

DEVELOPMENT AND VALIDATION OF BIOANALYTICAL METHOD FOR ESTIMATION OF DARIFENACIN HYDROBROMIDE IN DOSAGE FORM USING β -NAPHTHOL

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ABSTRACT

A sensitive visible spectrophotometric method was developed and validated for the estimation of darifenacin hydrobromide in the pharmaceutical dosage form using diazo coupling reaction with the chromogenic reagent β naphthol forms a yellow coloured diazo complex with darifenacin hydrobromide. Analysis was carried out at its λ_{\max} 384nm and the method was validated according to USFDA guidelines. The method obeys the Beer's law in the concentration range of 40-140ng/ml. The correlation coefficient was found to be 0.999. As the method was sensitive, it was successfully applied to the analysis of darifenacin hydrobromide in spiked rat plasma.

Keywords: β -naphthol, darifenacin hydrobromide, spectrophotometric determination and USFDA guidelines.

INTRODUCTION

Darifenacin Hydrobromide¹⁻³ chemically known as (*S*)-2-{1-[2-(2, 3-dihydrobenzofuran-5-yl)ethyl]-3-pyrrolidiny]-2,2-diphenylacetamide hydrobromide. Its empirical formula is $C_{28}H_{30}N_2O_2 \cdot HBr$ representing molecular weight of 507.5. It is white to almost white monomorphic crystalline solid, soluble in water (<1mg/mL at 25°C), chloroform (117mg/mL at 25°C) and methanol and ethanol (<1mg/mL at 25°C) and its melting point is 228-230°C. It is used to treat an overactive bladder by relaxing the muscles in the bladder.

Darifenacin is a selective muscarinic M3 receptor antagonist. This M3 receptor is the major subtype that controls urinary bladder muscle contraction. By blocking muscarinic receptors in the bladder, intravesicular pressure is lowered, bladder capacity is increased, and the frequency of bladder contractions is reduced.

Literature review¹⁹⁻²⁴ reveals that there are very few methods such as RP-HPLC, LC/MS-MS, UV and Visible spectrophotometric methods. The objective of the present study is to develop and validate new visible spectrophotometric method for analysis of darifenacin hydrobromide in spiked rat plasma using diazo coupling reagent β -naphthol.

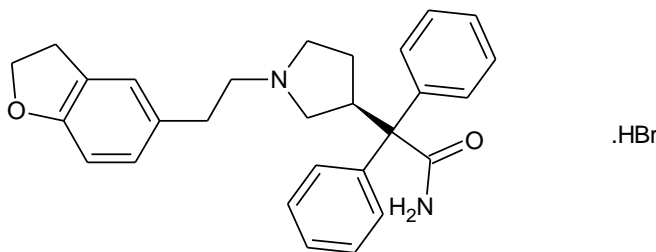


Fig.1: Chemical structure of Darifenacin Hydrobromide.

MATERIALS AND METHODS

Chemicals and reagents

The Darifenacin Hydrobromide⁴⁻⁶ was supplied as gift sample from Aurobindo Pharmaceuticals pvt Ltd. Hyderabad. The tablet dosage form (Darilong) 7.5mg manufactured by Sun Pharma laboratories ltd, Jammu was purchased from local pharmacy. β -naphthol, Sodium nitrite, Hydrochloric acid (Analytical reagent grade) were obtained from Sd fine chemicals limited (SDFCL), Mumbai. Ethanol was obtained from Merck specialties private limited, Mumbai.

Instruments

UV-visible spectrophotometer (Shimadzu 1800), pH meter (Elico LI-127), Digital balance (Shimadzu BL220H). Ultrasonic bath sonicator (PCI Analytics 6.5li200H), Refrigerated Centrifuge (EtekRC4100F), Hot Air Oven (Tempo Equipment Private Limited).

Experimental animals

Male Albino Wistar rats (180-200 g) were obtained from National Institute of Nutrition, Hyderabad.

Method development

Preparation of β -Naphthol (1%w/v)

Dissolve 100 mg of β -naphthol in 10 mL of distilled water.

Preparation of 0.1% sodium nitrite

Dissolve 100 mg of sodium nitrite in 100 mL of distilled water.

Preparation of stock solution

Standard darifenacin hydrobromide, 10mg was weighed and transferred to 10mL volumetric flask and dissolved in 0.1N HCl. The flask was shaken and was made upto the mark with 10mL 0.1N HCl to give a solution containing 1000 μ g/mL solution. From this stock solution 1mL was pipette out into another

10mL volumetric flask and the volume was made upto 10mL to give 100 μ g/mL. from this, 1mL was pipette out into another 10mL volumetric flask and volume made upto 10mL to give 10 μ g/mL.

The method was developed based on diazotization coupling reaction between Darifenacin hydro bromide and β -naphthol.

