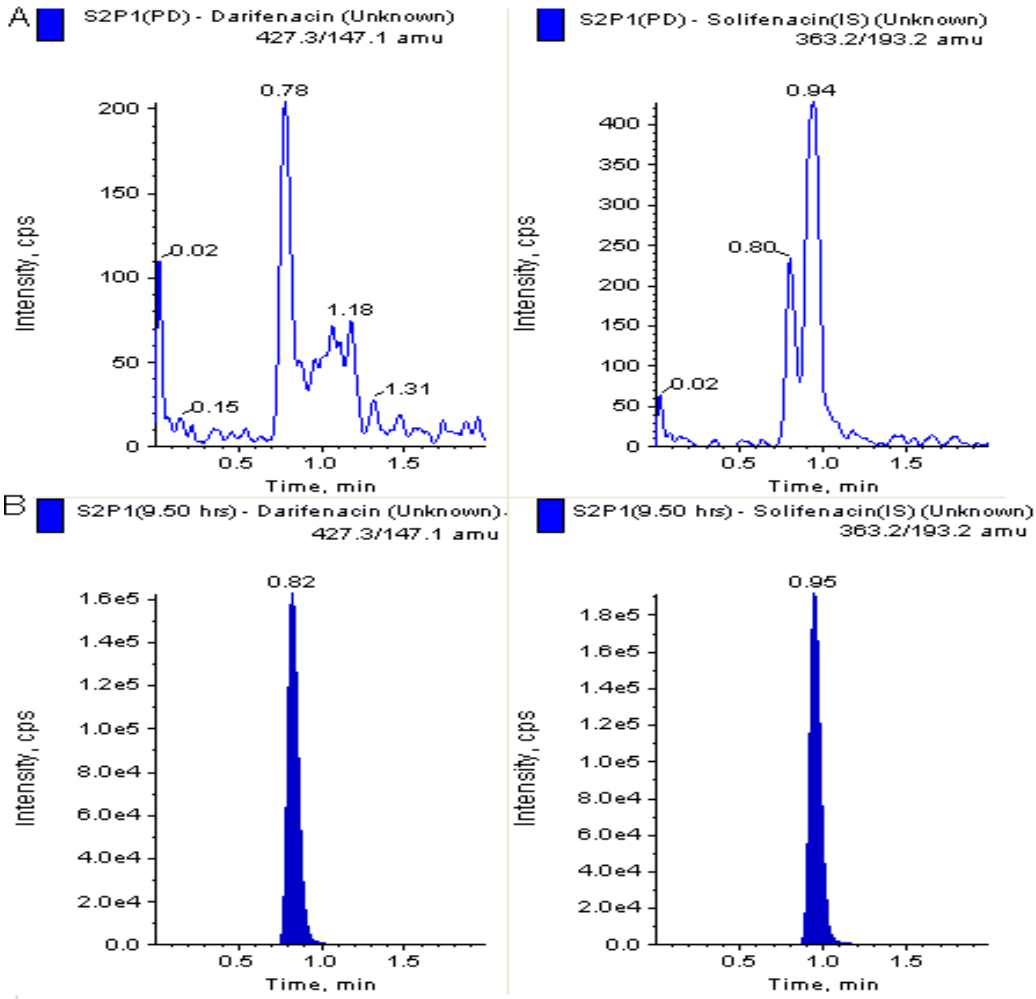


Figure 3.:MRM chromatograms resulting from the analysis of subject blank plasma sample (A) and 9.50 h subject plasma sample (B), after the administration of a 15 mg oral single dose of darifenacin extended release tablet. The sample concentration was determined to be 6.63 ng/mL.

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 precision and accuracy for darifenacin at LQC concentration were found to be 2.37% and 92.1%, and at HQC level they were 2.10% and 97.8%, respectively. Results revealed that no significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high quality control concentrations.

Linearity, precision and accuracy

The analyte showed good linearity in the concentration rage of 0.05–20.5 ng/mL. Both the regression models (1/x and $1/x^2$) were compared and best fit for the concentration–detector

response relationship was obtained with a weighting factor of $1/x^2$. The mean correlation coefficient was ≥ 0.99 for all the analytical runs generated during entire course of validation.

The intra–day and inter–day precision and accuracy results in plasma QC samples are summarized in Table 1. The precision (% CV) and accuracy values of darifenacin for intra– and inter–day ranged from 2.72–10.9% and 99.7–109%, and 3.02–9.96% and 97.4–106%, respectively. The results revealed good precision and accuracy.

