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Development and validation of a sensitive LC-MS/MS method for quantification of darifenacin in human plasma: application to pharmacokinetic and bioequivalence studies

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Abstract

Background: Darifenacin is a selective muscarinic M3 receptor antagonist used in the management of overactive bladder syndrome. Accurate quantification of darifenacin in biological matrices is essential for pharmacokinetic studies, bioequivalence assessments, and therapeutic drug monitoring.

Objective: The present study aimed to develop and validate a rapid, sensitive, and robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of darifenacin in human plasma using liquid-liquid extraction.

Methods: Darifenacin and an appropriate internal standard were extracted from human plasma using liquid-liquid extraction technique. Chromatographic separation was achieved using a reverse-phase column with gradient elution. Detection was performed using electrospray ionization in positive ion mode with multiple reaction monitoring (MRM). The method was validated according to US FDA and ICH guidelines for bioanalytical method validation, encompassing selectivity, linearity, accuracy, precision, recovery, matrix effect, and stability parameters.

Results: The validated method demonstrated excellent linearity over the concentration range with correlation coefficient (r^2) > 0.99. The lower limit of quantification (LLOQ) was adequate for pharmacokinetic studies. Intra-day and inter-day precision (% RSD) were within acceptable limits (< 15%). Accuracy ranged between 85-115%

at HQC, MQC, LQC quality control levels and 80-120 % for LOQQC. Recovery was consistent and reproducible. The analyte remained stable under various storage and processing conditions including freeze-thaw cycles, bench-top stability, autosampler stability, and long-term storage.

Conclusion: A simple, sensitive, and validated LC-MS/MS method has been successfully developed for the quantification of darifenacin in human plasma. The method is suitable for application in pharmacokinetic and bioequivalence studies, meeting all regulatory requirements for bioanalytical method validation.

Keywords: Darifenacin, LC-MS/MS, Method validation, Pharmacokinetics, Bioequivalence, Human plasma, Liquid-liquid extraction, Bioanalytical method.

1. INTRODUCTION

Overactive bladder (OAB) is a highly prevalent urological condition characterized by urinary urgency, with or without urge incontinence, usually accompanied by frequency and nocturia. The condition significantly impacts the quality of life of affected individuals, with an estimated prevalence of 12-17% in adults worldwide. Pharmacological intervention remains the primary therapeutic approach for managing OAB symptoms.

Darifenacin is a competitive muscarinic receptor antagonist with selectivity for the M3 receptor subtype. The M3 receptors are predominantly responsible for bladder smooth muscle contraction, making darifenacin an effective therapeutic agent for OAB. Unlike non-selective antimuscarinics, darifenacin's selectivity for M3 receptors over M2 receptors potentially reduces cardiovascular side effects, while its lower affinity for M1 receptors may minimize cognitive impairment and dry mouth.

The pharmacokinetic profile of darifenacin is characterized by extensive first-pass metabolism, primarily through cytochrome P450 enzymes CYP3A4 and CYP2D6. The drug exhibits moderate bioavailability (approximately 15-19%) and a terminal half-life of 13-19 hours, supporting once-daily dosing. Peak plasma concentrations are typically achieved within 6-8 hours following oral administration.

Accurate and reliable quantification of darifenacin in biological matrices is crucial for several purposes: (1) pharmacokinetic studies to establish absorption, distribution, metabolism, and elimination parameters; (2) bioequivalence studies to compare generic formulations with innovator products; (3) therapeutic drug monitoring to optimize individual dosing regimens; and (4) drug interaction studies to evaluate the impact of co-administered medications.

Various analytical techniques have been employed for the determination of darifenacin in biological samples, including high-performance liquid chromatography (HPLC) with UV or fluorescence detection, and liquid chromatography-mass spectrometry (LC-MS). However, LC-MS/MS has emerged as the method of choice due to its superior sensitivity, selectivity, and capability for high-throughput analysis. The technique combines the separation power of liquid chromatography with the detection specificity of tandem mass spectrometry, enabling the quantification of analytes at trace concentrations in complex biological matrices.

Bioanalytical Method validation is a critical component of bioanalytical research, ensuring that the analytical procedure is suitable for its intended purpose and generates reliable, reproducible data. Regulatory agencies, including the US Food and Drug Administration (USFDA), EMA, ANVISA and other relevant regulatory agencies have established comprehensive guidelines for bioanalytical method validation, specifying requirements for selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, and stability.

The present study describes the development and comprehensive validation of a rapid, sensitive, and robust LC-MS/MS method for the quantification of darifenacin in human plasma. The method employs liquid-liquid extraction for sample preparation and electrospray ionization with multiple reaction monitoring for detection. Complete validation was performed according to current regulatory guidelines, encompassing all critical parameters. The validated method is applicable to pharmacokinetic and bioequivalence studies of darifenacin.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Working standards of darifenacin hydrobromide and an appropriate darifenacin D-4 hydrobromide internal standard (ISTD) were procured from certified suppliers and stored according to manufacturer recommendations. HPLC-grade solvents including acetonitrile, methanol, and water were obtained from commercial sources. GR and HPLC grade formic acid and tertiary methyl butyl ether respectively were used. 0.1% formic acid and acetonitrile were used for mobile phase preparation. All reagents used for sample extraction were of analytical grade or higher.

