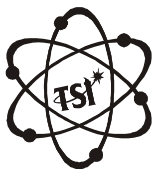

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Note

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A stereoselective, stability indicating validated LC-assay method for the separation and quantification of darifenacin and its enantiomer

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ABSTRACT

A simple, rapid isocratic stability indicating chiral HPLC method has been developed for the separation of R-Darifenacin from S-Darifenacin and quantitative determination of R-Darfenacin enantiomer and Darfenacin Hydrobromide assay in bulk forms and pharmaceutical dosage forms. Forced degradation studies were performed on bulk drug sample of Darifenacin using acid (2.0 N hydrochloric acid), base (1.0 N sodium hydroxide), oxidation (3.0% v/v hydrogen peroxide), water hydrolysis, Thermal (105^{0}C) and photolytic degradation. Considerable degradation was observed during oxidative stress condition. Mobile phase contains n-hexane, ethanol and diethyl amine in the ratio of 75: 25: 0.05 (v/v/v). Good resolution viz. 3 min between R- and S- forms of Darifenacin was achieved with Immobilized chiral stationary phase Chiralpak IC (250mm×4.6 mm ID, 5micron) column at 27^{0}C temperature. Flow rate was kept at 0.8 ml/min. This method was further used to determine the amount of R- Darifenacin, which was monitored by UV absorption at 230 nm. This method is capable of detecting R-Darifenacin to a level of 0.08μg/ml. The method was validated as per ICH guidelines.

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KEYWORDS

Chiral HPLC method;
Separation;
Quantification;
Darifenacin;
Forced degradation.

INTRODUCTION

Most of the pharmaceutical industries are now concentrating towards the study of the therapeutic effects of enantiomers of the existing drug molecules to have a detailed impurity profile. The determination of amounts of different enantiomeric forms in pharmaceuticals is essential in this connection and high performance liquid chromatographic method (HPLC)[1] is generally opted for this purpose. The chemical name of Darifenacin hydrobromide is (S)-2-{1-[2-(2,3-dihydrobenzofuran-5-yl)ethyl]-3-pyrrolidinyl}-2,2-diphenylacetamide hydrobromide is a potent muscarinic receptor antagonist developed by Novartis[2-3]. Its main avenue of effect is reduction in overactive bladder. Darifenacin hydrobromide is a new M3 selective receptor antagonist treatment for overactive bladder approved for use anywhere in the world. Darifenacin, a chiral diphenylacetamide tertiary amine derivative (Figure 1) marketed as the water-soluble hydrobromide salt, is a selective M3 receptor antagonist. The empirical formula for Darifenacin hydrobromide is $C_{28}H_{30}N_2O_2$.HBr, and the molecular weight is 507.5.

