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Development and Validation of HPLC Method for Determination of Potential Genotoxic Impurities in Darifenacin Hydrobromide Drug Substance

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ABSTRACT

Simple and sensitive RP-HPLC method was developed, optimized and validated for the determination of Potential Genotoxic Impurities like 5-(2-Bromoethyl)-2,3-dihydrobenzofuran, 5-(2-Chloroethyl)-2,3-Dihydrobenzofuran and Oxidized 5-(2-Bromoethyl) benzofuran contents in Darifenacin hydrobromide drug substance.The analysis was performed on Ascentis® Express C18, 2.7µ (100mm x 4.6mm) (Make: Supelco), maintained at temperature 30°C and UV detection at 205nm. The separation was achieved using Mobile phase, which is prepared by mixing a buffer (mixing 1 ml of orthophosphoric acid in 1000 ml of water) and acetonitrile in the ratio of 50:50%v/v. Flow rate was kept as 0.6 ml/min and injection volume was 20µl. The method was validated as per International Conference of Harmonization (ICH) guidelines in terms of limit of detection (LOD), Limit of quantitation (LOQ), linearity, precision, accuracy, specificity and robustness. The achieved limit of detection (LOD) values were 2, 3 and 5 µg/g, limit of quantification (LOQ) values were 5, 10 and 10 µg/g and the average accuracy values were 101.0, 107.1 and 96.0**%** for 5-(2-Bromoethyl)-2,3-dihydrobenzofuran, 5-(2-Chloroethyl)-2,3-Dihydrobenzofuran and Oxidized 5-(2-Bromoethyl)benzofuran respectively.

INTRODUCTION

Darifenacin hydrobromide [DFH] is chemically known as (*S*)-2-[1-[2-(2,3-Dihydrobenzofuran-5-yl)ethyl]-3-pyrrolidinyl]-2,2-diphenylacetamide hydrobromide. DFH is a novel antimuscarinic agent [1] and it is intended for symptomatic treatment of urge incontinence and increased urinary frequency and urgency as may occur in patients with overactive bladder syndrome [2-3]. In general, antimuscarinic agents work on the principle of blocking the binding of acetylcholine to muscarinic receptors. DFH is marketed under brand name *Enablex* in the form of extended release tablets [4].The empirical formula of DFH is $C_{28}H_{30}N_2O_2$.HBr and molecular weight is 507.5. The recommended maximum daily dose is 15mg /day. The chemical structure of DFH is shown in Figure.1.

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Figure 1: Chemical structure of DFH

Literature revealed that some of the analytical methods have been published for quantification of DFH pure and formulation with its related substances. By using HPLC techniques, Mohammed Nazeerunnis and et.al., Thenmozhi and et.al., and V Raja Kumar et.al. [5-7] and many, Murthy et.al. published UPLC method in 2013 [8] and TLC method also published in 2011 by Satish et.al.

During the preparation of drug substance, some of starting materials and its intermediates are reactive by design and may occur as impurities in the final API. The nature of this chemical reactivity can often be translated into biological reactivity and these materials can often be mutagens or carcinogens. Many times it has been established that due to high chemical relativities the fate of the several genotoxic agents precluded their retention within the final API especially if their formation was separated from the final API by several synthetic steps. Some of these known impurities are potential mutagens or carcinogens but can be difficult or impossible to eliminate completely from the synthetic scheme. 5-(2-Bromoethyl)-2,3-dihydrobenzofuran [BEDBF] is one of the raw materials used in the preparation of DFH. The possible impurities of BEDBF are 5-(2-Chloroethyl)-2,3-dihydrobenzofuran[CEDBF] and

Oxidized 5-(2-Bromoethyl)benzofuran [Oxidized BEDBF]. All of these three compounds are structurally alert and potential genotoxic impurities. Based on the current regulatory guidance's for genotoxic impurities, analytical methods should be developed to meet the required limit of 1.5mg/day daily intake of individual impurity [9-11]. Each impurity limit is considered as 100µg/g with respect to DFH maximum daily dose 15mg/day. To the best of our knowledge, no HPLC method is available in the literature for the trace level quantitative determination of these three genotoxic impurities in DFH. Further, the method is validated to comply the requirements of ICH Validation guidelines [12]. The chemical structures of impurities are shown in Figure 2.