Drug-free human plasma containing K2EDTA as anticoagulant was collected from designated blood centres. The plasma was stored at -20°C until use and screened for absence of interfering peaks before validation experiments.

2.2 Instrumentation

The LC-MS/MS system consisted of a high-performance liquid chromatography system (Shimadzu) coupled to a tandem mass spectrometer (AB Sciex triple quad 4000 mass detector) equipped with electrospray ionization (ESI) source. The HPLC system included a quaternary pump for gradient delivery, online degasser, autosampler with temperature control, and column oven. Data acquisition and processing were performed using dedicated Analyst software.

Chromatographic separation was achieved on a reverse-phase C18 column with appropriate dimensions and particle size, maintained at controlled temperature. Additional laboratory equipment included analytical balance, pH meter, vortex mixer, refrigerated centrifuge, and ultra-low temperature freezer for sample storage.

2.3 Chromatographic and Mass Spectrometric Conditions

Mobile phase composition was optimized to achieve adequate peak shape, retention time, and resolution. Gradient elution was employed with mobile phase (0.1% formic acid and Acetonitrile). The flow rate was maintained at an optimized value to balance analysis time and peak resolution. The column temperature was controlled to ensure reproducible retention times.

Mass spectrometric detection was performed in positive electrospray ionization mode using multiple reaction monitoring (MRM). Source parameters including ion spray voltage, temperature, gas flows, and focusing parameters were optimized for maximum sensitivity. The MRM transitions for darifenacin and internal standard were selected based on their respective fragmentation patterns. Collision energy and other compound-specific parameters were optimized to maximize signal intensity while maintaining specificity.

2.4 Preparation of Standard Stock Solutions and Calibration Standards

Primary stock solutions of darifenacin and internal standard were prepared by accurately weighing the working standards and dissolving in methanol and diluent (Water and Acetonitrile) respectively to achieve the target concentration. The stock solutions were stored at 2-8°C and were stable for the duration of the study.

Working solutions were prepared by serial dilution of the stock solutions in diluent. Calibration standards were prepared by spiking appropriate volumes of working solutions into blank human plasma to achieve the desired concentration range from 51.077-20035.860 pg/ml. The calibration curve typically consisted of 9 non-zero standards plus a blank sample (matrix processed without internal standard) and a zero sample (matrix processed with internal standard).

Quality control (QC) samples were prepared independently from calibration standards at four levels: lower limit of quantification quality control (LLOQ QC), low quality control (LQC), medium quality control (MQC), and high-quality control (HQC). These samples were aliquoted and stored at -70°C for use throughout the validation.

2.5 Sample Preparation

Plasma samples were processed using liquid-liquid extraction (LLE) technique, which was optimized to achieve high recovery and minimal matrix effects. The extraction procedure briefed below.

Retrieved the CC standards, QC samples and subject samples from the deep freezer and thaw in water bath maintained at room temperature. Vortexed to mix. Added 50 µL of ISTD dilution (about 75.000 ng/mL) into pre-labelled polypropylene tubes, except in STD blanks. Added 500 µL of CC standards, QC samples and subject samples into pre-labelled polypropylene tube containing ISTD dilution and vortexed to mix. Added 2.500 mL of extraction solvent (Tertiary methyl butyl ether) into the tubes and vortexed using vortex mixer for 10 minutes.

Centrifuged the samples at 4500 rpm and 5°C for 5 minutes. Transferred 2.000 mL of supernatant into pre-labelled polypropylene tubes. Evaporated the samples under gentle stream of nitrogen gas at 50°C till dryness. Reconstituted the dried residue with 0.300 mL of Mobile phase, vortexed and transferred to appropriate pre-labelled auto sampler vials. Noted the time of placement of samples in the auto sampler. The stock weightings, stock solution preparation, spiking solution preparation, spiking in plasma and sample processing has been done under yellow monochromatic light.

2.6 Method Validation

The developed method was validated according to US FDA guidance for industry on bioanalytical method validation and other relevant regulatory

guidelines. The following validation parameters were evaluated:

2.6.1 Selectivity and Specificity

Selectivity was assessed by analyzing blank plasma samples from at least six different sources and two lipemic and two haemolytic to evaluate potential interference from endogenous compounds at the retention times of darifenacin and internal standard. The interference response at Analyte and ISTD RT of LLOQ should not exceed 20% of the analyte response and 5% of the internal standard response.

Specificity was further evaluated by analyzing plasma samples containing common concomitant medications to ensure no interference with analyte quantification.

2.6.2 Linearity and Calibration Curve

Linearity was evaluated by analyzing calibration standards over the intended concentration range. The calibration curve was constructed by plotting the peak area ratio of analyte to internal standard against the nominal concentration of the calibration standards.

Linear regression with $1/x^2$ weighting was typically employed. The calibration curve was considered acceptable if the correlation coefficient (r^2) was ≥ 0.98 and the back-calculated concentrations were within ±15% of nominal values (±20% at LLOQ). At least 75% of calibration standards and at least six concentration levels must meet these criteria.

2.6.3 Lower Limit of Quantification (LLOQ)

The LLOQ was defined as the lowest concentration on the calibration curve that could be quantified with acceptable precision (≤20% CV) and accuracy (80-120% of nominal concentration). The LLOQ was validated by analyzing at least five replicates of the LLOQ samples.

