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Validated liquid chromatographic–fluorescence method for the quantitation of darifenacin in mice plasma and its application to a pharmacokinetic study

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ABSTRACT

A highly selective, sensitive, and rapid high-performance liquid chromatography (HPLC) method has been developed and validated for the quantification of darifenacin in mouse plasma. Bisoprolol was used as an internal standard (IS). Darifenacin and the IS were extracted using the deproteinisation technique, followed by injection of an aliquot of the supernatant into the chromatographic system. The chromatographic separation was achieved on a reversed phase C18 column with a mobile phase of acetonitrile: 0.1% diethyl amine (pH 3.5) (60:40, v/v) pumped at a flow rate of 1.0 mL min^{-1}. The analytes were detected at 210 and 314 nm for excitation and emission, respectively. The assay exhibited a linear range of 100–3000 ng mL^{-1}, with a lower detection limit of 35 ng mL^{-1}. The method was statistically validated for linearity, accuracy, precision, selectivity and stability according to the FDA guidelines. The intra- and inter-assay coefficients of variation did not exceed 13.5% from the nominal concentration. The accuracy for darifenacin was within ±15% of the theoretical value. The assay was successfully applied in a pharmacokinetic study.

1. Introduction

Darifenacin hydrobromide, which is chemically (*S*)-2-{1-[2-(2,3-dihydrobenzofuran-5-yl) ethyl]-3-pyrrolidinyl}-2,2-diphenyl acetamide hydrobromide (Fig. 1) [1], is a potent muscarinic M3 receptor antagonist [2,3] that is used to treat symptoms of over-active bladder, such as frequent or urgent urination, and incontinence (urine leakage). The recommended starting dose of darifenacin extended-release tablets is 7.5 mg once daily. On the basis of individual's response, the dose may be increased to 15 mg once daily, as early as two weeks after starting therapy. Darifenacin is approximately 98% bound to plasma proteins and is metabolised by the liver. Metabolism is mediated by cytochrome P450 enzymes CYP2D6 and CYP3A4. Elimination is 60% by urine and 40% through faeces. The elimination half-life of darifenacin is approximately 13–19 h [4–6].

A few analytical methods have been published for the quantification of darifenacin, using spectrophotometry [7,8], spectrofluorimetry [9], high-performance thin-layer chromatography (HPTLC) [10], high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [11–13], and liquid chromatography–mass spectrometry (LC–MS) [14–16].

A stability-indicating HPLC method and the isolation of process-related impurities and degradation products of darifenacin have been described in the literature in recent years [11,13,16]. The method reported for the determination of the enantiomers of darifenacin is mainly based on chiral HPLC, with the use of a Chiralpak-IC column [12]. However, the chromatographic methods reported for the determination of darifenacin in human plasma have been primarily LC–MS methods [14–15]. Although LC–MS offers excellent selectivity and sensitivity and faster analysis time than HPLC methods, it requires relatively expensive instrumentation and highly skilled technical expertise, either of which may not be readily available and/or affordable for most laboratories in resource-limited settings. In such settings, selective and sensitive HPLC methods are preferable to more expensive LC–MS techniques. The use of fluorescence detectors has allowed increased selectivity and sensitivity for the determination of the concentrations of darifenacin.

To the best of our knowledge, the combination of the validation parameters of: linearity, precision, accuracy, recovery and stability have not been reported in a single darifenacin HPLC–fluorescence

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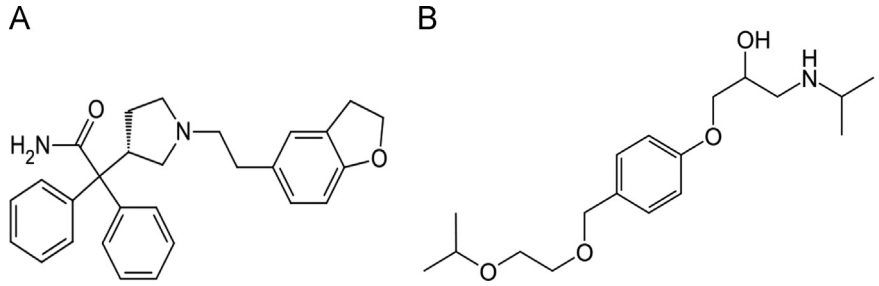

Fig. 1. Chemical structure of darifenacin (a) and bisoprolol (b) (IS).