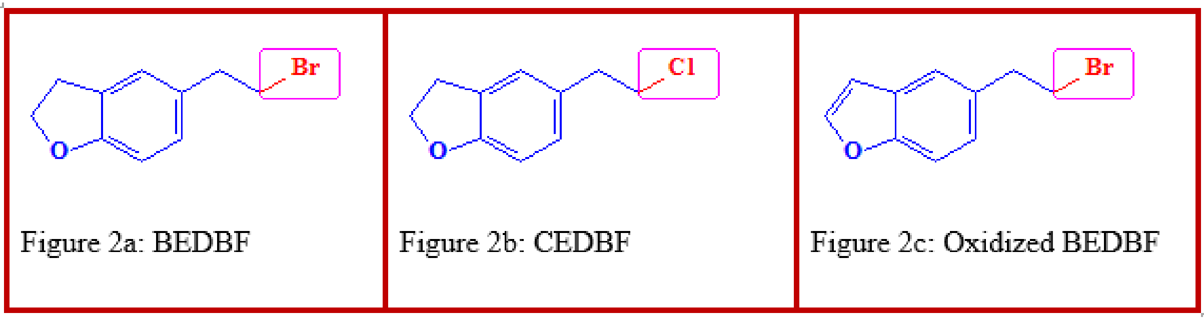

Figure 2: Chemical structures of impurities

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2.0 MATERIALS AND METHODS

2.1 Chemicals, reagents, standards and samples

The investigated samples of DFH drug substance, analyte impurities BEDBF, CEDBF, Oxidized BEDBF and DFH related substances (for specificity experiment) were gifted from APL Research Centre-II Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad). GR grade of Orthophosphoric acid was procured from Merck, India. Acetonitrile was procured from Merck, India and pure milli-Q water was used with the help of millipore purification system (Millipore®, Milford, MA, USA).

2.2 Instrumentation, buffers and chromatographic conditions

A Waters Alliance 2695 separation module equipped with 2996 photodiode array detector with Empower 3 data handling system [Waters Corporation, MILFORD, MA 01757, USA] was used.The analysis was carried out on a stainless steel column 100 mm long, 4.6 mm internal diameter filled with Octadecyl silane chemically bonded to porous silica particles of

2.7 µm diameter [Ascentis® Express C18, 2.7µ (100mm x 4.6mm) (Make: Supelco)] maintained at 30°C temperature. Buffer was prepared by mixing 1 ml of orthophosphoric acid in 1000 ml of water. Further, Mobile phase was prepared by mixing a buffer and acetonitrile in the ratio of 50:50%v/v. Diluent was prepared by a degassed mixture of water and acetonitrile in the ratio of 70:30 v/v. Flow rate was kept as 0.6 ml/min, injection volume was 20µl, chromatographic data acquisition time was 20 min and UV detection was carried out at 205 nm. Retention time of BEDBF was at about 9 minutes. The pump was in isocratic mode.

2.3 Preparation of solutions

2.3.1 Standard solution

0.5 µg/ml BEDBF reference standard was prepared using 20 mg of BEDBF in 100 ml clean, dry volumetric flask. 2 ml of acetonitrile was added to it and sonicated to dissolve. Volume was made up with diluents. 5 ml of this solution was diluted to 100 ml with diluents and filtered through 0.45 µ porosity membrane filter.

2.3.2 Sample solution

5 mg/ml DFH drug substance sample solution was prepared with diluents and filtered through 0.45 µ porosity membrane filter.

2.3.3 Suitability requirements

The column efficiency as determined from the BEDBF peak was not less than 7000 USP plate count and USP tailing for the same peak was not more than 1.5 Relative standard deviation (RSD) for peak areas of BEDBF obtained from six injections of the standard solution was not more than 5.0%.

3.0 RESULTS AND DISCUSSION

3.1 MethodValidation

The developed method was established through the validation experiments as per the ICH Guidelines [12], individually in terms of specificity or selectivity, LOD, LOQ, linearity, accuracy and Precision.