2.6.4 Accuracy and Precision

Intra-day (within-run) accuracy and precision were assessed by analyzing six replicates of QC samples at four concentration levels (LOQ QC, LQC, MQC, and HQC) within a single analytical run.

Inter-day (between-run) accuracy and precision were evaluated by analyzing QC samples at the same four levels on at least three different days.

Accuracy was expressed as percent bias (% bias) and should be within ±15% of nominal concentration for all QC samples (±20% at LOQQC). Precision was expressed as percent relative standard deviation (% CV) and should not exceed 15% for all QC samples (20% at LOQQC).

2.6.5 Recovery and Matrix Effect

Extraction recovery was determined by comparing the peak areas of extracted QC samples at three concentration levels (LQC, MQC, HQC) with those of post-extraction spiked samples representing 100% recovery. Recovery should be consistent, precise, and reproducible across the concentration range, although it need not be 100%. Dilution factor was used for calculating the recovery.

Matrix effect was evaluated by comparing the peak areas of post-extraction spiked samples with those of extracted QC samples at equivalent concentrations. The matrix effect should be consistent and reproducible across different lots of plasma. Both suppression and enhancement of ionization were evaluated.

2.6.6 Stability Studies

Comprehensive stability studies were conducted to ensure analyte stability throughout sample collection, processing, storage, and analysis. The following stability parameters were evaluated using QC samples at two concentration levels (LQC and HQC):

Stock Solution Stability:

Short-term and long-term stability for drug and ISTD was established by keeping drug and ISTD stability aliquots on bench at room temperature for intended time/duration and compared peak areas of stability stock aliquots and comparison stock aliquots and calculated % Accuracy and the % accuracy should be between 90-110 %.

Bench-top (BT) Stability: BT stability was established by keeping unprocessed HQC and LQC samples kept at room temperature for intended duration and compared with freshly spiked LQC and HQC samples and nominal concentrations used for calculating % Accuracy.

Freeze-Thaw (FT) Stability: FT stability was established by subjecting unprocessed HQC and LQC samples to multiple freeze-thaw cycles simulating anticipated freeze and thawing for 5 cycles and compared with freshly spiked LQC and HQC samples and nominal concentrations used for calculating % Accuracy.

Long-term (LT) Stability: FT stability was established by storing HQC and LQC samples at -70±20°C for extended periods covering the anticipated study duration and compared with freshly spiked LQC and HQC samples and nominal concentrations used for calculating % Accuracy.

Post-preparative Stability (Autosampler Stability-AS): Autosampler stability was established by keeping processed HQC and LQC samples in the autosampler at the set temperature and compared with freshly spiked LQC and HQC samples and nominal concentrations used for calculating % Accuracy.

Dry Extract Stability: Autosampler stability was established by storing dried extracts of HQC and LQC samples at 2-8°C before reconstitution and compared with freshly spiked LQC and HQC samples and nominal concentrations used for calculating % Accuracy.

Whole Blood Stability: Whole Blood Stability was established by unprocessed HQC and LQC level samples spiked with whole blood and placed on bench at room temperature for intended period and compared with freshly spiked LQC and HQC samples and nominal concentrations used for calculating % Accuracy.
HQC and LQC Samples were considered stable and above-mentioned experiments were considered acceptable if the mean concentration was within ±15% of the nominal concentration.

2.6.7 Additional Validation Parameters

Carryover: Evaluated by injecting blank samples immediately after the upper limit of quantification (ULOQ) standards. Carryover in the blank sample should not exceed 20% of LLOQ response for analyte and 5% for internal standard.

Dilution Integrity: Assessed by diluting samples with concentrations above ULOQ (about 1.5 times of ULOQ) with blank matrix and analyzing the diluted samples. The dilution factor should not affect accuracy and precision.

Reinjection Reproducibility: Evaluated by reinjecting a previously analysed run to ensure result reproducibility.

Extended P&A: Extended P&A was established by 30 sets of four level QCs anticipating long run to be used in the subject sample analysis. Calculated % accuracy and % CV for accepting the batch. 67% total and 50% at each level criteria by meting accuracy within ±15% for all QC levels and for LOQQC ±20 %. %CV less than 15% for all QCs and Less than 20% for LOQQC.

Ruggedness: Assessed by having different analysts perform the method using different instruments and reagent lots to demonstrate method robustness.

3. RESULTS AND DISCUSSION

3.1 Method Development and Optimization

The development of the LC-MS/MS method involved systematic optimization of multiple parameters to achieve optimal sensitivity, selectivity, and chromatographic performance. Mass spectrometric parameters were optimized by infusing standard solutions of darifenacin and internal standard directly into the mass spectrometer. The protonated molecular ions [M+H]+ were identified, and product ion scans were performed to select the most abundant and specific fragment ions for MRM transitions.

Chromatographic conditions were optimized to achieve adequate retention, peak shape, and resolution while maintaining reasonable analysis time. Several columns and mobile phase compositions were evaluated. The selected reverse-phase C18 column provided optimal retention and peak symmetry for both analyte and internal standard. The gradient program was designed to achieve efficient separation from matrix components while maintaining acceptable peak shape.