(FL) method with pharmacokinetic applications. Therefore, we developed a simple, rapid, sensitive and selective HPLC–FL method for the quantification of darifenacin in mouse plasma. The plasma sample containing the drug and IS was deproteinised with acetonitrile and an aliquot of the supernatant was injected into the analytical column without any further cleanup. The drug and internal standard were detected at 210 and 314 nm for excitation and emission, respectively. The lower limit of quantitation was 100.0 ng mL^{-1} using 50 μL of mouse plasma, with a lower limit of detection of 35.0 ng mL^{-1}; the total run time was 15.0 min. The method proved to be very robust and was successfully applied for the analysis of plasma samples from mice dosed with darifenacin.

2. Experimental

2.1. Reagents and materials

Darifenacin hydrobromide (> 99% purity) as a reference standard was purchased from Novartis International Pharmaceutical (Ringaskiddy, Cork, Ireland). Bisoprolol (> 99% purity) as an IS was obtained from Sigma Chemical (St. Louis, MO, USA). The structures of the standards are described in Fig. 1. HPLC-grade acetonitrile and analytical grade diethyl amine and, *ortho*-phosphoric acid were purchased from BDH Chemicals (City, UK). Bidistilled water was purified using a cartridge system (Milford, USA). Ultra pure water with a sensitivity of 18 μΩ was obtained from a Millipore Milli-Q plus purification system, Waters Milford, USA.

2.2. Apparatus

The HPLC analysis was performed on a Waters HPLC system (Milford, USA) equipped with a 1500 series HPLC pump; the system was operated at a flow rate of 1.0 mL min^{-1}. A dualwavelength fluorescence detector (2475) and an autosampler (717plus) were used. The data was collected with the Empower Pro Chromatography Manager software for data acquisition and analysis. The chromatographic separations were performed with an phenyl HYPERSIL C18 column (125 × 4.6 mm^2 i.d., 5 μm particle diameter) manufactured by Thermo Electron corporation, which was coupled to a Water Symmetry C18 Sentry Guard column (20 mm). All solutions were degassed by ultrasonication (Technal, Brazil) and filtered through a 0.45-μm Millex filter (Millipore).

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile: 0.1% diethyl amine (60:40, v/v) (pH = 3.5). The mobile phase was freshly prepared, then filtered and degassed. All separations were performed isocratically at a flow rate of 1 mL $min.^{-1}$ Column temperature was maintained at room temperature (25 ± 2 °C). The injection volume was 50 μL, and the detection wavelengths were set at 210 and 314 nm for excitation and emission, respectively. The excitation and emission wavelengths were set according to the fluorescence spectra of darifenacin.

2.4. Preparation of standard solutions

We prepared standard stock solutions of both darifenacin and bisoprolol as an IS by dissolving an appropriate amount of each compound in methanol to yield a concentration of 1 mg mL^{-1}. For the preparation of the working standard solutions of these drugs, were prepared by diluting 1.0 mL of the stock solutions were diluted to 10 mL in a measuring flask with methanol to give 100 μg mL^{-1} of each drug and were further diluted to 10 μg mL^{-1} of darifenacin and IS. The stock solutions were stable for at least two months when stored in a refrigerator, and no evidence of degradation of the analyte was observed in the chromatograms obtained during this period.

2.5. Preparation of samples

One hundred microliters of mouse plasma was spiked with darifenacin and the IS to yield darifenacin concentrations of 100, 200, 400, 800, 1000, 2000 and 3000 ng mL^{-1} of darifenacin and IS (1000 ng mL^{-1}) in 2.0 mL disposable polypropylene microcentrifuge tubes. Each tube was vortexed for 30 s and was then mixed with 500 μL of acetonitrile for deproteinisation each tube was subsequently, vortexed at high speed for 1 min, and centrifuged at 12,000 rpm for 12 min. The supernatants were loaded into the autosampler tray and 50 μL of the supernatant was injected into the HPLC system for analysis. Blank mouse plasma samples were processed in the same manner using methanol rather than darifenacin and IS. The accuracy and precision of the method was calculated.

