EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 9
Issue: 11
204-207
Year: 2022

DEVELOPMENT AND EVALUATION OF M-PHENYLENEDIAMINE AS A GENOTOXIC IMPURITY IN FLUCONAZOLE DRUG SUBSTANCE BY USING HPLC

Vinayak T. Vele*, Vishal Telvekar, Shivaji Kadam, Rupesh Kelaskar and Mohan A. Chandavarkar

R & D Synthetic API Analytical Development Laboratory, FDC Ltd., 142-148, S.V. Road, Jogeshwari (W), Mumbai-400102, Maharashtra, India.

***Corresponding Author: Dr. Vinayak T. Vele**
R & D Synthetic API Analytical Development Laboratory, FDC Ltd., 142-148, S.V. Road, Jogeshwari (W), Mumbai-400102, Maharashtra, India.

Article Received on 03/09/2022 **Article Revised on 24/09/2022** **Article Accepted on 14/10/2022**

ABSTRACT
A simple and accurate method was developed for the determination of M-Phenylenediamine in Fluconazole. Chromatographic separation between M-Phenylenediamine and Fluconazole was achieved using a C18 column using a mobile phase containing buffer and acetonitrile in gradient mode. The resolution between the between M-Phenylenediamine and Fluconazole was found to be more than 7.0. The limit of detection (LOD) and limit of quantification (LOQ) of the M-Phenylenediamine was 0.023 and 0.038 µg mL-1 respectively, for 50 µL injection volume. The percentage recoveries of the M-Phenylenediamine ranged from 93.2% to 115.7% in the samples of Fluconazole. The developed method was validated as per International Conference on Harmonization guidelines in terms of specificity, limit of detection, limit of quantification, precision, linearity, accuracy and ruggedness.

KEYWORDS: Development, Validation, Fluconazole, M-Phenylenediamine, Genotoxic, HPLC.

INTRODUCTION
Fluconazole[1,2] (trade name Diflucan), 2-(2,4-difluorophenyl)-1,3-bis(1,2,4-triazol-1-yl)propan-2-ol (Figure 1), is an antifungal medication used for a number of fungal infections. This includes candidiasis, blastomycosis, coccidiodomycosis, histoplasmosis, dermatophyosis and pityriasis versicolor.[2] It is also used to prevent candidiasis in those who are at high risk such as following organ transplantation, low birth weight babies and those with low blood neutrophil counts.[2,3] It is given either by mouth or by injection into a vein.[2,3]

Fluconazole involves the use of 1-(2,4-Difluoro phenyl)-2- (1H-1,2,4-triazol-1y-l)-ethanone as one of key starting material in the manufacturing process. M-Phenylenediamine is one of the key intermediate used in the manufacturing process of 1-(2, 4-Difluoro phenyl)-2-(1H-1,2,4-triazol-1y-l)-ethanone. So the study is proposed and conducted for the method development and further validation of method for determination of M-Phenylenediamine in Fluconazole drug substance. As per the IARC monograph of M-Phenylenediamine, it is listed in 2A group (Probably carcinogenic to humans) and also it is genotoxic as per the final evaluation.[4,5] The Recommended maximum daily dose for Fluconazole is 2000 mg. Based on daily dose limit of M-Phenylenediamine is proposed and evaluated at 1.5 µg/g level.[6] The limit for the M-Phenylenediamine obtained is 0.75 µg/mL. The results obtained after completion of validation are all within the set acceptance criteria. In previous studies, several analytical instruments were used to determine Phenylenediamines including gas chromatography coupled mass spectrometry (GC-MS)[8,9,10], derivative high-performance liquid chromatography (HPLC)[11,12] and capillary electrophoresis (CE).[13,14]

In the present work we have developed a new, simple precise method for determination of M-Phenylenediamine along with Fluconazole and its known impurities using C18 column by high performance liquid chromatography. The developed method was validated according to International Conference on harmonization (ICH) guidelines[7] for the quantitative determination of the M-Phenylenediamine in Fluconazole.

EXPERIMENTAL SECTION
Chemicals and Reagents
Samples of Fluconazole were obtained from R & D synthetic Department of FDC Ltd, Mumbai, India. HPLC-grade Acetonitrile, Methanol and Ammonium formate was procured from Fischer Scientific, USA. Also, M-Phenylenediamine with certified purity procured from Sigma-Aldrich.

Instrumentation
HPLC system used was Waters (2489 series, US) system equipped with auto sampler, quaternary pump, degasser, and a UV Detector. The output signal was monitored and processed using Empower software.