The liquid-liquid extraction procedure was optimized by evaluating different organic solvents and pH conditions. The selected extraction solvent provided high and consistent recovery with minimal matrix interference. The extraction procedure effectively removed endogenous plasma components while efficiently extracting darifenacin and internal standard.

3.2 Selectivity and Specificity

The method demonstrated excellent selectivity with no significant interference from endogenous plasma components at the retention times of darifenacin and internal standard. Analysis of blank plasma from six different sources and Lipemic and Haemolytic plasma showed zero interference (< 20% of LLOQ response for analyte and < 5% for internal standard), meeting regulatory acceptance criteria.

Additionally, commonly co-administered medications were evaluated for potential interference. No significant interference was observed, demonstrating the method's specificity and suitability for use in BA/BE studies where subjects may be receiving concomitant medications.

3.3 Linearity and Calibration Curve

The calibration curve demonstrated excellent linearity over the validated concentration range from 51.077-20035.860 pg/ml. Linear regression with $1/x^2$ weighting provided the best fit, with correlation coefficients (r^2) consistently exceeding 0.98 across all

validation runs.

Back-calculated concentrations of calibration standards were within ±15% of nominal values (±20% at LLOQ) in all runs, meeting acceptance criteria. The selected concentration range was adequate to cover expected plasma concentrations in pharmacokinetic studies following therapeutic doses of darifenacin.

3.4 Sensitivity (LLOQ)

The validated LLOQ was sufficiently sensitive to quantify darifenacin in plasma samples from pharmacokinetic studies. At the LLOQ, precision was ≤ 20% CV and accuracy was within 80-120% of nominal concentration across multiple validation runs. The signal-to-noise ratio at LLOQ was well above the minimum requirement of 5:1, and mean signal-to-noise ratio at LLOQ is 29.62 ensuring reliable quantification at the lowest calibration level.

3.5 Accuracy and Precision

The method demonstrated excellent accuracy and precision at all QC levels. Intra-day accuracy ranged from 98.8-105.5 % across all QC levels, with precision (% CV) ranging from 1.2-4.5 %. Inter-day accuracy ranged from 99.4-105.9%, with precision ranging from 1.9-5.1%.

All values were well within the regulatory acceptance criteria of ±15% for accuracy and ≤15% CV for precision (±20% and ≤20% at LLOQ), demonstrating the method's reliability and reproducibility for routine sample analysis.

3.6 Recovery and Matrix Effect

The extraction recovery of darifenacin was consistent across QC levels, ranging from 89.4-99.0% with precision ≤15% CV. The internal standard recovery was similarly consistent at approximately 82-88%. While recovery was not 100%, it was reproducible and consistent, which is the critical requirement for accurate quantification.

Matrix effect evaluation revealed minimal ion suppression or enhancement (matrix factor between 0.996-0.999) at all QC levels. The internal standard-normalized matrix factor was close to 1.0 with precision < 15%, indicating effective compensation of any matrix effects by the internal standard. The consistency of matrix effects across different plasma lots demonstrated the robustness of the sample preparation method.

3.7 Stability

Comprehensive stability studies demonstrated that darifenacin was stable under all evaluated conditions and all stabilities done under monochromatic light.

Stock Solution Stability: Stock solutions were stable for 58 days at 2-8°C and for 07 hours at room temperature. Bench-top Stability: Plasma samples were stable for 07 hours at room temperature. Freeze-Thaw Stability: Samples withstood for five freeze-thaw cycles without significant degradation. Long-term Stability: Samples were stable for at least 65 days at -70°C, covering typical bioanalytical study durations. Autosampler Stability: Processed samples were stable for at least 44 hours in the autosampler at 10°C. Dry Extract Stability: Dried extracts were stable for at least 38 hours at 2-8°C before reconstitution. Whole Blood Stability: Analyte in whole blood was stable for 03 hours at room temperature before centrifugation.

In all stability experiments, mean concentrations were within ±15% of nominal values, meeting acceptance criteria and ensuring reliable quantification throughout sample handling and analysis procedures.

3.8 Additional Validation Parameters

Carryover: No significant carryover was observed in blank samples injected after ULOQ standards, with response ≤ 20% of LLOQ for analyte and ≤5% for internal standard.

Dilution Integrity: Samples diluted about 1.5 folds with blank plasma showed accuracy between 103.5-109.6% and precision ≤10% CV, validating the dilution procedure for samples exceeding ULOQ.

Reinjection Reproducibility: Reinjection of a complete analytical run showed ≤10% difference from original results, demonstrating stability of processed samples and instrument reproducibility.

Ruggedness: Analysis by different analysts using different instruments demonstrated comparable results, confirming method robustness and transferability.

Extended P&A showed accuracy and %CV results within acceptable limits for all QC levels and 67% and 50% criteria met, % accuracy ranges from 95.3-104.6% and % CV ranges from 1.3 -4.1.

4. APPLICATION TO PHARMACOKINETIC STUDIES

The validated method has been successfully transferred and applied by designated clinical research lab to analyze plasma samples from pharmacokinetic and bioequivalence confirmatory/pilot studies of darifenacin. The

sensitivity, selectivity, and wide linear range make the method suitable for comprehensive pharmacokinetic characterization including determination of Cmax (maximum plasma concentration), Tmax (time to reach Cmax), AUC (area under the concentration-time curve), and terminal half-life.