It has S- and R-enatiomeric forms (Figure 1) and

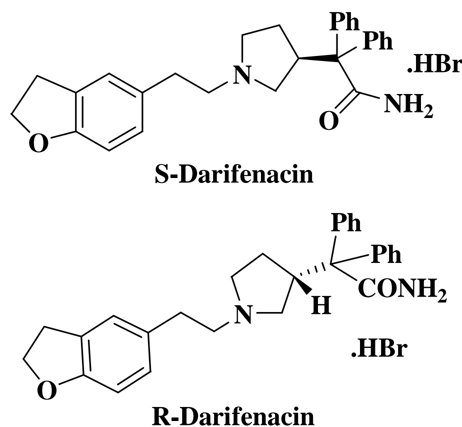


Figure 1: S- and R- forms of darifenacin

since S-enantiomer ((S)-2-[1-[2-(2,3-dihydrobenzo furan-5-yl)ethyl]-3-pyrrolidinyl]- 2,2-diphenylaceta mide hydrobromide) is able to cure the disease, pharmaceuticals containing S-enantiomer were released in the market. Since, there is a chance of formation of R-Darifenacin ((R)-2-[1-[2-(2,3-dihydrobenzofuran-5-yl)ethyl]-3-pyrrolidinyl]- 2,2-diphenylacetamide hydrobromide) during synthesis of S-Darifenacin, the separation of R-Darifenacin from S-Darifenacin followed by its determination is one of the key issues before S-Darifenacin is released to market in the form of pharmaceuticals. Enantiomers of racemic drugs often differ in pharmacokinetic behavior or pharmacological action[4]. The development of analytical methods for quantitative analysis of chiral materials and for assessment of enantiomeric purity is extremely challenging due to the fact that enantiomers posses virtually identical properties[5]. Although many analytical techniques like gas chromatography(GC), capillary electrophoresis (CE), liquid chromatography(LC) can be employed to achieve this, the most widely used is liquid chromatography employing a chiral stationary phase(CSP)[6-8]. In literature rapid solid phase extraction for high throughput assay of darifenacin in human plasma was reported[9]. A method for determination of enantiomeric purity of Darifenacin was reported[10]. However, these literature methods were not completely validated in view of specificity and suffering from interferences from degradation products, formed during stress study. Hence, we herewith are reporting a sterioselective, stability indicating, rapid isocratic HPLC method to separate and quantitate R-Darifenacin from S-Darifenacin and quantitative determination of S-Darifenacin in bulk active substances and finished dosage forms. This method was validated in accordance with ICH requirements[11] in terms of linearity, precision, accuracy, specificity and robustness.

The method development strategies adopted using chiral pack IC column involves different experiments based on nature and structure of compound. These trials including the addition of acid additives for acidic compounds and base additives for basic compounds to the mobile phase. The design of mobile phase consists of a combination of alkane or chlorinated solvents and polar alcohols based on normal or polar interactive modes. Initiated the screening analysis with the above combination of experiments to derive best suitable column and mobile phase conditions.

EXPERIMENTAL

Chemicals

HPLC grade n-hexane and ethanol were procured from Merck, India. Diethylamine was purchased from Fluka, India. S-and R- isomers of Darifenacin and Reference standard of Darifenacin Hydrobromide were obtained from process development laboratory of Dr. Reddy's Laboratories Ltd., API, IPDO, Hyderabad, India.

Chromatographic conditions

A Waters Model Alliance 2690 separation module equipped with an auto sampler and waters 2996-photodiode array UV detector (Waters Corporation, Milford, USA) was used for the analysis. The data was recorded using Waters Empower software. The separations and quantification were performed on Chiralpak IC column (250x4.6mm, Make: Diacel Chemical Industries Ltd. Japan).

Sample preparation

20 mg each of S- and R- forms of Darifenacin were dissolved in 10 ml of ethanol to get individual concentrations of 2.0 mg/ml in the mixture. This solution was used for the enantiomeric separation. 20 mg of R-Darifenacin was dissolved in 10 ml of ethanol to get 2.0 mg/ml solution and this solution was diluted further to get the required concentrations for the validation of the

developed method for the determination of R-darifenacin.

Prepared solutions of Darifenacin hydrobromide by dissolving 100mg each of sample and standard in separate 50ml volumetric flasks and dilute to volume with ethanol. Dilute these stock solutions to 50ml separately to get a final concentration of 200µg per ml. These solutions were used for assay determination.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities[8]. The specificity of the developed HPLC method for Darfenacin Hydrobromide was carried out in the presence of its impurities. Forced degradation studies were also performed for bulk drug to provide an indication of the stability-indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions of UV light (254 nm and 365 nm), thermal degradation (drug substance exposed to 105^{0}C), acid hydrolysis (using 2.0 N HCl), base hydrolysis (using 1.0 N NaOH), water hydrolysis and oxidative degradation (using 3% H_2O_2) to evaluate the ability of the proposed method to separate Darfenacin from R-Darfenacin and degradation products. For Thermal and light studies, study period was 7 days where as for acid, base and oxidation, it was 48 hours. Peak purity test was carried out on the stressed samples of Darfenacin by using PDA detector.

Assay studies were carried out for stressed samples against qualified reference standard and the mass balance (% assay+ % degradation) was calculated.