Chromatographic condition

The chromatographic column used was Inert sustain, Make- C18 column (250 mm x 4.6 mm, 5µm), (GL Science Inc., Japan). The mobile phase used was 0.01M Ammonium formate buffer, was used as a mobile phase A and acetonitrile was used as a mobile phase B. The gradient program time (minutes) % mobile phase B (T%B) was set as 0/18, 10/18, 15/90, 25/90, 35/18 and 50/18 respectively. The flow rate of the mobile phase was 1.0 mL/min. The column and autosampler temperature was maintained at 35°C and 10°C, and the eluent was monitored at a wavelength of 215 nm. The injection volume used was 50 µL. The diluent used was Mobile phase A: Methanol (30:70 v/v).

Preparation of Standard Solutions

The stock solution of the M-Phenylenediamine and Fluconazole were prepared individually by dissolving an appropriate amount of the substances in diluent. For quantitation of M-Phenylenediamine in Fluconazole a solution of 0.075 µg/mL concentration was used. The target analyte concentration was fixed as 100.0 mg mL-1.

RESULTS AND DISCUSSION

Method Development

A solution of Fluconazole and M-Phenylenediamine (100mg/mL & 0.075µg/mL) prepared in diluents for method establishment. To develop a rugged and suitable HPLC method for the separation, different stationary phases and mobile phases were employed. Preliminary column screening involved different types of C18, C8, Cyano, Amino and Phenyl columns were employed. On Inert sustain C18 (250 mm x 4.6 mm, 5µm) column provided selectivity between the Fluconazole peak and the M-Phenylenediamine peak using a mobile phase consisting of buffer-methanol (70:30, v/v), but the resolution between Fluconazole, known impurities and M-Phenylenediamine were closed. We continued to select the best mobile phases that would give optimum resolution and selectivity for the M-Phenylenediamine and Fluconazole. Good separation was achieved on C18 column and buffer-acetonitrile (82:18 v/v) as the mobile phase.

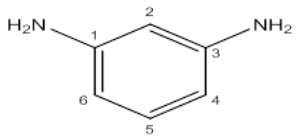
Optimized Chromatographic Conditions

Due to the better chromatographic results obtained on the C18 column, further method optimization and quantification of the M-Phenylenediamine were carried out on this column. Based on the data obtained from method development and optimization activities, the Inert sustain C18 (250 mm×4.6 mm, 5µm) column with the mobile phase of buffer-acetonitrile in gradient was selected for the final method. The flow rate of the experimental method was 1.0 mL/min with an injection volume of 50µL. The column temperature was 35°C, and the detection wavelength was 215 nm. Under these conditions, M-Phenylenediamine and Fluconazole were separated well and the peak of the M-Phenylenediamine eluted before the peak of Fluconazole. In the optimized method, the typical retention times of the Fluconazole and M-Phenylenediamine were approximately 4.88 and 13.42 min, respectively. Baseline separation of Fluconazole and M-Phenylenediamine was obtained with a total run time of 50 min. The system suitability results were given in Table 1.

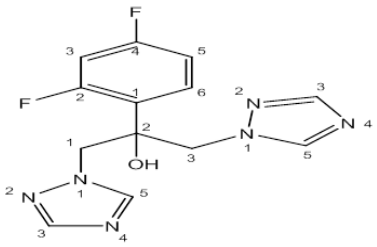
Table 1: System suitability criteria.

Component	Retention time (min)	Relative retention time (min)	Resolution	Tailing factor
M-Phenylenediamine	4.88	0.36	---	1.1
Fluconazole	13.42	1.00	9.5	---

The structure of M-Phenylenediamine and Fluconazole are displayed in Fig. 1. The typical chromatogram of the Fluconazole spiked with M-Phenylenediamine displayed in Fig. 2.