2.6. Animals

Adult male and female Swiss albino mice aged 10–14 weeks and weighing 25–30 g were obtained from the Experimental Animal Care Center at King Saud University. The animals were maintained in an air-conditioned animal house at a temperature of 25–28 °C, a relative humidity of ~50% and a photo-cycle of 12:12 h light and dark periods. The animals were provided with standard diet pellets and water *ad libitum*. All experiments were conducted according to the Guidelines of the Animal Care and Use Committee at King Saud University. Each treatment and control group consisted of 6 randomly assigned mice.

2.7. Darifenacin treatment and plasma sampling

Darifenacin was dissolved in 10% ethanol in saline and administered intraperitoneally at a dose of 2 mg kg^{-1}. Blood was collected from the heart in tubes containing heparin at 0.5, 1, 2, 4, 8, 12 h after drug administration. The experiment included a control group of mice administered ethanol in saline to provide

the blank mice plasma. The blood samples were centrifuged and the plasma was separated and frozen at −20 °C until analysed.

2.8. Method of validation

2.8.1. Calibration curves

The linearity of the method was evaluated using a calibration curve over the concentration range 100–3000 ng mL^{-1} of darifenacin, including the LOQ. We constructed the calibration curve by plotting the area ratios of darifenacin and IS versus the concentration of darifenacin by least-squares regression analysis. The calibration curve requires a correlation coefficient (r^2) of 0.999. The acceptance criterion of each back-calculated standard concentration should be within 15% of the nominal value. For the calibration plots for darifenacin the working solutions were diluted to yield eight concentration levels (100, 200, 400, 800, 1000, 2000 and 3000 ng mL^{-1}).

2.8.2. Recovery

We determine the recovery of darifenacin from plasma at concentration ranges of 100, 200, 400, 600, 800, 1000, 2000 and 3000 ng mL^{-1} by comparing each peak area of eight extracted samples with the mean peak area of six unextracted standard solutions that contained the corresponding concentrations in the mobile phase that represented 100% recovery.

The quality control (QC) samples at three concentration levels 150, 1250, and 2500 ng mL^{-1} were prepared by spiking the drug-free plasma with appropriate volumes of darifenacin and IS. Before being spiked, the drug-free plasma was tested to ensure that there no endogenous interferences were present at the retention times of darifenacin and the IS.

2.8.3. Precision and accuracy

Intra-day accuracy and precision were determined by replicate analysis of eight sets of samples spiked with eight different concentrations of darifenacin (100, 200, 400, 600, 800, 1000, 2000 and 3000 ng mL^{-1}) within one day or over 6 consecutive days. Acceptable intra-day accuracy is 85–115% and, the coefficient of variation (CV) values should be less than 15% over the calibration range. The accuracy and precision of the method were determined for darifenacin according to the United States Food and Drug Adminstration (FDA) guidance for bioanalytical method validation [17].

3. Results and discussion

3.1. Optimisation of the chromatographic conditions

3.1.1. Method development

The proposed HPLC method provides a simple procedure for the determination of darifenacin in biological samples. A straightforward and accurate HPLC method to determine darifenacin in mouse plasma was initially developed. The chromatographic conditions, on the basis of isocratic separation, gave a good profile in plasma when a reversed-phase C18 column was used. We studied different ratios of the mobile phase to shorten the retention times of the analyte and to improve peak symmetry. The best results with respect to peak symmetry and retention time were obtained using acetonitrile: 0.1% diethylamine(pH 3.5) (60:40), as the mobile phase. The pH of the mobile phase was adjusted with 20% *o*-phosphoric acid to pH 3.5. The darifenacin detection was performed at 210 and 314 nm for excitation and emission, respectively, because the maximum response and the absence of endogenous interfering peaks from the blank matrix are achived at this wavelength. The samples of darifenacin were successfully extracted from interfering plasma using an acetonitrile-based deproteinisation technique, with excellent recoveries. In the present study, bisoprolol was selected as an internal standard because physicochemical characteristics are similar to those of darifenacin. Both drugs possess native fluorescence character, similar solubilities in alcohols, and nearly similar log P values. Furthermore,with the proposed HPLC method, bisoprolol exhibited high recovery with a short retention time and with good validation results.

The drug produce two maximum absorbances at 210 and 294 nm and the corresponding emmission at 314 nm for both wavelengths. In this study, the excitaion wavelength measured at 210 nm show was more sensitive than that at 294 nm. We therefore used 210 and 314 nm for excitaion and emission wavelengths, respectively.