Method validation

Precision

Chiral method precision was evaluated by carrying out six independent preparations of test sample of Darfenacin spiked with 0.15% of R-Darfenacin with respect to analyte concentration. The % R.S.D of area for R-Darfenacin was calculated. Assay precision was carried out on six independent preparations of standard and test solutions of Darifenacin hydrobromide at 200µg/ml concentration. The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for R-Darfenacin was estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration. Precision study was also carried at the LOQ level by injecting six individual preparations of R-Darfenacin and calculated the %R.S.D of the area.

Linearity

Linearity test solutions for chiral method were prepared by diluting the impurity stock solution to the required concentrations. The solutions were prepared at seven concentration levels from LOQ to 150% with respect to the impurity specification level of 0.15% (i.e. LOQ, 0.0375%, 0.075%, 0.1125%, 0.15%, 0.1875% and 0.225%).The calibration curve was drawn by plotting the peak areas of R-Darfenacin versus its corresponding concentration.

Linearity test solutions for assay method were prepared from stock solution at six concentration levels from 25% to 150% of assay concentration (50µg/mL, 100µg/mL, 150µg/mL, 200µg/mL, 250µg/mL and 300µg/mL). The peak area versus concentration data was performed by least-squares linear regression analysis. The calibration curve was drawn by plotting Darifenacin average area for triplicate injections and the concentration expressed in percentage.

Linearity test was performed for two consecutive days in the same concentration range for R-Darfenacin content method. The % R.S.D value of the slope and Y-intercept of the calibration curve was calculated.

Accuracy

The accuracy study of R-Darfenacin was carried out in triplicate at 0.075, 0.15 and 0.225 % of the Darfenacin analyte concentration (200µg/mL). The percentages of recoveries for impurity were calculated.

The accuracy of the assay method was evaluated in triplicate at three concentration levels i.e. 100µg/mL, 200µg/mL and 300µg/mL in bulk drug sample. The % recoveries were calculated.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately al-

tered and the resolution between R-Darfenacin impurity and Darfenacin was evaluated.

The flow rate of the mobile phase was 0.8 mL/min. To study the effect of flow rate on the resolution, it was changed by 0.2 units from 0.6 mL/min to 1.0 mL/min. The effect of column temperature on resolution was studied at 22^{0}C and 32^{0}C instead of 27^{0}C .In the all above varied conditions, the components of the mobile phase were held constant as per the method.

Solution stability and mobile phase stability

The solution stability of Darfenacin and its enantiomer R-Darfenacin in the chiral method was carried out by leaving spiked sample solution in tightly capped volumetric flask at room temperature for 48hours. Content of R-Darfenacin were determined for every 6h interval up to the study performed. Mobile phase was also carried out for 48 hours by injecting the freshly prepared sample solutions for every 6h interval. Content of R-Darfenacin were checked in test solutions. Mobile phase prepared was kept constant during the study period.

The % RSD of assay of Darifenacin was calculated for the study period during mobile phase and solution stability experiments.

RESULTS AND DISCUSSION

Method development and optimization of chromato graphic conditions

Initially, experiments were carried out by using chiral columns in the reverse phase for the separation of S- and R- forms of Darifenacin, but it was not achieved. Later several experiments were carried out in the normal phase using various chiral columns. It was observed that the R and S-forms of Darifenacin were eluted as broad peaks with long retention times by using a Chiral Pak-AD column at 0.8 ml/min flow rate and a mixture of n-hexane, isopropanol, diethylamine (75: 25: 0.5) mobile phase. However an improvement in peak shapes were observed when Chiral pak AD-H column was used with the same mobile phase and same flow rate. However, relatively longer retention times and improper separation between enantiomers were observed with this column. The next trial was made on cellulose based CSP viz Chiral cel OD-H column was used for further