Table 1: **Precision and accuracy data for darifenacin**

Quality control	Run	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
Intra–day variations (*n*=6 at each concentration)				
LLOQ	1	0.056 ± 0.003	4.64	103
	2	0.057 ± 0.003	4.64	105
	3	0.056 ± 0.003	5.48	103
LQC	1	0.14 ± 0.02	10.7	92.3
	2	0.17 ± 0.01	5.01	107
	3	0.14 ± 0.01	5.19	92.7
MQC1	1	3.18 ± 0.08	2.65	103
	2	3.08 ± 0.06	1.83	100
	3	3.14 ± 0.12	3.84	102
MQC2	1	11.3 ± 0.39	3.42	110
	2	10.9 ± 0.36	3.26	107
	3	10.3 ± 0.30	2.92	99.9
HQC	1	18.9 ± 0.85	4.52	108
	2	18.6 ± 0.32	1.73	107
	3	18.3 ± 0.68	3.71	105
Inter–day variations (*n*=18 at each concentration)				
LLOQ		0.056 ± 0.003	4.73	104
LQC		0.15 ± 0.01	9.96	97.4
MQC1		3.13 ± 0.09	3.02	102
MQC2		10.8 ± 0.56	5.18	106
HQC		18.6 ± 0.67	3.59	106

Spiked concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 0.05, 0.15, 3.08, 10.3 and 17.4 ng/mL, respectively.

Recovery and dilution integrity

The mean overall recovery of darifenacin was 92.4±3.96% with the precision range of 4.10–6.29% and the recovery of IS was 84.4% with the precision range of 4.00–6.93%. Good and reproducible recoveries were obtained for the analyte and the IS. Thus, the assay has been proved to be robust in high throughput bioanalysis.

The upper concentration limit of darifenacin can be extended to 33.8 ng/mL for by 1/2 and 1/4

dilutions with screened human blank plasma. The precision (%CV) for dilution integrity of 1/2 and 1/4 dilution was found to be 2.71% and 3.25%, while the accuracy results were found to be 96.4% and 103%, respectively.

Stability studies

The drug stability at various conditions was evaluated. In the different stability experiments carried out viz. bench top stability (12 h), autosampler stability (41 h), wet extract stability (37 h), repeated freeze–thaw cycles (4 cycles), reinjection stability (29 h) and long term stability at –70 °C for 30 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 (Table 2). Therefore, the results were found to be within the acceptable limits during the entire validation.

Stock solutions of darifenacin and the IS were found to be stable for 14 days at 2–8 °C. The percentage stability (with the precision range) of darifenacin and the IS was 99.3% (3.39–8.72%) and 99.4% (5.83–5.84%), respectively.

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

Stability test	QC spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Process[a]	0.15	0.17 ± 0.01	3.75	109
	17.4	18.2 ± 0.70	3.83	105
Process[b]	0.15	0.16 ± 0.00	1.69	106
	17.4	16.7 ± 0.44	2.65	95.5
Bench top[c]	0.15	0.17 ± 0.01	6.21	113
	17.4	19.2 ± 0.70	3.64	110
FT[d]	0.15	0.15 ± 0.01	9.50	98.1
	17.4	19.6 ± 0.51	2.59	112
Reinjection[e]	0.15	0.15 ± 0.00	2.26	99.1
	17.4	19.0 ± 0.67	3.53	109
Long–term[f]	0.15	0.16 ± 0.01	4.04	104
	17.4	18.4 ± 0.35	1.91	105

[a] after 41 h in autosampler at 10°C; [b] after 37 h at 2–8°C; [c] after 12 h at room temperature; [d] after 4 freeze and thaw cycles; [e] after 29 h of Reinjection;

[f] at –70°C for 30 days

Pharmacokinetic study and incurred samples reanalysis

The present method was successfully used to quantify darifenacin plasma concentrations for a pharmacokinetic study in healthy South Indian adult male subjects (*n*=6). Figure. 4 depicts the mean plasma concentration *vs* time profile of darifenacin after administration of a single 15 mg oral dose of darifenacin extended release tablet under fasting condition. The maximum

concentration (C_{max}) in plasma (9.12±2.22 ng/mL) for darifenacin was attained at 8.33±2.21 h (t_{max}).). The area under the plasma concentration–time curve from time zero to last measurable time point (AUC_{0-t}) and area under the plasma concentration time curve from time zero to infinity time point (AUC_{0-inf}) for darifenacin were 147±33.9and 154±33.2 ng*h/mL, respectively. The terminal half–life (t½) was found to be 6.56±1.17 h.