The step by step reaction scheme is as follows

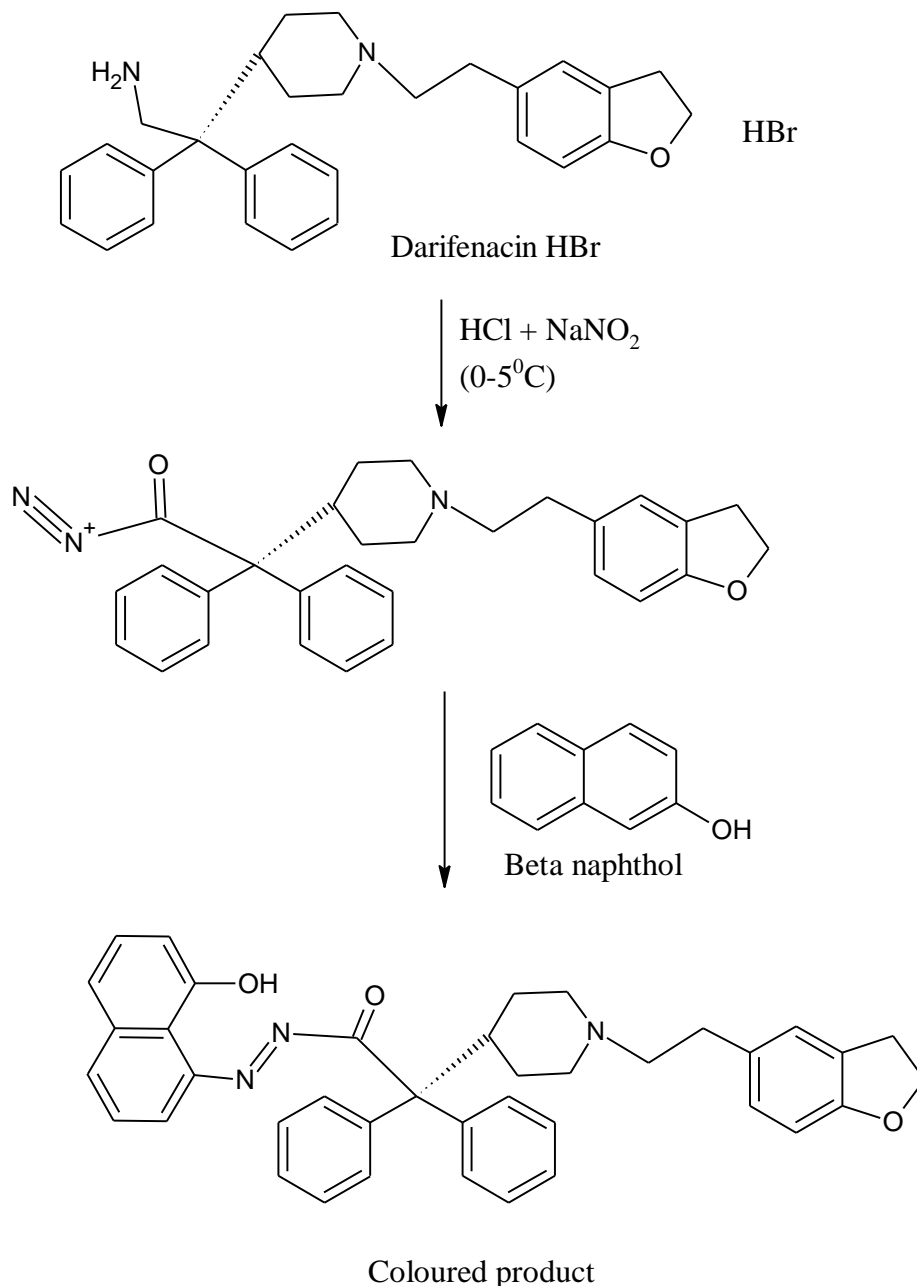


Fig .2: Proposed reaction scheme of darifenacin hydrobromide with β -Naphthol

Plasma sample preparation

The blood samples were collected from retro-orbital puncture into disodium EDTA vials (20mg disodium EDTA in 1mL water, 1mL of blood requires 50 μ L of disodium EDTA). Plasma was separated from blood samples by centrifugation at 1400 rpm for 7 minutes. After centrifugation, plasma layer gets separated and it is collected and stored at -20⁰C for further use.

Extraction procedure

- A pool of blank rat plasma was obtained.
- 100 μ L of plasma sample in was spiked with appropriate volume of the stock solution and 400 μ L of methanol was added.
- The mixture was vortexed for 1 minute.
- Centrifuged at 3000 rpm for 12minutes at 4⁰C.
- The organic layer was separated & filtered through a 0.2 μ m cellulose acetate filter.
- The organic layer was evaporated on a Savant vacuum evaporator at about 60⁰C. The residue was reconstituted in 10mL of solvent and used for the measurement of the absorbance at the absorption maxima of three drugs.

Determination of absorption maximum (λ_{max})

0.8mL was taken in a 10mL volumetric flask to which 1mL of sodium nitrite (0.1% w/v) was added. This reaction was carried out by keeping the flask in an ice tray. Later, to this 1mL of β -naphthol (1% w/v) was added and the volume was made up to 10mL with distilled water to give a solution of 80ng/mL solution. The absorbance of resulting coloured solution was measured against respective blank solution in visible region i.e., 300-800 nm which shows a maximum absorbance at 384nm and the spectrum is shown in **fig.3**.