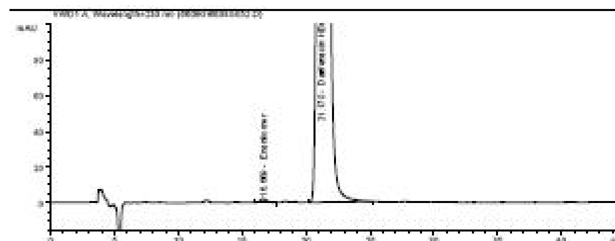


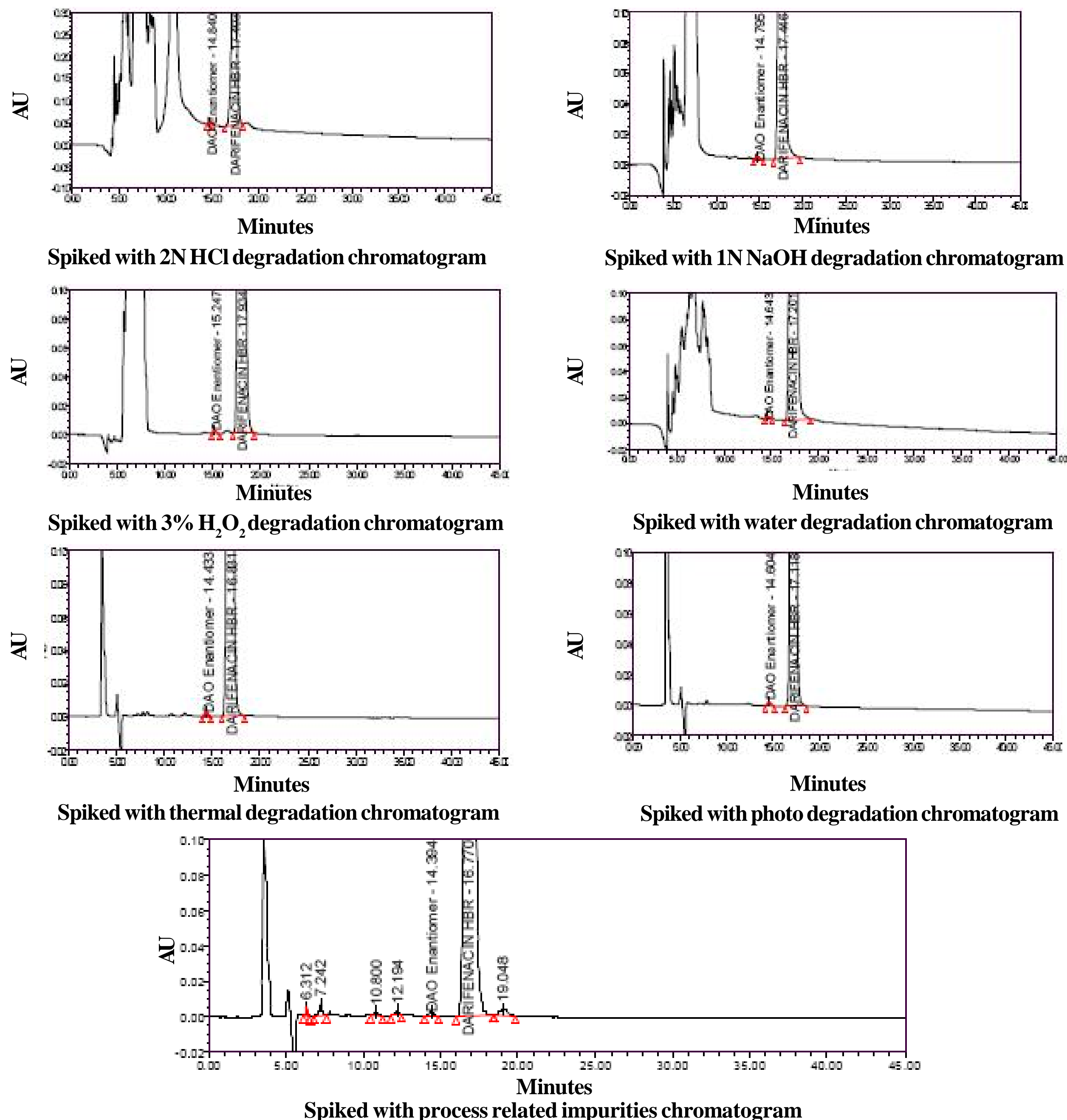
Figure 2: Chromatogram indicating the enantiomeric separation of darifenacin