The methods robustness and reproducibility ensure reliable data generation in BA/BE studies. The comprehensive stability validation supports various sample collection scenarios and allows flexible scheduling of sample analysis without compromising data integrity.

The high throughput capability, with a short run time per sample, enables efficient analysis of large sample sets typical of bioequivalence studies, reducing overall study timelines and costs.

Table 1: System Suitability

Table 1a: System suitability experiment for Darifenacin

Injection No.	Retention time (minutes)		Area ratio
	Analyte - Darifenacin	ISTD - Darifenacin D4	
1	1.373	1.370	2.877
2	1.375	1.370	2.866
3	1.375	1.371	2.873
4	1.375	1.371	2.875
5	1.375	1.370	2.857
6	1.375	1.371	2.928
Mean	1.3747	1.3705	2.8793
SD	0.00082	0.00055	0.02494
%CV	0.1	0.0	0.9

Table 1b: System suitability experiment (Ruggedness batch) for Darifenacin

Injection No.	Retention time (minutes)		Area ratio
	Analyte - Darifenacin	ISTD - Darifenacin D4	
1	1.332	1.328	2.675
2	1.331	1.327	2.686
3	1.334	1.330	2.685
4	1.335	1.331	2.669
5	1.334	1.330	2.686
6	1.333	1.329	2.660
Mean	1.3332	1.3292	2.6768
SD	0.00147	0.00147	0.01080
%CV	0.1	0.1	0.4

Acceptance Criteria

%CV for retention time should be ≤ 2%

%CV for area ratio should be ≤ 5%

Table 2: Matric Effect for Darifenacin

S.No.	Analyte - Darifenacin						
	Response of aqueous samples		Plasma Lot No.	Response of post extracted samples		Matrix factor	
	HQC	LQC		HQC	LQC	HQC	LQC
1	5117008	44603	K-1251	5039100	43225	0.970	0.969
2	5199790	42919	K-1252	5069645	43557	0.976	0.976
3	5140017	44420	K-1253	5151790	43177	0.992	0.968
4	5248466	45291	K-1254	5084379	43069	0.979	0.965
5	5157908	44425	K-1255	5096934	43843	0.982	0.982
6	5158127	44985	K-1256	5091744	43636	0.981	0.978
7	5240941	44611	LLC-1551	5148528	44118	0.991	0.989
8	5281698	45758	LT-2321	5188984	43497	0.999	0.975
9	-	-	HD-213	5157094	43254	0.993	0.969
10	-	-	HK-1157	5187960	44454	0.999	0.996
Mean	5192994.4	44626.5	Mean	5121615.8	43583.0		

S.No.	ISTD - Darifenacin D4						
	Response of aqueous samples		Plasma Lot No.	Response of post extracted samples		Matrix factor	
	HQC	LQC		HQC	LQC	HQC	LQC
1	1979862	2256942	K-1251	2015385	2070447	0.995	0.927
2	2030203	2203935	K-1252	2075724	2096807	1.024	0.939
3	2017759	2218617	K-1253	2037341	2242011	1.005	1.004
4	2055980	2227187	K-1254	2110605	2095561	1.042	0.939
5	2022910	2240121	K-1255	2598395	2166831	1.282	0.971
6	2017003	2248177	K-1256	2280858	2577674	1.126	1.155
7	2033786	2219352	LLC-1551	2217025	2149215	1.094	0.963
8	2054486	2245311	LT-2321	2298030	2151314	1.134	0.964
9	-	-	HD-213	2267125	2177995	1.119	0.976
10	-	-	HK-1157	2226134	2179642	1.099	0.976
Mean	2026498.6	2232455.3	Mean	2212662.2	2190749.7		

Plasma Lot No.	Matrix factor-Analyte-HQC	Matrix factor-ISTD-HQC	ISTD Normalised Matrix factor-HQC	Matrix factor-Analyte-LQC	Matrix factor-ISTD-LQC	ISTD Normalised Matrix Factor-LQC
K-1251	0.970	0.995	0.975	0.969	0.927	1.045
K-1252	0.976	1.024	0.953	0.976	0.939	1.039
K-1253	0.992	1.005	0.987	0.968	1.004	0.964
K-1254	0.979	1.042	0.940	0.965	0.939	1.028
K-1255	0.982	1.282	0.766	0.982	0.971	1.011
K-1256	0.979	1.126	0.869	0.965	1.155	0.835
LLC-1551	0.991	1.094	0.906	0.989	0.963	1.027
LT-2321	0.999	1.134	0.881	0.975	0.964	1.011
HD-213	0.993	1.119	0.887	0.969	0.976	0.993
HK-1157	0.999	1.099	0.909	0.996	0.976	1.020
Mean			0.9073	Mean		0.9973
SD			0.06380	SD		0.06164
% CV			7.0	% CV		6.2

$$\textbf{Matrix factor} = \frac{\text{Response of post extracted sample}}{\text{Mean response of aqueous spiked samples}}$$

ISTD Normalized Matrix Factor = Matrix factor of Analyte / Matrix factor of ISTD

Acceptance Criteria

The %CV for ISTD normalized factor at both HQC and LQC levels should not be greater than **15%**.

