VALIDATION AND APPLICATION OF REVERSED PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTIFICATION OF PIZOTIFEN MALATE IN PHARMACEUTICAL SOLID DOSAGE FORMULATIONS

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ABSTRACT

The aim of this study was to develop and validate an isocratic reversed phase high-performance liquid chromatographic method for quantification of pizotifen malate in pharmaceutical solid dosage formulations. Good chromatographic separation of pizotifen malate was achieved by using an analytical column, C_{18} ODS column. The system was operated at 40°C oven temperature using a mobile phase consisting of acetonitrile and acetate buffer pH 7.0 (60:40) at a flow rate of 2 ml/min. The method showed high sensitivity with good linearity ($r^2 = 0.99997$) over the tested concentration range of 0.0020 - 0.0300 mg/ml for pizotifen malate. Detection was carried out at 231 nm and retention time was 2.838 min. Placebo and blank studies were performed and no peak was observed at the retention time of pizotifen malate. The intermediate precision and accuracy results (mean ± RSD, n=3) were (99.11±0.21) % and (99.19±0.55) % respectively with tailing factor (1.26±0.19). The proposed method was validated in terms of selectivity, linearity, accuracy, precision, range, detection and quantitation limit, system suitability and solution stability. This method can be successfully employed for simultaneous quantitative analysis of pizotifen malate in pharmaceutical solid dosage formulations.

Keywords: Chromatography, pizotifen malate, placebo study, sedating antihistamine, system suitability, ultraviolet detection.

INTRODUCTION

Pizotifen Malate is 9, 10-dihydro-4-(1-methylpiperidin-4-ylidene)-4H benzo [4,5] cyclohepta [1, 2-b] thiophene hydrogen malate (fig. 1). Its molecular weight is 429.5. It is white or slightly yellowish white, crystalline powder, very slightly soluble in water; slightly soluble in ethanol (96%); sparingly soluble in methanol (British Pharmacopoeia, 2009).

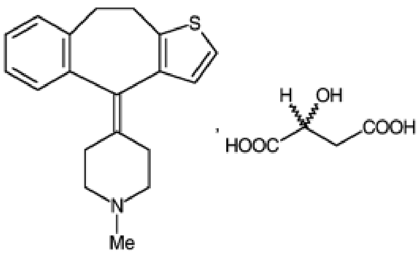

Fig. 1: Chemical Structure of Pizotifen Malate

Pizotifen is a sedating antihistamine that has strong serotonin antagonist and weak antimuscarinic properties. It also antagonises the action of tryptamine. Pizotifen is used, usually as the malate, for the prophylaxis of migraine and for the prevention of headache attacks during cluster periods. It is not effective in treating an acute attack (Martindale, 2009).

Chromatography is a technique applied to separate individual components in a mixture (Raghavan and Joseph, 2002). Reversed-phase HPLC is by far the most popular mode of chromatography. Almost 90% of all analyses of low molecular weight samples are carried out by RP-HPLC (Kazakevich and Lobrutto, 2007).

There were several spectroscopic methods like atomic absorption spectroscopy, colorimetric, spectrophotometric and spectrofluorimetric methods used for the estimation of pizotifen malate in pharmaceutical dosage forms (El-Kousy and Bebawy, 1999; Dessouky, *et al*., 2004). However, all these spectroscopic techniques have number of limitations to accurately determine the pizotifen malate in pharmaceutical dosage forms. On the other hand, RP-HPLC is very simple, fast, highly reproducible and cost effective method to estimate pizotifen malate in pharmaceutical dosage forms (Stout, and Dorsey, 2001).

Here, we reported the development of simple, specific and sensitive and cost effective isocratic RP-HPLC method that will be used for the routine analysis of pizotifen malate in pharmaceutical formulations. The established

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**Corresponding author:* e-mail: mukidpharma@yahoo.com method was validated with respect to selectivity, linearity, accuracy, precision, range, detection and quantitation limit, system suitability and solution stability.