3.1.2. System suitability

The chromatographic separation using the proposed HPLC method was perfromed to evaluate the chromatographic parameters (e.g., the capacity factor, K'), the asymmetry of the peaks, the tailing factor and the resolution between two consecutive peaks. Fig. 2 shows a representative chromatogram that corresponds to the chromatographic separation of these substances. The peaks of darifenacin and IS were well resolved, with retention times of 6.69 and 10.96 min, respectively. No endogenous peak from plasma was found to interfere with the elution of either darifenacin or IS. Analysis was achieved within 15 min for a total chromatography run. The capacity factor (K') of the first peak was 2.221, and that of the second peak was 3.20; the resolution factor was 6.85. The results obtained for the asymmetry of the peak and the tailing-factor parameters were 1.0 for IS and darifenacin.

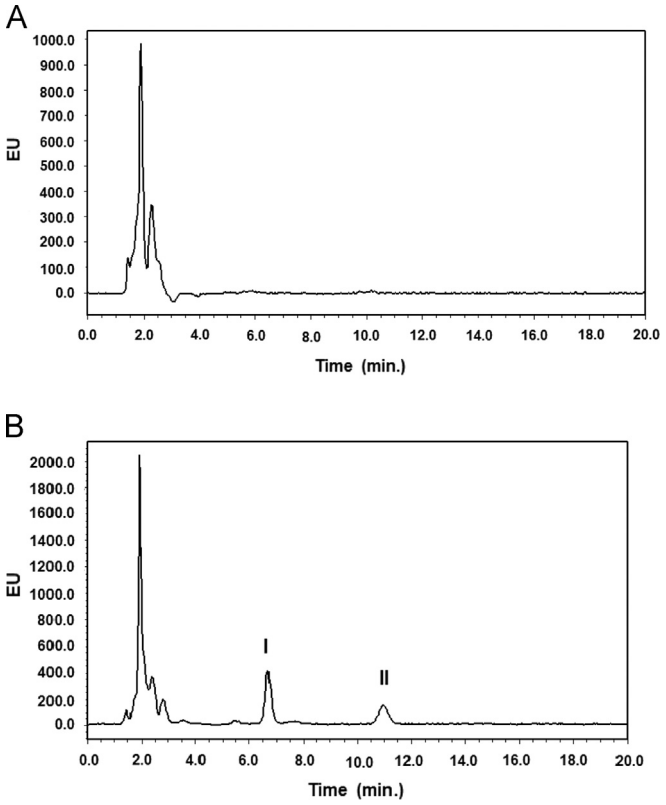

Fig. 2. Represented HPLC chromatogram for the analysis of darifenacin in drug-free plasma: (A) blank plasma (B) mouse plasma spiked with 100 and 1000 (ng mL^{-1}) of darifenacin (II) and IS(I) respectively (retention time was 6.69 and 10.96 (min)).

The selectivity factor between the darifenacin and IS was 1.45. We concluded that the developed method is the optimum according to the studied parameters. The capacity factor obtained is within the accepted values: – greater than 2 for the first peak and less than 10 for the second peak. Lastly, good resolution was obtained between two consecutive peaks in the developed method. Therefore, this method can be applied to its intended purpose with no problems, its suitability being proved.

3.2. Validation of the method

3.2.1. Specificity of the chromatographic method

To serve as blanks, plasma samples were obtained from six different sources and were assayed to evaluate the selectivity of the method and the detection of interference. Representative chromatograms of mouse blank plasma and mouse plasma spiked with 100 and 1000 ng mL^{-1} of darifenacin and IS, respectively, are shown in Fig. 2. The peaks of darifenacin and IS were well resolved, with retention times of 10.96 and 6.69 min, respectively. No endogenous peak from plasma was found to interfere with the elution of either darifenacin or IS. Analysis was achieved within 15 min for a total chromatography run. The peaks were completely resolved one from another at therapeutic concentration of darifenacin. The deproteinisation of the plasma sample was sufficient to isolate the darifenacin and IS from plasma without any interfering endogenous peaks at 210 and 314 nm for excitation and emission, respectively.