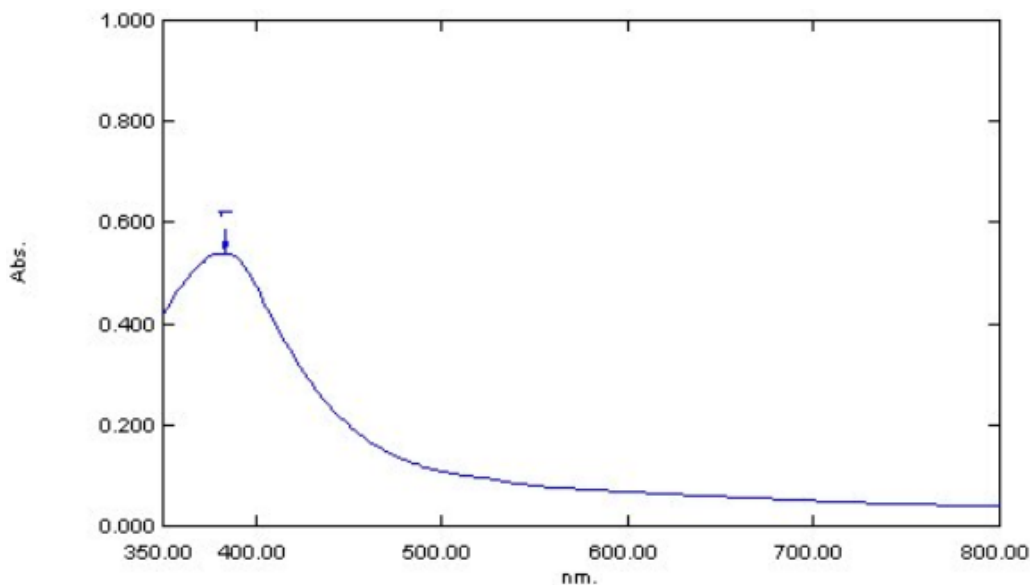


Fig.3: Absorption spectrum of darifenacin Hydrobromide and β naphthol.

Construction of calibration curve in spiked rat plasma

From the 1000 $\mu\text{g/mL}$ solution of darifenacin hydrobromide, 100 $\mu\text{g/mL}$ solution was prepared by transferring 1 mL into a 100mL volumetric flask and volume was made up to the mark with 0.1N HCl. From this, 10 $\mu\text{g/mL}$ solution was prepared by transferring 1mL into a 10mL volumetric flask. From this 1000ng/mL solution was prepared by transferring 1mL into a 10mL volumetric flask followed by the addition of 1mL of 0.1% sodium nitrite and 1mL of 1% β -naphthol. From this, 0.4, 0.6, 0.8, 1.2, and 1.4mL were transferred to ependroff tubes to which 100 μL of plasma and 400 μL of methanol were added which gives 40, 60, 80, 120, 140ng/mL and centrifuged at 3000 rpm for 12min. The supernatant organic layer was collected and made up to the volume to 5 mL with suitable solvent i.e., distilled water and the absorbance of these concentrations was measured spectrophotometrically at 384nm against blank plasma extracted solution which was illustrated in **fig. 4** and absorbance values against concentrations in **table 1**.

Table 1: Calibration curve data

| S.No | Concentration (ng/mL) | Absorbance* |
|------|-----------------------|-------------|
| 1 | 40 | 0.061 |
| 2 | 60 | 0.089 |
| 3 | 80 | 0.110 |
| 4 | 120 | 0.160 |
| 5 | 140 | 0.184 |

* Average of three determinations

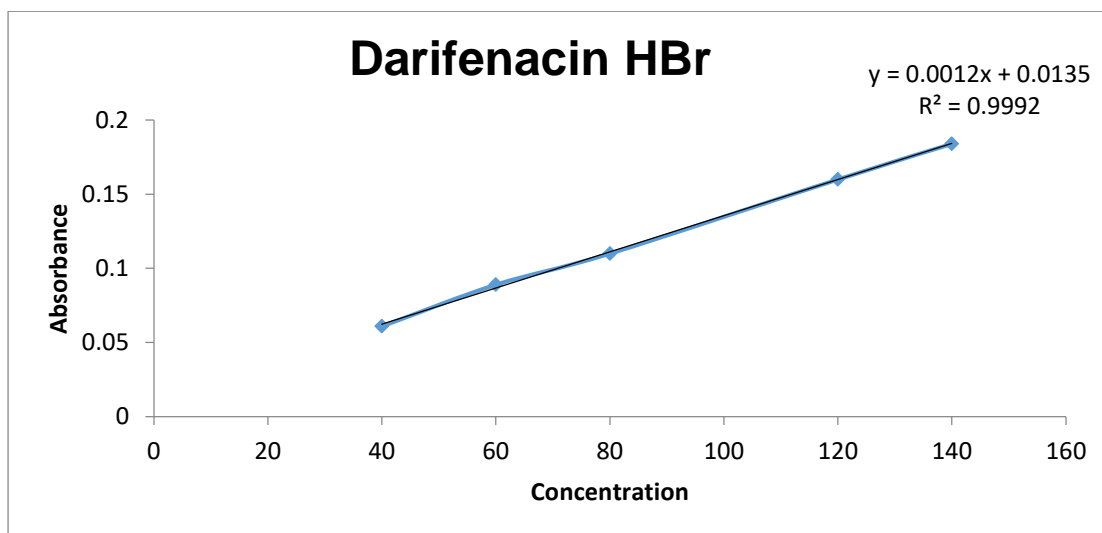


Fig.4: Calibration graph of darifenacin hydrobromide solution with plasma

Bioanalytical Method Validation⁷⁻¹¹

All analytical procedures were validated according to USFDA guidelines 'Bioanalytical Method Validation'.