EXPERIMENTAL

Materials and reagents

Pizotifen malate was obtained from Square Pharmaceuticals Ltd, Bangladesh. Acetonitrile, Triethylamine, Ammonium Acetate, Phosphoric acid were obtained from Merck (Germany). All chemicals and reagents were of HPLC grade quality.

Instrumentation

A Shimadzu PC Based Binary Gradient and Isocratic High Performance Liquid Chromatography System equipped with Pump (LC-10ATvp), UV-VIS Detector (SPD 10Avp), Refractive Index Detector (RID-10A), Column C_{18} (ODS HYPERSIL, 10 cm X 4.6 mm, 5 µm), Pre-column (ODS HYPERSIL, 1 cm X 4.0 mm, 5 µm), Temperature Column Oven (CTO-10Avp), System controller (SCL-10Avp), online Degasser, computer and Printer (Model No. LC2010AHT).

Chromatographic conditions

Column C_{18} (ODS HYPERSIL, 10 cm X 4.6 mm, 5 µm) was used for separation and the chromatographs were recorded using Empower2 software. The mobile phase, acetonitrile and buffer (60:40), was pumped at a flow rate of 2 mL/min. It was filtered through 0.20/0.45 µm filter and degassed before use. The elution was monitored at 231 nm and the injection volume was 100 µL. The oven temperature was 40°C.

Preparation of standard and sample solutions

Preparation of buffer solution

25 ml triethylamine was added in 1000 ml 0.1M ammonium acetate (dissolved 7.708 gm ammonium acetate in 1000 ml purified water and mixed) and the solution was adjusted to pH 7.0 with concentrated phosphoric acid and mixed well.

Preparation of mobile phase

A mixture of acetonitrile and buffer at a ratio of 60:40 was prepared with vigorous shaking. The solution was filtered through 0.20/0.45 µm membrane filter and degas before use.

Preparation of standard solution

72.50 mg pizotifen malate WS (equivalent to 50 mg pizotifen) was weighed accurately and kept into 100 ml volumetric flask. It was dissolved in and diluted with mobile phase. 10 ml of the mixture was diluted to 250 ml with mobile phase, mixed well and filtered through Whatman GF/C or equivalent filter paper.

Preparation of sample solution

10 tablets were weighed and recorded individually. One tablet was placed into 25 ml volumetric flask containing about 20 ml mobile phase, and dissolved in mobile phase with the aid of sonication (15 minutes or until completely disintegrated) and shook it for 30 minutes. Volume was diluted with mobile phase and mixed well. Then this mixture was centrifuged for 5 minutes at 4000 rpm. The desired concentration for the drug was obtained by accurate dilution and the analysis was followed up as in the general analytical procedure.

Method validation

The method validation assays were carried out by following International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) Q2B (1996). The following parameters were determined for the validation of analytical assay method of pizotifen malate in pharmaceutical formulation: selectivity, linearity, accuracy, precision, range, detection and quantitaition limit, system suitability and solution stability.

Selectivity

Selectivity is the ability of a method to discriminate between the analyte(s) of interest and other components that are present in the sample. Studies are designed to evaluate the degree of interference, if any, which can be attributed to other analytes, impurities, degradation products, reagent "blanks" and placebo (Lister AS, 2005). Interference of placebo and blank (reagent) in determination of analyte were evaluated by comparing the chromatograms of six placebo and blank samples (figs. 2, 3, 4).

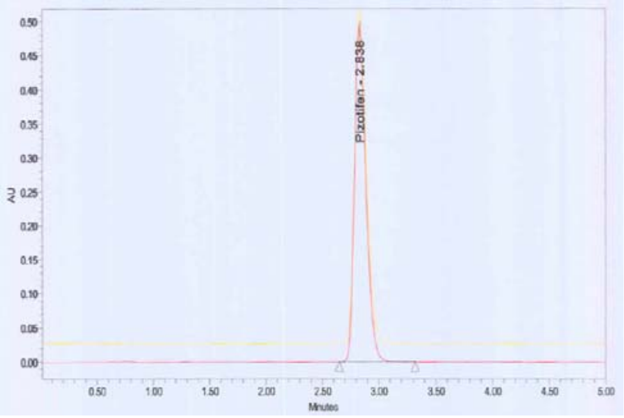

Fig. 2: Typical chromatogram of a 0.02 mg/ml pizotifen solution using the final optimized method Column (C_{18} (ODS HYPERSIL, 10 cm X 4.6 mm, 5 µm) kept at 40 °C, mobile phases: acetonitrile and buffer (60:40), flow rate: 2.0 ml/min, injection volume: 100 µl, UV detection: 231 nm.