Table 3: Sensitivity
Table 3a: Sensitivity of the method for Darifenacin

Analysed with P&A 01		
LLOQ		
Nominal concentration (pg/mL)		51.077
Maximum limit (pg/mL)		61.292
Minimum limit (pg/mL)		40.862
Batch ID	S.No.	Back calculated concentration (pg/mL)
P&A 01	1	50.434
	2	49.213
	3	52.445
	4	48.525
	5	53.693
	6	48.038
	Mean	50.3913
	SD	2.25966
	% CV	4.5
	% Accuracy	98.7
	% Bias	-1.3

Acceptance Criteria

The Limit of Quantification is acceptable if mean of 6 determinations is within ±20% of the nominal concentration and precision (CV) is ≤ 20%.

Table 3b: Signal of Noise Ratio for Darifenacin

Sample name	Signal to Noise Ratio
Sensitivity LOQ - 001	23.6
Sensitivity LOQ - 002	30.0
Sensitivity LOQ - 003	27.9
Sensitivity LOQ - 004	21.4
Sensitivity LOQ - 005	30.3
Sensitivity LOQ - 006	44.5
Mean	29.62

Acceptance Criteria
Signal to noise ratio should be ≥ 5.

Table 4: Intra and Inter day precision and accuracy of quality control samples of Darifenacin in human plasma

Quality control samples		HQC	MQC	DQC	LQC	LOQ QC
Nominal concentration (pg/mL)		16600.651	9960.390	30053.790	135.461	51.204
Maximum limit (pg/mL)		19090.749	11454.449	34561.859	155.780	61.445
Minimum limit (pg/mL)		14110.553	8466.332	25545.722	115.142	40.963
Batch ID	S.No.	Back calculated concentrations (pg/mL)				
P&A I (Intra day)	001	16700.738	10405.432	30558.213	139.198	51.530
	002	17929.035	10205.487	30975.311	143.350	50.903
	003	16772.125	10431.914	33099.347	144.530	50.631
	004	17281.741	10407.998	32774.823	144.735	49.311
	005	17533.336	10442.701	31787.521	141.898	51.855
	006	17546.668	10601.801	29347.525	143.735	49.337
	Mean	17293.9405	10415.8888	31423.7900	142.9077	50.5945
	SD	479.34453	126.47496	1416.07309	2.08001	1.07603
	% CV	2.8	1.2	4.5	1.5	2.1
	% Accuracy	104.2	104.6	104.6	105.5	98.8
	% Bias	4.2	4.6	4.6	5.5	-1.2
P&A III	007	16275.088	10328.583	30092.359	137.943	48.815
	008	17798.558	10135.650	31765.078	139.102	50.033
	009	17287.930	10259.803	34450.732	141.334	48.611
	010	17500.844	9948.907	33172.171	140.120	49.119
	011	17057.082	10388.551	33825.096	142.559	50.989
	012	16860.162	10028.597	34338.807	135.263	53.265
	Mean	17129.9440	10181.6818	32940.7072	139.3868	50.1387
	SD	532.62389	173.32959	1706.79632	2.59088	1.76826
	% CV	3.1	1.7	5.2	1.9	3.5
	% Accuracy	103.2	102.2	109.6	102.9	97.9
	% Bias	3.2	2.2	9.6	2.9	-2.1
P&A IV (Different column with different analyst)	013	16172.987	10390.316	29981.955	139.712	51.056
	014	17683.230	10046.060	31765.629	142.272	52.234
	015	17339.793	10192.992	30142.596	141.084	50.256
	016	17393.554	9895.857	31571.376	139.843	52.648
	017	17125.152	10230.587	29947.886	142.867	53.239
	018	16735.008	9992.463	33262.416	136.855	52.134
	Mean	17074.9540	10124.7125	31111.9763	140.4388	51.9278
	SD	542.82095	180.25965	1329.24440	2.16333	1.08896
	% CV	3.2	1.8	4.3	1.5	2.1
	% Accuracy	102.9	101.6	103.5	103.7	101.4
	% Bias	2.9	1.6	3.5	3.7	1.4
Global Statistics (Inter day)	Mean	17166.2795	10240.7611	31825.4912	140.9111	50.8870
	SD	496.83217	199.76857	1625.37924	2.63229	1.48968
	% CV	2.9	2.0	5.1	1.9	2.9
	% Accuracy	103.4	102.8	105.9	104.0	99.4
	% Bias	3.4	2.8	5.9	4.0	-0.6

Note: CC02 was rejected, due to standard zero's were not within the acceptance criteria.

Acceptance Criteria

The back calculated values of at least 67% (20 out of 30) of total QC samples and 50% (3 out of 6) at each QC level (HQC, MQC, DQC, LQC & LOQ QC) should be within ±15% of the nominal concentration, except LOQ QC where the back calculated value should be within ±20% of the nominal concentration.

The intra and inter day batch mean concentration should be within ±15% of the nominal value at low, dilution, medium and high QC concentrations and should not deviate by more than ±20% at the LOQ QC concentration.

The intra and inter day batch precision (%CV) for low, dilution, middle and high QC concentrations should be ≤15% and for LOQ QC should be ≤20%.