experiments. In this experiment, S- and R isomers were eluted with good peak shape and resolution. But this experiments suffers with interference of known impurity with R-Darifenacin peak. Several attempts were made to remove the interference but could not succeeded. Keeping the peak shape and resolution between the enantiomers in view, the recent advancement in chiral CSPs viz immobilized stationary phases were selected for further experimentation. Chiral pak IC column was selected for this experimentation with n-Hexane and IPA as initial mobile phase. However, several modifiers were tested to achieve a good resolution and peak shapes. A good resolution of all the isomers was achieved by using diethylamine in ethanol as a modifier. With high percentage of ethanol, merging of related impurities viz. acid impurity and alkenyl impurity peaks with Darifenacin analyte peak. This interference was eliminated by optimization of mobile phase conditions. Finally, the resolution was found to be 4.0 min (Figure 2) for the separation S- and R- forms of Darifenacin with the mobile phase consisting of n-Hexane, ethanol and 0.5% diethyl amine in the ratio of 75 : 25 : 0.05 (v/v/v). The elution was monitored at wavelength $\lambda = 230$ nm. Now, the same conditions were maintained for the determination of R-Darifenacin and assay of Darifenacin.

Method validation

Results of forced degradation studies

Degradation was not observed in Darfenacin samples during stress conditions like UV light and Thermal studies. The degradation of drug substance was observed in acid, base, water hydrolysis and in oxidation condition (Figure 3). Darifenacin was degraded under oxidation condition (treated with 3.0% hydrogen peroxide for 3hrs) and it was confirmed by co-injection with a qualified standard. Peak purity test results



Spiked with 2N HCl degradation chromatogram

Spiked with 1N NaOH degradation chromatogram

Spiked with 3% H_2O_2 degradation chromatogram

Spiked with water degradation chromatogram

Spiked with thermal degradation chromatogram

Spiked with photo degradation chromatogram

Spiked with process related impurities chromatogram

Figure 3: Chromatograms indicating the degradation of darfenacin hydrobromide in acid, base, peroxide, water, thermal, photo and spiked with process related impurities

confirm that the Darifenacin and its enantiomer peak is homogeneous and pure in all the analyzed stress samples of Darifenacin. There is no interference of degradation products and related impurities with R-Darifenacin peak indicate the specificity of the method. The peak purities of both the forms were studied by using PDA detector and found to be homogeneous with no detectable impurities embedded in them.

The mass balance (%Assay + degradation products) is achieved >98%. The assay of Darifenacin is unaffected in the presence of impurities and degradants which confirm the stability-indicating power of the method.

Limit of detection and quantification

The limit of detection (LOD) represents the con-

Note

TABLE 1: Regression characteristics of the determination method for R-darifenacin

Parameter	Values
LOD (μg mL^{-1})	0.08
LOQ (μg mL^{-1})	0.315
Linear Dynamic Range (μg mL^{-1})	0.08-4.6
Slope	20182.7
Intercept	0.148
Correlation coefficient	0.9995

TABLE 2: Precision of the determination method for R-darifenacin

Injection no.	R-darifenacin area	
	0.315 μg/ml(LOQ level)	1.5μg/ml
1	7.228	65.523
2	7.994	65.504
3	7.243	60.011
4	7.043	60.047
5	8.631	64.371
6	7.758	61.377
% RSD	7.8	4.2

TABLE 3: Accuracy of the determination method for R-darifenacin

S. no.	% Concentration	% Recovery
1	50	102.9
2	75	100.8
3	100	99.6
4	150	101.2

centration of analyte that would yield an S/N (signal to noise) ratio of 2.0. LOD for R-Darifenacin was found to be 0.08 μg/ml. The limit of quantification (LOQ) represents the concentration of analyte that would yield an S/N ratio of 10. LOQ for R-Darifenacin was found as 0.315 μg/ml. The % RSD value of precision at LOQ level is 7.8. The percent recovery values at LOQ (96 to 102) were obtained in acceptable range as per ICH guidelines.