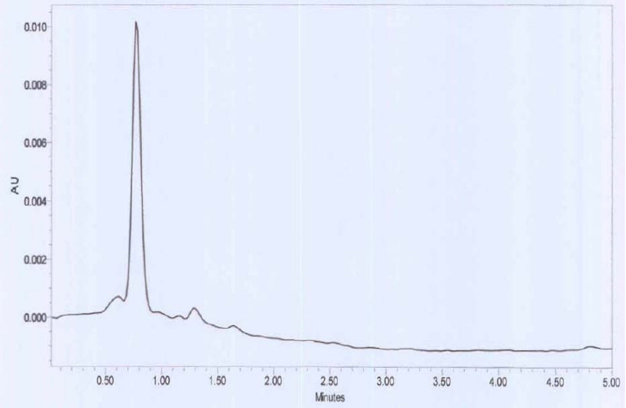

Fig. 3: Chromatogram of blank (acetonitrile and buffer at a ratio of 60:40). Chromatographic conditions are the same as for fig. 2.

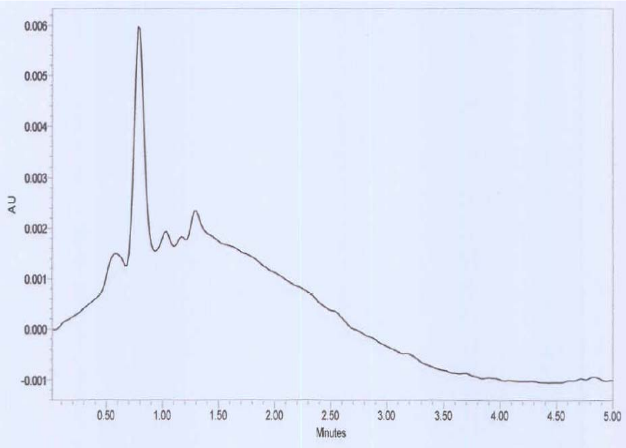

Fig. 4: Chromatogram of Placebo, retention time 0.75 minutes, run time 5.0 minutes.

Linearity

The linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range. For HPLC methods, the relationship between sample concentration and detector response (peak area or height) was used to make this determination. A minimum of five concentration ranges should be investigated and a plot of the detector response vs. the sample concentration should be generated. The seven concentrations (minimum of five concentration ranges) of the standard solution were subjected to regression analysis carried out by ANOVA to calculate calibration equation and correlation coefficients (tables 1, 2).

Range

The specified range is normally derived from the linearity studies. The range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample (Guidelines for the Validation of Analytical Methods for Active Constituent, Agricultural and Veterinary Chemical Products, Australian Pesticides and Veterinary Medicines Authority, 2004). The linearity, precision and accuracy of the HPLC method were determined over a range of concentrations. It was determined by performing six replicate injections.

Table 1: Linearity characteristics of the RP-HPLC method

Linearity		
Concentration in %	Concentration (mg/ml)	Area
10	0.0020	370299
20	0.0040	716252
30	0.0060	1043990
40	0.0080	1404307
50	0.0100	1752770
100	0.0200	3497761
150	0.0300	5188654
slope		172595083
y-intercept		+23775
Correlation coefficient, r		0.99997

Accuracy

Accuracy is the closeness in agreement between the accepted true value or a reference value and the actual result obtained. Accuracy studies were evaluated by determining the recovery of a spiked sample of the analyte into the matrix of the sample to be analyzed. Solutions were prepared containing known amounts of pizotifen in the presence of the excipients in the tablet formulation, at 50%, 100% and 150% of the assay concentration. Sample solutions were prepared triplicates for each spiked level .These solutions were analyzed by the HPLC method against standard at 100% theory (table 3).