3.1.1 Specificity: According to ICH, Specificity is the ability of the method to determine the individual analyte in presence of other related substances of drug substance. For specificity determination, the analytes (i.e BEDBF, CEDBF, Oxidized BEDBF), DFH related substances RS-1, RS-2 and RS-3 solutions were prepared individually and injected into HPLC to confirm the retention times. Subsequently diluent, solutions of DFH drug substance, DFH drug substance spiked with BEDBF, CEDBF and Oxidized BEDBF (Spiked sample), DFH drug substance spiked with BEDBF, CEDBF, Oxidized BEDBF, RS-1, RS-2 and RS-3(All Spiked samples) were prepared and injected into HPLC to confirm any co-elution with analyte peaks from respective diluents, any of related substances peaks and the peak homogeneity was verified for each analyte using waters empower software and found to be pure (purity angle should be less than purity threshold). The typical HPLC chromatograms of specificity experiments were shown in Figure 3 and results are tabulated in Table 1.

Table 1: Specificity Results

			RT (min)		RRT (w.r.t BEDBF)
Analytes	BEDBF		9.80		1.0
	CEDBF		8.26		0.84
	Oxidized BEDBF		13.07		1.33
Other impurities for information	RS-1		1.56		0.16
	RS-2		1.97		0.20
	RS-3		2.80		0.29
	Spiked Sample		**All Spiked Sample**		
	Purity Angle	Purity Threshold	Purity Angle	Purity Threshold	
BEDBF	0.336	1.702	0.262	1.165	
CEDBF	0.382	1.324	0.451	0.897	
Oxidized BEDBF	0.785	1.846	0.643	1.319	

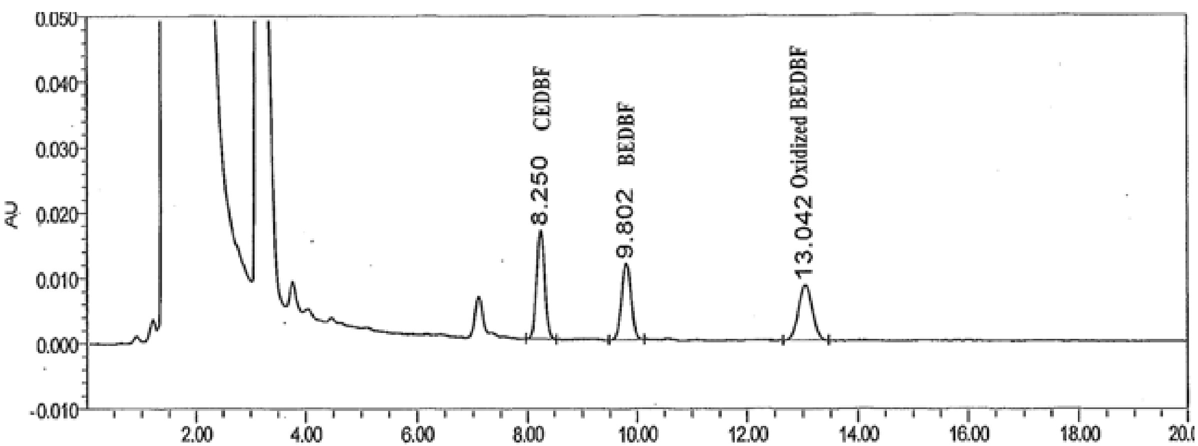

Figure 3: A Typical HPLC Chromatogram of All Spiked sample spiking with BEDBF, CEDBF, Oxidized BEDBF and Related substances

3.1.2 LOD and LOQ

To quantify the limit of detection (LOD) and limit of quantification (LOQ) for BEDBF impurity was determined by using the values of Slope (S), Standard deviation and responses (SN) that have been obtained from linearity studies and using the formula 3.3 x S/SN for LOD and 10 x S/SN for LOQ. For CEDBF and Oxidized BEDBF impurities, LOD and LOQ were determined based on response of analytes. The predicted concentrations of LOD and LOQ for these three impurities were verified for precision by analyzing the solutions containing BEDBF, CEDBF and Oxidized BEDBF at about predicted concentrations and injected each solution six times into HPLC system as per method conditions. Results are tabulated in Table 2.

3.1.3 Linearity

The linearity of the detector was determined by preparing a series of solutions using BEDBF, CEDBF and Oxidized BEDBF at concentration levels from about LOQ to 150% level. The obtained data was subjected to statistical analysis by using a linear regression model. The statistical evaluations like slope, intercept, STEYX and correlation coefficient values of linearity data is given in Table 2.