Following parameters were performed for method validation:

Sensitivity (Lloq): It is the lower limit of quantification (LLOQ). The lowest concentration of the calibration curve where the analyte response was more than five times the blank response was selected as LLOQ.

Linearity

The linearity of the method was estimated by preparing calibration samples which were prepared by spiking appropriate amount of sample and organic solvent (methanol) into control plasma to get 40, 60, 80, 120, 140ng/mL. The results are reported in **table 6.1 and fig.6.2**.

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

The sensitivity of the proposed method for measurement of darifenacin hydrobromide was estimated in terms of LOD & LOQ. The Limit of detection (LOD) and limit of quantification (LOQ) was determined according to the ICH guidelines for the validation of analytical procedure. The formulae used were:

$$\text{LOD} = 3.3\sigma/S$$

$$\text{LOQ} = 10\sigma/S$$

Where, σ = standard deviation of the response (intercept)

S = slope of the calibration curve

Recovery

Recovery was determined at three levels LQC (96ng/mL), MQC (120ng/mL), HQC (144ng/mL) .the obtained results were reported in **table 8.1**.

Accuracy

Accuracy of the method was determined at three different concentration levels (96, 120, and 144ng/mL) Mean and %RSD values were calculated and are shown in the **table 8**. The results indicate that the method was accurate.

Precision

Precision of the data was reported in terms of Repeatability, Intra-day precision, and Inter-day precision. The %RSD values were calculated and are shown in the **table 9, 9.1 and 9.2**. The results indicate that the method is precise.

STABILITY STUDIES

Freeze and Thaw Stability

Drug stability was determined after three freeze-thaw cycles. Three aliquots of each higher and lower concentration were frozen for 24 hours and then thawed unassisted at room temperature, when thawed completely the samples were refrozen under same conditions as before. Freeze and thaw cycles were repeated twice or more and analysed on third cycle. The obtained results were reported in **table 10.1**.

Short-Term Stability

Three aliquots of each low and high concentration were thawed at room temperature for 24 hours and then analyzed. The obtained results were reported in **table 10.2**.

Stock Solution Stability

The stability of stock solutions of both drug and internal standard were evaluated at room temperature for 6 hours. The obtained results were reported in **table 10.3**.

RESULTS AND DISCUSSION:

Validation of Developed Method for Darifenacin Hydrobromide in Spiked Rat Plasma

The optimized method was validated according to USFDA guidelines 'Bioanalytical Method Validation'.

Selectivity and Sensitivity (LLOQ)

There was no interference in absorbance obtained in blank plasma. The spectrum of blank plasma and darifenacin hydrobromide solution with β -naphthol spiked in plasma were represented in **figures 5.1** and **5.2** respectively.