Precision

The precision of the method was determined by repeatability (intra day) and intermediate precision (inter day). Repeatability was carried out by performing six repeated analysis of the same working solution of pizotifen, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on three different days (inter-day) and also by another analyst performing the analysis in the same laboratory (between-analysts).

Detection and quantitaition limit

The detection limit of an analytical procedure is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated as an exact value (Lister, 2005). It may be expressed as:

$LOD = 3.3 * SD/S$

Where SD = the standard deviation of the response, S = the slope of the calibration curve

Table 2: Regression statistics by ANOVA of linearity of the RP-HPLC method

Linear Regression					
Regression Statistics					
R	0.999974859				
R Square	0.999949718				
Adjusted R Square	0.999939662				
Standard Error	13470.8220				
Total Number Of Cases	7				
B = 23775.1887 + 172595083.4906 * A					
ANOVA					
	d.f.	SS	MS	F	p-level
Regression	1	18043660923327.100	18043660923327	99434.36	00000
Residual	5	907315222.635	181463044.5		
Total	6	18044568238549.7			
	Coefficients	Standard Error	t Stat	p-level	
Intercept	23775	8065.530953	2.947752457	0.031968	
A	172595083	547343.7814	315.3321356	00000	

Table 3: Accuracy of the test method (Recovery)

Accuracy of the test method (Recovery studies)				
Spiked level (%)	Theoretical Concentration (mg/ml)	Determined Concentration (mg/ml)	% Recovery	Mean Recovery (%) and RSD (%)
50	0.00992	0.00986	99.65	99.55; 0.09
50	0.01000	0.00995	99.50	
50	0.00996	0.00991	99.50	
100	0.01984	0.01971	99.33	99.08; 0.37
100	0.01992	0.01965	98.65	
100	0.01988	0.01973	99.24	
150	0.02988	0.02939	98.38	98.96; 0.87
150	0.02984	0.02941	98.55	
150	0.03000	0.02998	99.95	
Mean and RSD				99.19; 0.55

The quantitation limit is the lowest amount of the analyte in the sample that can be quantitatively determined with defined precision under the stated experimental conditions. The limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products or low levels of active constituent in a product (Lister, 2005). It may be expressed as:

LOQ = 10* SD/S

Where SD = the standard deviation of the response, S = the slope of the calibration curve

System suitability

To ensure the validity of the analytical procedure, a system suitability test was performed. Data from five injections of 100 μL of the working standard solution were used for the evaluation of the system suitability parameters like retention time, area, tailing factor and theoretical plates by the Empower2 software.

RESULTS AND DISCUSSION

Development and optimization of isocratic HPLC conditions

A UV scan of pizotifen malate showed a maximal absorbance at or near 231 nm. Initial method development was conducted on a C_{18} (ODS HYPERSIL, 10 cm X 4.6 mm, 5 μm) column used for separation at oven temperature, 40°C. This column provides efficient and reproducible separations of nonpolar compounds. Consequently, it was selected for method development

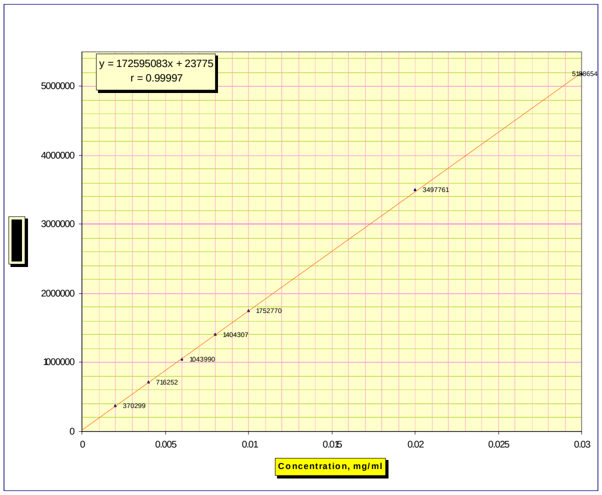

Fig. 5: Linearity curve for Assay Method validation.