Table 5: Observed recovery

Table 5a: Observed recovery of Darifenacin in human plasma

Analysed with P&A 03		
	Post extracted area	Extracted area
HQC	5600271	3902788
	5527857	4045929
	5575558	4069008
	5653110	4063943
	5721391	4032790
	5756553	4075020
Mean	5639123.3	4031579.7
SD	87958.60	65007.68
% CV	1.6	1.6
% Recovery	89.4	
MQC	2897611	2429528
	2871677	2443549
	2921687	2481956
	3226607	2342457
	3255524	2454954
	3307355	2364413
Mean	3080076.8	2419476.2
SD	202839.73	54418.47
% CV	6.6	2.2
% Recovery	98.2	
LQC	41402	35931
	41588	34897
	41997	34251
	44150	33233
	44503	34321
	45672	32766
Mean	43218.7	34233.2
SD	1788.16	1138.79
% CV	4.1	3.3
% Recovery	99.0	
%Global recovery	95.5	

$$\textbf{\% Recovery} = \frac{\text{Mean Peak area of extracted sample} \times \text{Total volume of extraction solvent} \times 100}{\text{Mean Peak area of Post extracted sample} \quad \text{Actual volume transferred for estimation}}$$

Dilution factor: 2.5 / 2.0 = 1.25
Total volume of extraction solvent: 2.5 mL
Actual volume transferred for estimation: 2.0 mL

Acceptance Criteria

The recovery for Analyte(s) is deemed acceptable if %CV is ≤ 15% for individual low, middle and high QC peak areas, individual recovery (L, M & H) and Global recovery should not be more than 115%.

Table 5b: Observed recovery of Darifenacin D4 (ISTD) in human plasma

Analysed with P&A 03		
	Post extracted area	Extracted area
HQC	2218035	1721934
	2176562	1632285
	2209935	1690086
	2207656	1667445
	2194612	1697714
	2211848	1735529
MQC	2235488	1689123
	2228659	1731212
	2181436	1737143
	2217324	1690744
	2219030	1696947
	2255460	1693030
LQC	2229410	1883852
	2281706	1814286
	2260391	1752348
	2280270	1715121
	2224803	1740726
	2231410	1752198
Mean	2225779.7	1724540.2
SD	29340.80	56089.65
% CV	1.3	3.3
% Recovery	96.9	

$$\% \text{ Recovery} = \frac{\text{Mean Peak area of extracted sample x Total volume of extraction solvent} \times 100}{\text{Mean Peak area of Post extracted sample} \quad \text{Actual volume transferred for estimation}}$$

Dilution factor: 2.5 / 2.0 = 1.25
Total volume of extraction solvent: 2.5 mL
Actual volume transferred for estimation: 2.0 mL

Acceptance Criteria
Global recovery should not be more than 115% and %CV should be ≤15%.

Table 6: Intermediate term stability, Bench top stability, Autosampler stability, Post extract stability, Freeze thaw stability and Calibration curve standard stability

Table 6a: Stability experiment results of Darifenacin
Analysed with freshly spiked CC

Quality control samples	Freshly Spiked		Intermediate term 03 days 05 hours -20 ± 10° C		Bench top 09 hours 04 minutes 24 ± 4° C		Autosampler 44 hours 44 minutes 10 ± 1°C	
	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC
Nominal concentration (pg/mL)	16589.914	135.374	16600.651	135.461	16600.651	135.461	16600.651	135.461
Maximum limit (pg/mL)	19078.401	155.680	19090.749	155.780	19090.749	155.780	19090.749	155.780
Minimum limit (pg/mL)	14101.427	115.068	14110.553	115.142	14110.553	115.142	14110.553	115.142
S.No.	Back calculated concentrations (pg/mL)							
1	17401.929	138.160	17109.356	139.594	17405.050	141.307	17241.235	138.591
2	17284.966	133.498	16930.910	138.323	17368.651	136.195	17135.131	136.973
3	17051.970	141.148	17210.606	138.494	17520.980	122.704	16796.306	134.906
4	17519.158	139.107	17222.095	136.620	16937.974	141.148	17187.344	141.196
5	17207.808	135.641	16626.737	142.568	17325.124	137.405	16992.298	140.011
6	17208.948	137.515	17197.289	138.546	17047.862	134.120	16963.161	135.532
Mean	17279.1298	137.5115	17049.4988	139.0242	17267.6068	135.4798	17052.5792	137.8682
SD	163.91528	2.67572	234.07719	1.98317	225.18984	6.86109	166.05587	2.49888
% CV	0.9	1.9	1.4	1.4	1.3	5.1	1.0	1.8
% Accuracy	104.2	101.6	102.7	102.6	104.0	100.0	102.7	101.8
% Bias	4.2	1.6	2.7	2.6	4.0	0.0	2.7	1.8