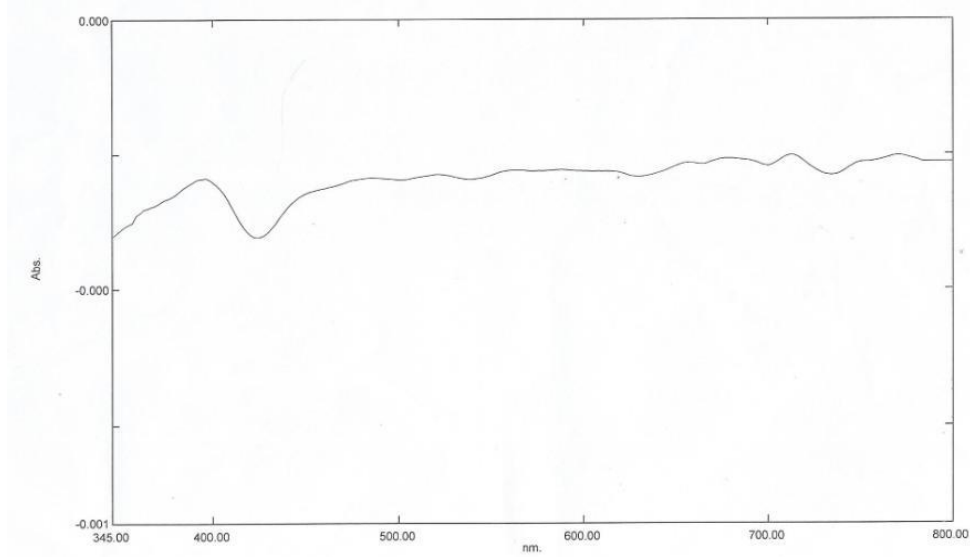


Fig.5.1 Absorption Spectrum of blank plasma

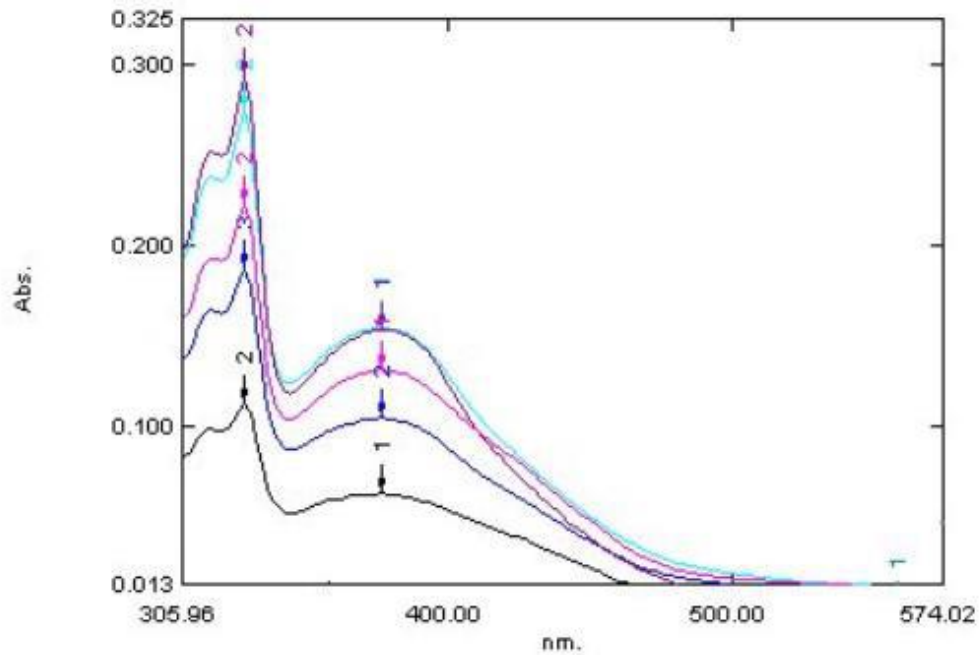


Fig.5.2 Overlay spectrum of darifenacin hydrobromide solution spiked in rat plasma (40-140ng/mL)

Sensitivity(LLOQ)

LLOQ is the least concentration in the calibration curve which is 40ng/mL.

Linearity

The linearity of the method was found to be in the concentration range of 40-140ng/mL. The linearity graph was plotted between concentration vs. absorbance.

The linear regression equation for darifenacin hydrobromide was $y=0.001x-0.0135$ with r^2 0.999.

The calibration curve data is shown in **table 6.1** and the calibration curve is shown in **fig. 6.2**

Table 6.1 Calibration curve data Darifenacin Hydrobromide

| S.No. | Concentration (ng/mL) | Absorbance |
|-------|--------------------------|------------|
| 1 | 40 | 0.061 |
| 2 | 60 | 0.089 |
| 3 | 80 | 0.110 |
| 4 | 120 | 0.160 |
| 5 | 140 | 0.184 |

*Average of three determinations

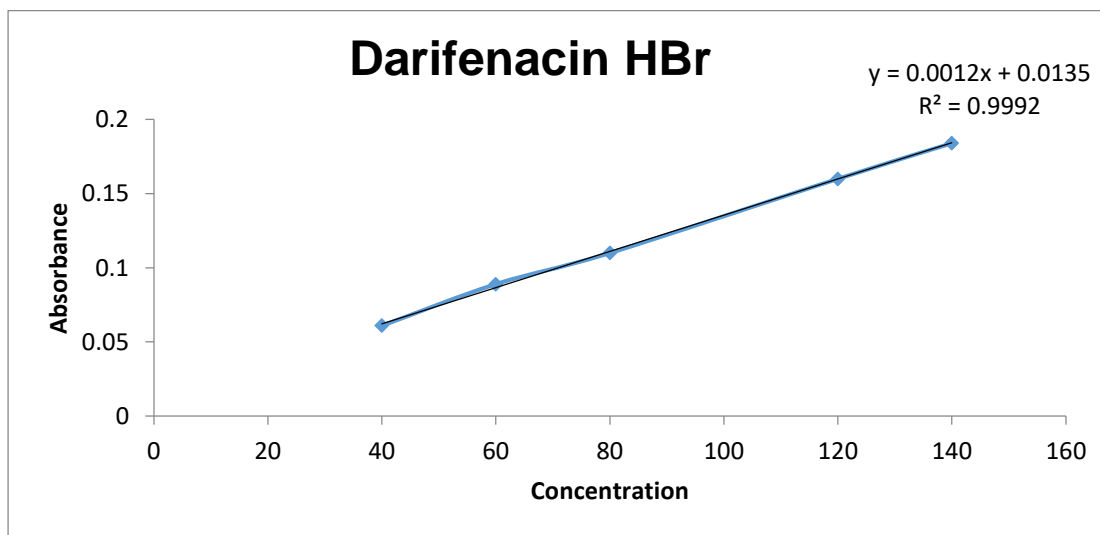


Fig.6.2 Calibration graph of darifenacin hydrobromide solution with plasma

Limit of Detection and Limit of Quantification

The sensitivity of proposed method for measurement of darifenacin hydrobromide was estimated in terms of LOD and LOQ. The calculated values of LOD and LOQ were found to be 5.6 and 17ng/mL respectively.