The reproducibility of the present method was established by reanalysis of incurred samples (ISR). For incurred samples analysis two plasma samples from each subject were selected and re–assayed in a single bioanalytical run. The differences in concentrations between the ISR and the initial values for all the tested samples were less than 20% (Table 3), indicating good reproducibility of the present method.

Table 3 : Incurred samples re–analysis data of darifenacin

Sample	Initial conc. (ng/mL)	Re–assay conc. (ng/mL)	Difference [a] (%)
1	8.43	7.92	6.21
2	3.23	3.31	–2.39
3	6.28	6.45	–2.73
4	0.15	0.14	3.51
5	12.1	13.3	–9.27
6	0.14	0.15	–4.23
7	9.99	10.2	–2.37
8	0.18	0.16	11.2
9	6.97	6.29	10.3
10	0.33	0.32	3.74
11	8.04	8.24	–2.52
12	0.16	0.17	–7.23

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

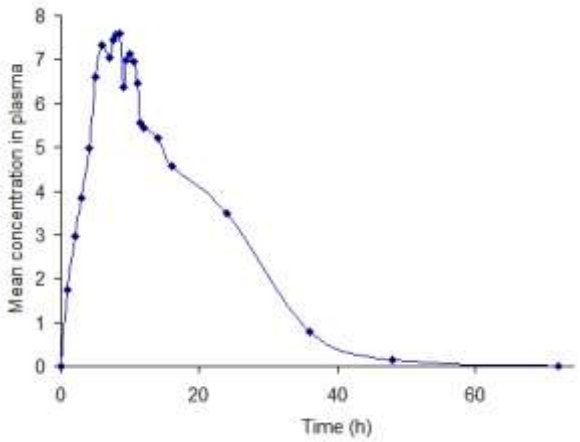


Figure 4: Mean plasma concentration–time profile of darifenacin in human plasma following oral administration of darifenacin hydrobromide (15 mg extended release tablet) to healthy volunteers (*n*=6).

CONCLUSION

The LC–MS/MS method presented here is simple, rapid and enough sensitive for the determination of darifenacin in human plasma. This method was validated as per the US FDA guidelines and employs low plasma volume (200 µL) for processing, therefore volume of blood sample collected from an individual during the study is reduced significantly—this allows inclusion of additional points. A simple protein precipitation technique was employed for the sample preparation, thereby significantly reduces the sample processing time. Moreover, the total analysis time (chromatography and extraction) is the shortest. Thus, the advantage of this method is that a relatively more number of samples can be analyzed in short time, thus increasing the output. 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.

ACKNOWLEDGEMENT

The authors gratefully acknowledge PCR Laboratories, Hyderabad for providing necessary facilities to carry out this work.

REFERENCES

1. Skerjanec A. The clinical pharmacokinetics of darifenacin. Clin Pharmacokinet 2006; 45: 325–350.
2. Kaye B, Herron WJ, Macrae PV, Robinson S, Stopher DA, Venn RF and Wild W. Rapid, solid phase extraction technique for the high–throughput assay of darifenacin in human plasma. Anal Chem. 1996; 68: 1658–1660.
3. Karavadi T and Challa BR. Method development and validation of darifenacin in rat plasma by liquid chromatography–tandem mass spectrometry: Application to a pharmacokinetic study. ISRN Spectroscopy 2012; doi:10.5402/2012/659257.
4. US DHHS, FDA and CDER. Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research and Center for Veterinary Medicine, 2001. Available at: http://www/ fda.gov/cder/guidance/index.htm.
5. Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J, Shah VP, Skelly JP, Swann PG and Weiner R. Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. Pharml Res 2007; 24: 1962–1973.

6. De Boer T and Wieling J. Incurred sample accuracy assessment: design of experiments based on standard addition. Bioanalysis 2011; 3: 983–992.
7. Macek J, Ptáček P, Klíma J. Determination of solifenacin in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B 2010; 878: 3327-3330.
8. Karra VK, Pilli NR, Inamadugu JK, and Rao JVLNS. Simultaneous determination of pioglitazone and candesartan in human plasma by LC–MS/MS and its application to a human pharmacokinetic study. J Pharm Anal 2012; 2: 167–173.