Quality control samples	Freshly Spiked		Post extract 06 hours 30 minutes 24 ± 4°C		Dry extract 38 hours 52 minutes 2 - 8°C		Freeze thaw 5 cycles -70 ± 20°C	
	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC
Nominal concentration (pg/mL)	16589.914	135.374	16600.651	135.461	16600.651	135.461	16600.651	135.461
Maximum limit (pg/mL)	19078.401	155.680	19090.749	155.780	19090.749	155.780	19090.749	155.780
Minimum limit (pg/mL)	14101.427	115.068	14110.553	115.142	14110.553	115.142	14110.553	115.142
S.No.	Back calculated concentrations (pg/mL)							
1	17401.929	138.160	17268.772	140.027	17119.430	140.829	17194.377	141.365
2	17284.966	133.498	17364.040	137.978	17096.906	136.566	17316.865	133.581
3	17051.970	141.148	17508.874	126.170	17263.736	142.605	17488.965	137.253
4	17519.158	139.107	17043.813	140.045	16975.134	141.106	17152.815	140.145
5	17207.808	135.641	17295.347	137.279	17276.035	140.425	17328.898	135.427
6	17208.948	137.515	17179.375	135.143	16715.055	138.760	16764.268	142.275
Mean	17279.1298	137.5115	17276.7035	136.1070	17074.3827	140.0485	17207.6980	138.3410
SD	163.91528	2.67572	158.52578	5.20426	208.81647	2.10680	247.19942	3.47010
% CV	0.9	1.9	0.9	3.8	1.2	1.5	1.4	2.5
% Accuracy	104.2	101.6	104.1	100.5	102.9	103.4	103.7	102.1
% Bias	4.2	1.6	4.1	0.5	2.9	3.4	3.7	2.1

$$\textbf{\% Accuracy} = \frac{\text{Mean concentration} \times 100}{\text{Nominal concentration}}$$

% Bias = % Accuracy − 100

Acceptance Criteria

The percent accuracy of the Analyte(s) should be within **85–115%** and precision (CV) should be **≤15%** at HQC and LQC levels.

Table 7: Whole blood stability experiment results

(At ambient temperature 24 ± 4°C)

Analysed with freshly spiked CC and QC

Quality control samples	Freshly Spiked		03 hours 15 minutes	
	HQC	LQC	HQC	LQC
Nominal concentration (pg/mL)	16600.651	135.461	16600.651	135.461
Maximum limit (pg/mL)	19090.749	155.780	19090.749	155.780
Minimum limit (pg/mL)	14110.553	115.142	14110.553	115.142
S.No.	Back calculated concentrations (pg/mL)			
1	16291.082	137.347	16150.842	135.971
2	17721.257	136.369	17766.146	139.232
3	17555.225	141.616	17367.673	142.514
4	17344.424	140.137	17498.559	138.959
5	17107.885	135.970	17118.937	142.007
6	16766.948	138.142	16157.101	133.600
Mean	17131.1368	138.2635	17009.8763	138.7138
SD	531.49794	2.21148	695.10186	3.44352
% CV	3.1	1.6	4.1	2.5
% Accuracy	103.2	102.1	102.5	102.4
% Bias	3.2	2.1	2.5	2.4

Acceptance Criteria

The percent accuracy of the analyte(s) should be within **85–115%** and precision (CV) should be **≤15%** at HQC and LQC levels.

Table 8: Precision and accuracy of quality control samples of Darifenacin in human plasma for reinjection reproducibility

Quality control samples		HQC	MQC	DQC	LQC	LOQ QC
Nominal concentration (pg/mL)		16600.651	9960.390	30053.790	135.461	51.204
Maximum limit (pg/mL)		19090.749	11454.449	34561.859	155.780	61.445
Minimum limit (pg/mL)		14110.553	8466.332	25545.722	115.142	40.963
Batch ID	**S.No.**	**Back calculated concentrations (pg/mL)**				
	001	16163.790	10393.079	30471.699	142.139	51.002
	002	17787.203	10078.055	31948.575	142.077	53.301
	003	17632.897	10369.661	33889.194	146.368	51.511
	004	17526.645	10081.663	34491.511	141.283	51.662
P&A1	005	17083.088	10364.207	30080.638	143.859	53.577
	006	16941.711	10125.845	32114.851	137.482	53.243
	Mean	17189.2223	10235.4183	32166.0780	142.2013	52.3827
	SD	598.71863	154.83846	1768.99290	2.94088	1.11313
	% CV	3.5	1.5	5.5	2.1	2.1
	% Accuracy	103.5	102.8	107.0	105.0	102.3
	% Bias	3.5	2.8	7.0	5.0	2.3

Acceptance Criteria

Accuracy of the analyte(s) concentration should be within ±15% of the nominal value at low, diluted, medium, and high QC concentrations and should not deviate by more than ±20% at the LLOQ QC concentration.

Batch precision (%CV) for low, diluted, middle, and high QC concentrations should be ≤ 15%, and for LLOQ QC should be ≤ 20%.

DISCUSSIONS

5. CONCLUSION

A simple, rapid, sensitive, and specific LC-MS/MS method has been successfully developed and validated for the quantification of darifenacin in human plasma. The method employs liquid-liquid extraction for sample preparation and achieves excellent chromatographic separation with short analysis time.

Comprehensive validation according to current regulatory guidelines demonstrated that the method meets all acceptance criteria for selectivity, linearity, accuracy, precision, recovery, matrix effect, and stability. The LLOQ is adequate for pharmacokinetic studies, and the linear range covers expected plasma concentrations following therapeutic doses.

The validated method is suitable for application in pharmacokinetic studies, bioequivalence assessments, and therapeutic drug monitoring of darifenacin. The method's high throughput capability, robustness, and reproducibility make it an ideal tool for routine bioanalytical support of clinical studies.

This work contributes to the growing body of validated bioanalytical methods for antimuscarinic agents and provides a reliable platform for future research on darifenacin pharmacokinetics and clinical applications.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this work.

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