Accuracy

Accuracy of the method was determined at three different concentrations levels. Mean and %RSD were calculated and shown in **Table 8**.

Table.8 Accuracy data of darifenacin hydrobromide in plasma

| S.No | Accuracy level | Concentration (ng/mL) | Absorbance | Mean* \pm SD | %RSD |
|------|----------------|-----------------------|-------------------------|-------------------|------|
| 1 | 80% | 96 | 0.140 0.142 0.143 | 0.141 \pm 0.001 | 0.7 |
| 2 | 100% | 120 | 0.159 0.156 0.158 | 0.157 \pm 0.001 | 0.6 |
| 3 | 120% | 144 | 0.162 0.161 0.159 | 0.160 \pm 0.001 | 0.6 |

*Average of three determinations

Recovery

Table 8.1 Recovery data of darifenacin hydrobromide tablets

| Formulation | Sample (ng/mL) | Pure (ng/mL) | Amt of plasma added | Amt of methanol added | Total | | |
|-------------------|----------------|--------------|---------------------|-----------------------|-------------------------|---------------------|----------------|
| | | | | | amount of added (ng/mL) | Total amt recovered | Recovered pure |
| Darilong 7.5mg | 120 | 96 | 100 | 400 | 216 | 213 | 99 |
| | 120 | 120 | 100 | 400 | 240 | 237.84 | 99.1 |
| | 120 | 144 | 100 | 400 | 264 | 258.7 | 98.8 |

*Average of three determinations

Precision

Precision of the data was reported in terms of Repeatability, Intra-day and Inter-day precision

Table.9 Repeatability data

| S.No. | Concentration (ng/mL) | Absorbance | %RSD |
|-------|--------------------------|------------|------|
| 1 | 120 | 0.161 | 1.88 |
| 2 | 120 | 0.160 | |
| 3 | 120 | 0.158 | |
| 4 | 120 | 0.160 | |
| 5 | 120 | 0.158 | |
| 6 | 120 | 0.150 | |

Table 9.1 Intra-day precision

| S.No. | Concentration(ng/mL) | Mean absorbance* | | Mean \pm Standard deviation* | %RSD |
|-------|----------------------|------------------|-----------|--------------------------------|------|
| | | Morning | Afternoon | | |
| 1 | LLOQ (40) | 0.061 | 0.064 | 0.063 \pm 0.001 | 1.5 |
| 2 | LOC (80) | 0.116 | 0.114 | 0.114 \pm 0.002 | 1.7 |
| 3 | MQC (120) | 0.165 | 0.163 | 0.163 \pm 0.002 | 1.2 |
| 4 | HQC (140) | 0.189 | 0.187 | 0.184 \pm 0.002 | 1.0 |

*Average of three determination

Table 9.2 Inter-day precision data

| S.No. | Concentration (ng/mL) | Mean absorbance* | | Mean \pm Standard deviation* | %RSD |
|-------|--------------------------|------------------|-------|--------------------------------------|------|
| | | Day 1 | Day 2 | | |
| 1 | LLOQ (40) | 0.061 | 0.063 | 0.062 \pm 0.001 | 1.6 |
| 2 | LOQ (80) | 0.114 | 0.011 | 0.115 \pm 0.002 | 1.7 |
| 3 | MQC (120) | 0.164 | 0.166 | 0.165 \pm 0.001 | 0.6 |
| 4 | HQC (140) | 0.184 | 0.184 | 0.184 \pm 0.002 | 1.0 |

*Average of three determinations

Stability Studies

Freeze and thaw Stability

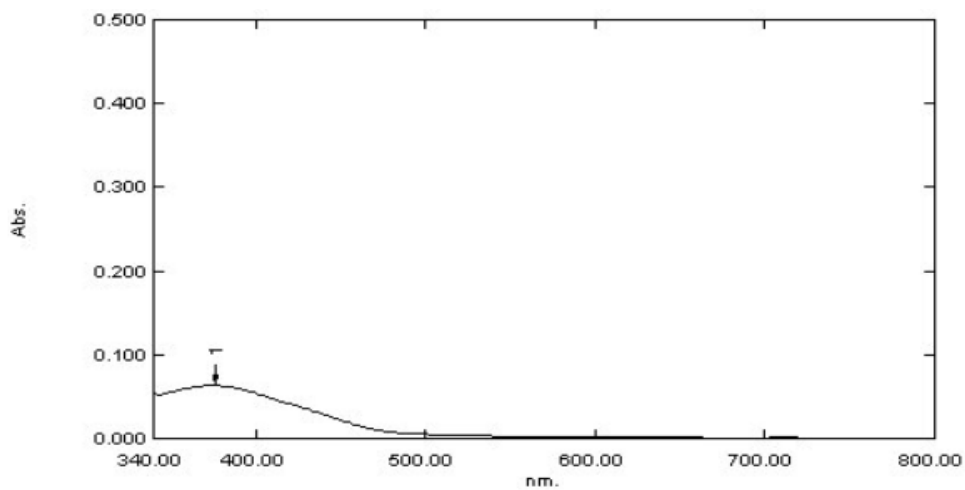


Fig.10. Spectrum of darifenacin HBr after freeze and thaw stability (40ng/mL)