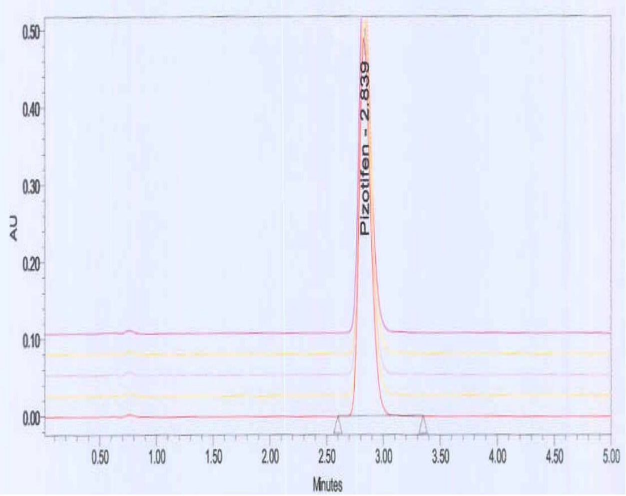

Fig. 6: Typical chromatogram of the system suitability test.

and validation of assay. Preliminary method development of suitable isocratic conditions to resolve pizotifen malate on the column C_{18} (ODS HYPERSIL, 10 cm X 4.6 mm, 5 µm) was conducted with acetonitrile and buffer as the mobile phase for initial method development. A mobile phase of with Acetonitrile and buffer with following ratio 60:40 was found to provide a reproducible, baseline resolved peak (fig. 2). These conditions allowed for separation of pizotifen malate from tablet formulation.

The chromatographic conditions were optimized with respect to specificity, resolution and time of analysis. The specificity of the method was established through the placebo study on common additives used for tablets which shows no peak with placebo (figs. 3, 4). For validation of analytical methods, the guidelines of the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use [ICH 1996] and [USP 2003] have recommended the accomplishment of accuracy tests, precision, specificity, linearity, and robustness of the method.

Method validation

Selectivity

Chromatograms of blank (fig. 3) and placebo (fig. 4) showed the retention time of 0.8 min and 0.75 min respectively where the retention time of analyte was 2.838 min. The absence of a peak eluting at the retention time of the active ingredient is sufficient to demonstrate specificity for excipients (Lister AS, 2005).

Linearity

The linearity of the response of the HPLC method for pizotifen was determined at 10%, 20%, 30%, 40%, 50%, 100%, and 150% of the target concentration. Regression analysis was carried out by ANOVA (table 2). The representative linear equation was y = 172595083x + 23775 where slope 17259508, y intercept +23775, correlation coefficient, R 0.99997(Acceptance criteria, R ≥ 0.999) and $S_{y/x}$ (standard error of estimate) 13470.8220 indicated good positive linearity (Lister AS, 2005). Similar results of correlation coefficient were obtained by Ganesh *et al.* (2009) and Frozna *et al.* (2006). F-ANOVA=99434.36, large number implied significant linearity (table 2). The results indicate that the method is linear over the concentration range studied (fig. 5).

Range

The HPLC method was shown to be linear and have an intercept of zero over a concentration range of 0.0020 to 0.030 mg/ml for pizotifen Malate. The precision of the HPLC system was determined at concentration 100%. The accuracy of the HPLC method was determined over the range of 0.010 to 0.030 mg/ml. This corresponds to a concentration range of 50 to 150% sample concentrations.

Accuracy

The accuracy of the method was carried out by spiked - placebo recovery method. Mean recovery of actual versus theoretical concentration was calculated, 99.19% ± 0.55% (limit 98-102%) (table 3) (Lister, 2005). Since this range includes a recovery of 100%, the HPLC method is considered accurate, and this accuracy is considered to extend to the analysis method for the lower strength.