3.2.2. Linearity and sensitivity

The method was extensively validated as per the FDA guidelines [17,18] and was found to be rugged and adequately sensitive for the routine analysis of samples. The peak-area ratios of darifenacin and IS corresponding to plasma concentrations over a range of 100–3000 ng mL^{-1} for darifenacin demonstrated an excellent linear relationship. The mean linear regression equation of the peak ratio (y) versus the drug concentration (ng mL^{-1}) in mouse plasma samples (x) showed a correlation coefficient (r^2)= 0.9951 ($y=0.0039x+0.1371$). The good linearity of the calibration graphs and negligible scatter of the experimental points are evident from the values of the correlation coefficient and the standard deviation of the obtained data [19]. The correlation coefficient (r^2) of the calibration graph generated during the validation was 0.9951 for the analysis. Table 1 summarises the accuracy of the calibration curve. The LOQ of darifenacin in plasma was verified as 100 ng mL^{-1}, at which the accuracy was between 80.15% and 119.7%, and the precision was less than 15%. The lower limit of detection (LOD) was 35.0 ng mL^{-1} at a signal-to-noise ratio of 3 [19].

Table 1
Analytical parameters for the determination of darifenacin using the proposed method.

Parameter	Darifenacin[a]
Slope ± SD	0.0039 ± 0.0002
Intercept ± SD	0.01371 ± 0.0012
Correlation coefficient (r^2)	0.9951
Linearity range (ng mL^{-1})	100.0–3000.0
LOD (ng mL^{-1})	35.0
LOQ (ng mL^{-1})	100.0
Retention time for darifenacin (min)	10.96
Retention time for IS (min)	6.69

[a] Values are the mean of six determinations.

3.2.3. Accuracy and precision

The results for accuracy and precision at concentrations of 100–3000 ng mL^{-1} for darifenacin are presented in Table 2. The intra-day accuracy and precision varied between 93.97% and 112.2% and between 2.45% and 12.5% respectively. All values of accuracy and precision including the LOQ, were within the limits considered as acceptable [19,20]. The precision of the method was evaluated in terms of repeatability (intra-day) and intermediate precision (inter-day). Three different concentrations of QC samples were analysed in six independent series during the same day and on different days (six days); each sample was injected in triplicate. The RSD% values of intra- and inter-day studies for darifenacin showed that the precision of the method was satisfactory; the results are shown in Table 3 and 4, where the intra- and inter-day relative standard deviations were less than 13.5%. The accuracies of the analytical method, which expresses the closeness between the reference value and the found value were 108.79% and 114.85%, for intra- and inter- day analyses, respectively [19,20].

Table 2
Recovery of calibration standard of the method for determining the concentration of darifenacin in plasma sample ($n=6$).

Conc. (ng mL^{-1})	Methanol[a] (peak area ratio)	Plasma[b] (peak area ratio)	Recovery (%)[c]
100	0.53	0.57	107.54
200	1.26	1.01	80.15
400	1.52	1.82	119.70
800	3.12	3.16	101.28
1000	3.90	3.96	101.53
2000	8.65	8.35	96.53
3000	12.13	12.53	103.29

[a] Sample dissolved in mobile phase and injected directly to HPLC.
[b] Drug extracted from plasma and injected directly to HPLC.
[c] Absolute recovery, 101.24%.

Table 3
Intra-day reproducibility of standard plasma calibration curve of darifenacin obtained by HPLC.

Conc. (ng mL^{-1})	Mean[a]	SD	CV (%)	Accuracy (%)
100	112.2	9.88	8.80	112.20
200	219.2	6.87	3.13	109.6
400	440.0	55.03	12.50	110.0
800	890.0	24.94	2.80	111.25
1000	937.6	32.74	3.49	93.76
2000	2008.2	49.23	2.45	100.5
3000	3010.5	77.56	2.57	100.35

[a] Average of three determinations

Table 4
Intra-day and inter-day reproducibility of quality control samples of darifenacin obtained by HPLC.

Conc. (ng mL^{-1})	Intra-day				Inter-day[b]			
	Mean[a]	SD	CV (%)	Accuracy (%)	Mean	SD	CV (%)	Accuracy (%)
150.0	167.77	15.59	9.3	111.85	175.0	23.6	13.5	116.67
1250.0	1458.13	109.35	7.49	116.65	1480.3	170.2	11.5	118.42
2500.0	2449.25	85.73	3.5	97.97	2735.0	218.8	8.0	109.42

[a] Average of three determinations.
[b] Average of six days.