Table 10.1 Stability data darifenacin HBr

| S.No. | Concentration (ng/mL) | Time (hr) | Mean absorbance | Mean | %RSD |
|-------|-----------------------|-----------|-----------------|--|------|
| | | | | Assay ^b ±Standard deviation | |
| 1 | LLOQ (40) | 0 | 0.062 | 0.062±0.001 | 1.6 |
| | | 24 | 0.061 | | |
| 2 | HQC (140) | 0 | 0.183 | 0.184±0.001 | 0.5 |
| | | 24 | 0.185 | | |

Short term temperature stability

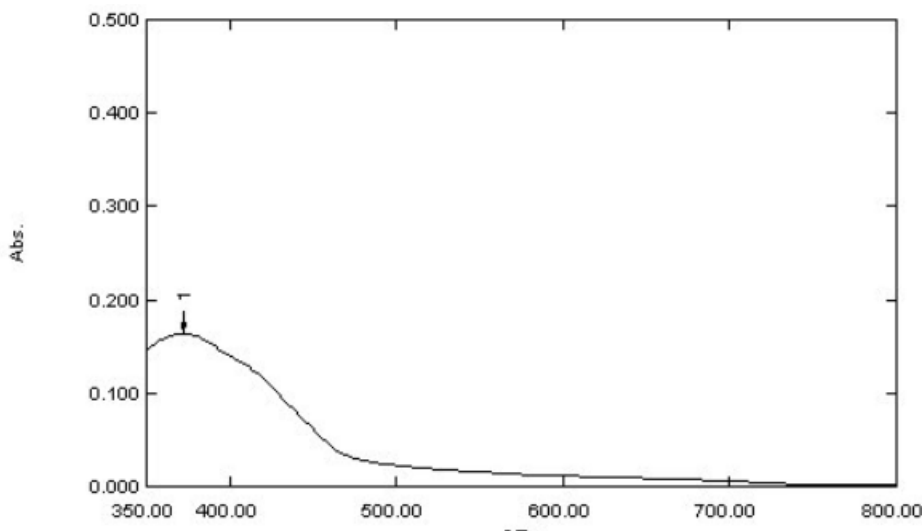


Fig.10.2 Spectrum of darifenacin HBr after short-term temperature stability (140ng/mL)

Table 10.2 Stability data darifenacin HBr

| S.No. | Concentration (ng/mL) | Time (hr) | Mean absorbance | Mean | %RSD |
|-------|-----------------------|-----------|-----------------|--|------|
| | | | | Assay ^b ±Standard deviation | |
| 1 | LLOQ (40) | 0 | 0.061 | 0.060±0.001 | 0.6 |
| | | 72 | 0.059 | | |
| 2 | HQC (140) | 0 | 0.186 | 0.184±0.002 | 1.0 |
| | | 72 | 0.183 | | |

Stock solution stability

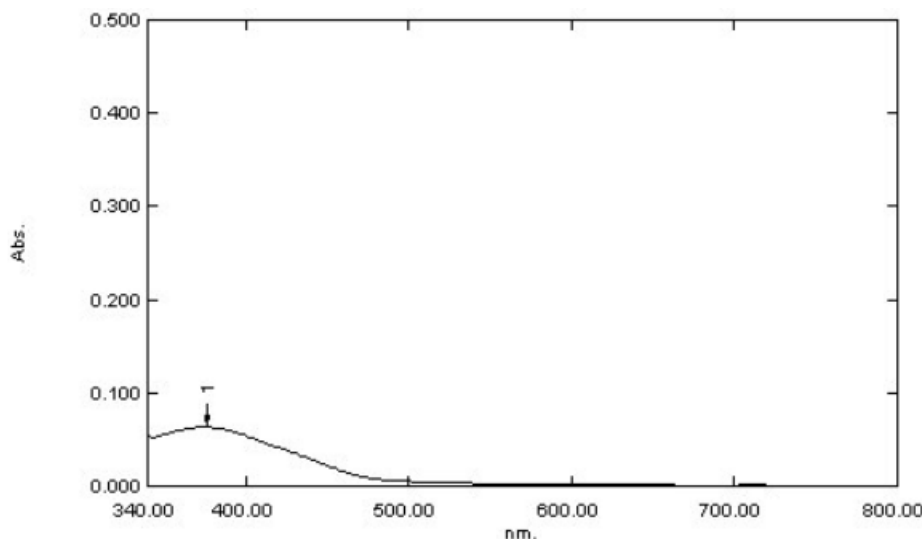


Fig.10.3 Spectrum of darifenacin HBr after Stock solution stability (140ng/mL)

Table 10.3 Stability data

| S.No. | Concentration (ng/mL) | Time (hr) | Mean absorbance | Mean Assay ^b ±Standard | %RSD |
|-------|--------------------------|-----------|--------------------|--------------------------------------|------|
| 1 | LLOQ (40) | 0 | 0.058 | 0.159±0.002 | 1.2 |
| | | 6 | 0.061 | | |
| 2 | HQC (140) | 0 | 0.183 | 0.185±0.002 | 1.0 |
| | | 6 | 0.187 | | |