Linearity

Linearity test solutions of R-Darifenacin ranging from 0.3 and 4.6μg/ml were prepared by diluting the stock solution of R-Darifenacin. The calibration curve was drawn between the peak areas of R-Darifenacin versus its concentration. The slope, intercept and correlation coefficient were derived from liner least-square regression analysis. The results revealed that an excellent correlation existed between the peak area and concentration of the analyte. The data is presented in TABLE 1.

Linear calibration plot for assay method was obtained over the calibration ranges tested, i.e. 50 μg/mL to 300 μg/mL and the correlation coefficient obtained was greater than 0.999. Linearity was checked for assay method over the same concentration range for two consecutive days. The percentage R.S.D values of the slope and Y-intercept of the calibration curves were 1.2 and 2.4 respectively. The results show that an excellent correlation existed between the peak area and concentration of the analyte.

Precision

The precision was evaluated by calculating the relative standard deviation of six replicate determinations using the same solution containing R-Darifenacin at two levels viz. 0.315 and 1.5 μg/ml and the percentage relative standard deviation values of injection repeatability for R- Darifenacin were found to be 7.8 and 4.0 respectively which is in the acceptable range as per the ICH guidelines. The results are given in TABLE 2.

The R.S.D of assay results obtained in intermediate precision study was within 0.1%.

Accuracy

The accuracy of method was evaluated by assaying freshly prepared solutions in triplicate at four concentration levels of 50%, 75%, 100% and 150%. The percent recovery values are in the acceptable range as per ICH guidelines. The data is presented in TABLE 3.

The percentage recovery of Darifenacin in bulk drug samples was ranged from 99.1 to 99.7% w/w.

Robustness

In the varied chromatographic conditions viz. flow rate and column temperature, the resolution between the peaks of S- and R- forms of Darifenacin was found to be 4.0 min illustrating the robustness of the method. However the retentions of R and S forms were varied within a range of 3 to 4 minutes with the variation of column oven temperature indicates the requirement of constant column oven temperature throughout the analysis.

Solution stability and mobile phase stability

No significant change was observed in the content of R-Darifenacin during solution stability and mobile phase stability experiments when performed using chiral method. The solution stability and mobile phase stabil-

TABLE 4: Summary of forced degradation results

Degradation condition	Time	Assay (%w/w on anhydrous basis)	Mass balance (%Assay + % degradation products)	Remarks
Thermal(105°C) solid	7 days	99.5	99.8	No significant degradation observed
Acid hydrolysis 2.0 N HCl	36 h	91.6	99.5	Degraded into acid impurity and some unknown degradation products were formed.
Base hydrolysis, 1.0 N NaOH	48 h	99.4	99.8	No significant degradation observed
Water hydrolysis	48 h	99.2	99.6	No significant degradation observed
Oxidation by H_2O_2 (3.0%)	4 h	93.6	99.5	Degraded into some unknown degradation products.
UV at 254 nm	7 days	99.6	99.8	No significant degradation observed

ity experiments data confirms that sample solutions and mobile phase used during R-Darifenacin content determination were stable up to 48 hours.

The RSD of assay of Darifenacin during solution stability and mobile phase stability experiments was within 0.5%.

CONCLUSION

An isocratic, stability indicating, sterioselective chiral liquid chromatographic method developed for the enantiomeric separation and quantitative determination of Assay and R-Darifenacin content is found to be precise, accurate and specific in bulk active substance and finished dosage forms. The method is fully validated as per ICH guidelines, showing satisfactory results for all method validation parameters tested. Hence, the method is stability indicating and can be used for the routine analysis of plant batches of Darifenacin hydrobromide in quality control laboratories and also to check the stability of bulk samples and tablet samples of Darifenacin hydrobromide.

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