3.2.4. Stability

Quality control samples of darifenacin were used for stability experiments. Stability was assessed under various conditions. The deviation of the mean test responses were within ± 15% of appropriate controls of darifenacin concentration. No evidence of degradation was observed during sample processing and storage for at least one month in a freezer at −80 °C. No effect on quantification of darifenacin was observed for the short-term stability of the frozen samples kept at room temperature for 6 h. The results of these studies suggest that the mouse plasma samples containing darifenacin can be handled under normal laboratory conditions without significant loss of compound.

3.2.5. Robustness and ruggedness

To measure the extent of the method robustness, the most critical parameters were interchanged while the other parameters were kept unchanged, and the chromatographic profile was observed and recorded in parallel. The chromatographic parameters were interchanged within a range of 1–10% of the optimum recommended conditions. The studied parameters were: – the composition of the mobile phase, the pH, the flow rate, and the column temperature. To evaluate the ruggedness of the HPLC method we performed the analysis using two different analysts (operators) and different instruments on different days. The results obtained using two different analysts and different instruments were found to be reproducible. RSD values of less than 15.0% were observed for repetitive measurements and operators. These results indicate that the method is capable of producing results with high precision.

3.2.6. Pharmacokinetic study

The described method was further applied to a pharmacokinetic study of darifenacin in mice. The concentration of darifenacin in mice plasma at different times after dosing was determined individually. After the determination, we constructed a plasma concentration–time curve (AUC) for darifenacin, as shown in Fig. 4. The main pharmacokinetic parameters of darifenacin were calculated from the curve. After drug administration, the mean values of T_{max} and C_{max} were 0.5 h and 125.3 ng mL^{-1}, respectively. No peaks similar to those obtained after the darifenacin treatment were observed in samples collected from the vehicletreated control animals (Fig. 3). In this study, no metabolite was detected after intraperitoneal administration of darifenacin in mouse plasma.

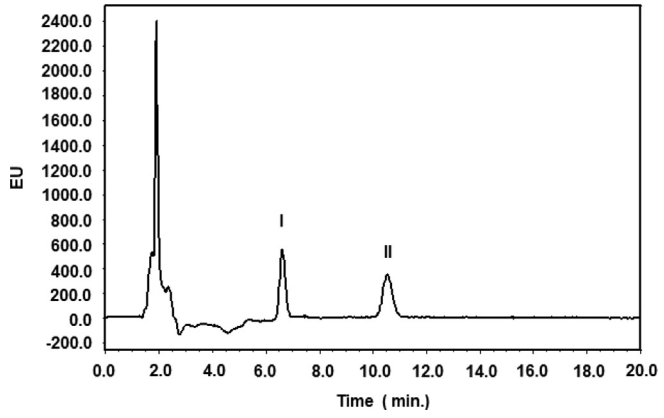

Fig. 3. Representative HPLC chromatograms of plasma taken from mouse after 20.0 (min).

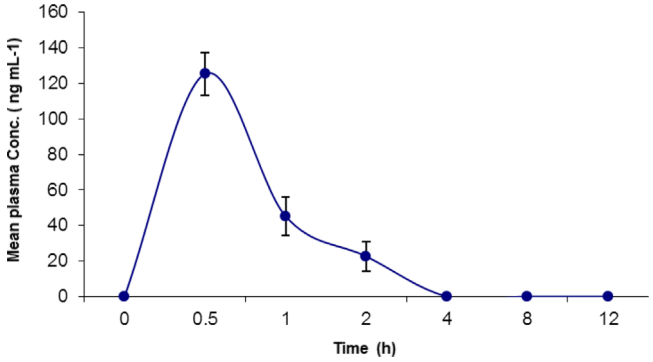

Fig. 4. Concentration–time profile of darifenacin in mouse plasma after single intraperitoneal administration of 2 (mg kg^{-1}) of darifenacin. Each point represents the mean ± S.D of six mice.

4. Conclusion

In conclusion, we have developed a simple, sensitive and reliable HPLC–FL method for measuring darifenacin in mouse plasma. A validated RP-HPLC procedure for the assay of darifenacin in mouse plasma is described for the first time. The method shows acceptable precision and, accuracy and adequate sensitivity for use in pharmacokinetic studies and appears to be suitable for use in all laboratories, irrespective of whether they are equipped with sophisticated or unsophisticated instruments.

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