Table 2: LOD/LOQ and Linearity results

	BEDBF	CEDBF	Oxidized BEDBF
No. of points covered	10	10	7
Concentration range (µg/mL)	0.026 – 0.764	0.051 -0.776	0.052-0.755
Slope	250926	288300	266682
Intercept	388	734	717
STEYX	450	492	759
Correlation coefficient	0.999	0.9999	0.9999
Limit of detection(µg/g)	2	3	5
Limit of quantification(µg/g)	5	10	10
Precision for Limit Of Detection (%RSD)	10.9	0.4	0.9
Precision for Limit Of Quantification (%RSD)	1.8	0.2	0.4

3.1.3 Accuracy

Accuracy of the method was performed by recovery experiments using standard addition technique. The recoveries were determined by spiking BEDBF, CEDBF and Oxidized BEDBF at four concentration levels from about LOQ to 150% levels (i.e LOQ, 50µg/g, 100µg/g and 150µg/g) into DFH drug substance. These samples were prepared as per respective test procedure and analyzed in triplicate and the percentage recoveries were calculated. The % recovery values for analytes ranged from 98.4 – 111.0, 104.0 – 113.8 and 92.7 – 100.7 and the average % recovery of four levels (twelve determinations) were 101.0, 107.1 and 96.0 for BEDBF, CEDBF and Oxidized BEDBF respectively.The fully validated accuracy results are shown in Table 3.

Table 3: Accuracy results

Accuracy	**BEDBF**			
(Average of 3 replicates)	LOQ level	50µg/g level	100µg/g level	150µg/g level
Added (µg/g)	5.00	50.01	99.98	150.47
Recovered (µg/g)	5.51	49.75	101.56	154.77
Recovery (%)	110.2	99.5	101.6	102.9
RSD (%)	0.7	0.6	0.6	0.2
Accuracy	**CEDBF**			
(Average of 3 replicates)	LOQ level	50µg/g level	100µg/g level	150µg/g level
Added (µg/g)	10.11	50.48	100.93	151.89
Recovered (µg/g)	11.35	106.67	106.67	161.93
Recovery (%)	112.26	105.69	105.69	106.61
RSD (%)	2.0	1.0	1.0	0.1
Accuracy	**Oxidized BEDBF**			
(Average of 3 replicates)	LOQ level	50µg/g level	100µg/g level	150µg/g level
Added (µg/g)	10.20	50.97	101.92	153.39
Recovered (µg/g)	9.51	47.76	100.33	147.05
Recovery (%)	93.23	93.70	98.43	95.87
RSD (%)	9.5	1.0	0.6	0.6

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3.1.4 Precision

System precision was established by preparing the standard solutions of individual analytes as per methodology and analyzed by injecting six replicates. Repeatability was the intra-day variation (method precision) and the intermediate precision was the inter-day variation (ruggedness). Method precision was demonstrated by preparing six sample solutions individually using a single batch of DFH drug substance spiked with BEDBF, CEDBF and Oxidized BEDBFseparately at a known concentration level (about 100µg/g) and injected each solution and determined the content of analytes. Achieved results like %RSD and 95% confidence interval for six determinations are summarized in Table 4.

Table 4: Precision results

Experiment	BEDBF	CEDBF	Oxidized BEDBF
	System precision		
%RSD	0.2		
95% Confidence interval(CI)	±265		
	Method precision		
Sample-1	101.3	105.6	100.2
Sample-2	102.1	107.0	100.8
Sample-3	101.5	107.5	100.0
Sample-4	101.5	107.4	100.1
Sample-5	100.5	106.1	100.0
Sample-6	103.1	107.9	100.8
Mean	101.7	106.9	100.3
SD	0.9	0.9	0.4
%RSD	0.9	0.8	0.4
95% Confidence interval(CI)	0.94	0.94	0.42

4.0 CONCLUSION

The HPLC chromatography method was developed, optimized and validated for the determination of BEDBF, CEDBF and Oxidized BEDBF contents in Darifenacin hydrobromide drug substance and the results of various validation parameters demonstrated that the methods are specific, sensitive, linear, precise and accurate and these methods can be introduced into routine use.

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