Precision

Repeatability (intra-day)

Repeatability was studied by calculating the relative standard deviation (RSD) for six determinations of the concentration of about 0.02 mg/mL, performed on the same day and under the same experimental conditions. The results of pizotifen determinations in the working standard solution with the relative standard deviation calculated as 0.12% are shown in table 4.

Table 4: Repeatability (intra day) characteristics of RP-HPLC method

Repeatability (intra day)				
Sample	Theoretical Concentration (mg/ml)	Determined Concentration (mg/ml)	Average recovery (%)	RSD (%)
1	0.02000	0.01988	99.40	0.12
2	0.01996	0.01986		
3	0.01988	0.01977		
4	0.01984	0.01974		
5	0.02000	0.01984		
6	0.01992	0.01979		

Table 5: Intermediate precision (inter-day) data of RP-HPLC method

Intermediate precision (inter day)									
Precision between day					Precision between analyst				
Sample	Day	Recovery[a] (%)	Mean	RSD (%)	Sample	Analyst	Recovery[a] (%)	Mean	RSD (%)
1	1	99.24	99.11	0.21	1	A	99.99	99.48	0.42
	2	99.29				B	98.96		
	3	98.80			2	A	100.79	99.88	0.44
2	1	98.85	98.96	0.18		B	99.96		
	2	98.81			3	A	99.96	99.10	0.40
	3	99.21				B	98.24		

[a] Values are means of three determinations

Table 6: System suitability parameters of the RP-HPLC method

	Retention Time	Area	Tailing Factor	Theoretical Plate
Mean[a]	2.842	3474743	1.26	3650
RSD[a]%	0.130	0.300	0.19	0.870

[a] Values are means of three determinations

Intermediate precision (inter-day)

Intermediate precision includes the estimation of variations in analysis when a method is used within laboratories, on different days, by different analysts, and on different equipments. The intermediate precision was assessed by analyzing two working standard solutions on three different days; the RSD values obtained were 0.21% and 0.18%, respectively (table 5). Between-analysts precision was determined by calculating the RSD for the analysis of three working standard solutions by two analysts; the values were found to be 0.42%, 0.44% and 0.40 %, respectively (table 5).

Detection and quantitaition limit

LOD and LOQ calculated from the data analyzed by ANOVA were 0.0002 mg/ml and 0.0008 mg/ml respectively.

System suitability

The system suitability tests were also carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed (fig. 6). Results of system suitability tests are given in table 6 showing that the parameters are within the suitable range.

Solution stability

Stability of standard solution and sample solutions were assessed at different storage condition. Bench-top stability was evaluated by exposing the sample at room temperature for 12 hours. It also assessed at -15°C for 3 and 6 days. Results, as shown in table 7, demonstrated a good stability of standard solution and sample solutions during all storage conditions and confirmed the applicability of the method for routine analysis.

CONCLUSIONS

This study is the first reported analytical method in which a new RP-HPLC method was developed and validated for the quantification of pizotifen malate in the solid pharmaceutical products. The analytical method is a simple, sensitive, short run time, low volume of injection and selective method suitable for application in a drug manufacturing quality control or regulatory analysis

Table 7: Stability of standard and sample solutions

Sample condition	Theoretical Concentration[a] (mg/ml)	Found[a] (mg/ml)	Recovery (%)	RSD%
Bench-top (room temperature for 12 hrs)	0.0200	0.0198	99.00	0.15
-15^0 C for 3 days	0.0195	0.0192	98.46	0.22
-15^0 C for 6 days	0.0198	0.0193	97.47	0.43

[a] Values are means of three determinations

laboratory of pizotifen malate either in the pure powdered form or available pharmaceutical dosage forms. At retention time of 2.838 min, pizotifen malate was identified. The results of the analysis (accuracy and precision) of tablet dosage forms by the proposed methods are highly reproducible and reliable and are in good agreement with the label claim of the drug. The additives usually present in the pharmaceutical formulations of the assayed samples did not interfere with determination of pizotifen malate by the proposed method. So the system meets the required system suitability and the area RSD% indicates a good degree of precision for the analytical system.

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