CONCLUSION

A new sensitive visible spectrophotometric method has been developed for the estimation of darifenacin hydrobromide using coupling reagent, β -naphthol in spiked rat plasma. The method was developed using diazo coupling reaction with the chromogenic reagent, β -naphthol for the estimation of darifenacin hydrobromide. Darifenacin after reaction with sodium nitrite under acidic condition formed a highly reactive intermediate which upon reaction with β -naphthol forms a yellow colored diazo complex. The color intensity range was found to be linear in concentration range of 40-140ng/mL. Analysis was carried out at its λ_{max} 384nm and was optimised and validated according to USFDA guidelines. The developed method was sensitive and the LOQ value was found to be 40ng/mL. All the validation parameters were found to be within the acceptable

limits. As the method was sensitive, it was successfully applied to the analysis of darifenacin hydrobromide in spiked rat plasma.

REFERENCES

1. Rang HP, Dale MM, Ritter JM., FlowerRJ, HendersonG. Rang and Dale's Pharmacology. 7th edition. Spain: Elsevier; 2012.
2. Material Safety Data Sheet of darifenacin hydrobromide.p.1-9. Available from: Datasheets.scbt.com/sc-204708.pdf.
3. Product block of darifenacin hydrobromide. Available from: www.scbt.jp/datasheet-204708-darifenacin-hydrobromide.html.
4. The Merck Index. 14th edition. USA: Merck Research Laboratories; 2006. 477p.
5. Remington The science and Practice of Pharmacy. Volume-II. 21st edition. New Delhi: Wolters Kluwer India Pvt Ltd; 2006.
6. Burgess C and Knowles A, Practical Absorption Spectrometry Techniques in visible and UltraViolet Spectrometry. Volume-3. London: Chapman and Hall;1984.
7. Berry RI and Nash AR. Pharmaceutical Process validation, Analytical method validation, International 3rd edition. New York: Marcel Dekker Inc; 2008.
8. Bioanalytical Method Validation, Guidance for Industry, United states Food and Drug Administration, May 2001
9. ICH, Q2 (R1) Validation of Analytical Procedures: Text and Methodology. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH harmonized Tripartite Guideline, Nov 2005.
10. International Conference on Harmonization, "Q2A: Text on Validation of Analytical Procedures", Federal Register 60(40), 11260-11262 (1995).
11. International Conference on Harmonization, "Q2B: Validation of Analytical procedures: Methodology, 1997; 62 (96), 27463-27467.
12. ChatwalGR and AnandSK. Instrumental Methods of Chemical Analysis. 5th edition. Mumbai: Himalaya Publishing House pvt ltd.;2011.
13. BeckettAH and StenlakeJB. Practical Pharmaceutical Chemistry. 4th edition. Part-Two. New Delhi: CBS Publishers & Distributors; 2004.
14. Micheal E, Schartz IS Krull. Analytical method development and validation Japan: Elsevier Sciences; 2001.

15. USP 24th revision/NF. In: Trustees Bo, editor 2000, 2000.
16. USP28, NF23. The United State Pharmacopoeial Convention. Asian Edition. 2005; 2389.
17. Berry RI and Nash AR. Pharmaceutical Process validation, Analytical method validation, International 3rd edition. New York: Marcel Dekker Inc; 2008.
18. Sharma PP. How to Practice GLP, 3rd edition, Delhi: Vandana Publications Pvt. Ltd; 2012.
19. Sathish NK and Pradeep V. UV Spectrophotometric determination of darifenacin hydrobromide in bulk and pharmaceutical dosage forms. *Der Pharmacia Sinica*. 2011; 2(2): 169-76.
20. Sai PP, Jagathi V, Devala Rao G and Sudhakar G. Visible Spectrometric method for the determination of darifenacin. *Research J Pharm Bio Chem Sci*. 2010; 1(2): 254-56.
21. Sridharan D, Umarani L, Thenmozhi A, Pavan kumar, Aswani Dutt C, Venkata Ramanaiah Mand Phanikishore Y. Development and Validation of UV spectrophotometric method of darifenacin hydrobromide in bulk and tablet dosage form. *Asian J pharm Ana*. 2011; 1(3): 43-45.
22. Siva Rama Krishna GV, Janardhan M and Santhosh Raja M. A simple sensitive UV-spectrophotometric method for the estimation of darifenacin hydrobromide in bulk and pharmaceutical dosage form. *Int J Pharm Inv*. 2012; 2(5):1-5.
23. Radhakrishnanand P, Subba Rao DV and Himabindu V. A Validated liquid chromatographic method for the determination of enantiomeric purity of darifenacin [(S)-enantiomer] in bulk drug and extended release tablets. *Chromatographia*. 2008; 68(11):1059-62.
24. SS Panda, Ravi Kumar BVV, Patel PK and Mohanta G. New stability indicating RP-UPLC method for determination of darifenacin hydrobromide in extended release tablet dosage form. *J Pharm Educ Res*. 2012 Dec; 3(2): 7-12.