meta phenylene diamine



Fluconazole

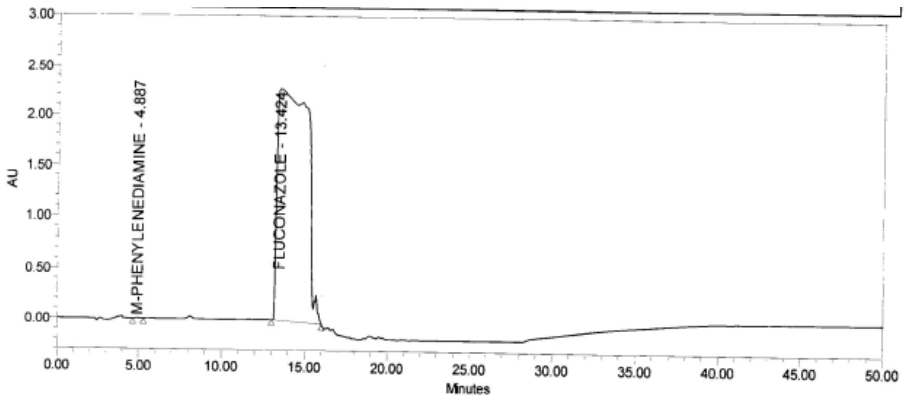


Fig. 2: A typical HPLC chromatogram of Fluconazole spiked with M-Phenylenediamine at specification level.

METHOD VALIDATION

Precision

The precision of an analytical procedure expresses the closeness of agreement among a series of measurements obtained from multiple samplings of the same homogenous sample under prescribed conditions. The system and method precision for the M-Phenylenediamine were checked at its specification level (i.e. 0.075µg/ml with respect to analyte concentration, 100.0 mg mL-1). The percentage RSD of method repeatability and system repeatability for the M-Phenylenediamine were found to be 1.76% and 1.02%, respectively, which confirms good precision of the method.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample. The linearity of the method for the M-Phenylenediamine was checked at six concentration levels, i.e. from limit of quantitation (LOQ) (50%) to 150% of the M-Phenylenediamine specification level (0.075µg/ml), which is with respect to of Fluconazole analyte concentration. The coefficient of regression of the calibration curve was found to be 0.9935, thus confirming the excellent correlation between the peak area and concentration of the M-Phenylenediamine.

Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and limit of quantification were achieved by injecting a series of dilutions of M-Phenylenediamine.[7] The precision of the developed method for M-Phenylenediamine at LOD and LOQ was checked by analyzing six test solutions prepared at the LOD and LOQ level and calculating the percentage relative standard deviation of area. The limit of detection and quantification for M-Phenylenediamine was found to be 0.02 µg mL-1 and 0.04 µg mL-1 respectively for the 50 µL of injection volume.

Ruggedness and Robustness

The ruggedness[7] of a method was defined as degree of reproducibility of results obtained by analysis of the same sample under a variety of normal test conditions such as different laboratories, different analysts, different instruments and different days. The standard addition and recovery experiments carried out for the M-Phenylenediamine in Fluconazole samples at the same concentration levels tested. The data obtained from both the experiment was well in agreement with each other, thus proving the method ruggedness. The robustness[7] of an analytical procedure is measured by its capability to remain unaffected through small, but deliberate, variations in method parameters and provide an indication of its reliability during normal usage. In the varied chromatographic conditions like flow rate, mobile phase ratio and column temperature, the resolution between the peaks of M-Phenylenediamine and Fluconazole was found to be >7.0 illustrating the robustness of the method.

Recovery of M-Phenylenediamine

The standard addition and recovery experiments were conducted for the M-Phenylenediamine in bulk samples of Fluconazole in triplicate at LOQ (50%) (0.04µg/mL), 100% (0.075µg/mL) and 150% (0.112µg/mL) with respect to test concentration. The percentage recovery ranged from 93.2% to 115.7% (Table 2).

Table 2: Summary of method validation data.

Parameter	µg mL^{-1}	r	% Mean recovery	% RSD
LOD	0.02	-	-	3.28
LOQ	0.04	-	-	1.79
Linearity				
(LOQ to 150%)	-	0.9935	-	-
Accuracy				
LOQ (50%) % spiking	-	-	103.1	1.05
100 % spiking	-	-	93.2	0.61

150 % spiking	-	-	115.7	0.44
Precision				
System precision	-	-	-	1.76
Method precision	-	-	-	1.02
Intermediate pre (Ruggedness)	-	-	-	3.90

CONCLUSION

A simple, rapid and accurate High Performance Liquid Chromatography (HPLC) method in ordered to separate M-Phenylenediamine and Fluconazole. Method validation was carried out using a C18 column due to the better chromatographic results achieved on the column. The validated method was demonstrated to be specificity, accurate, precise, selective, sensitive, rugged and robust. The developed and validated method can be implemented for the determination and quantitative of M-Phenylenediamine in Fluconazole bulk drug.

ACKNOWLEDGMENTS

The authors wish to thank the management of FDC's group for supporting this research work. Authors wish to acknowledge the R & D synthetic group for providing the samples for our research.

REFERENCES

1. www.wikipedia.com.
2. www.rxlist.com.
3. European pharmacopeia, 2101; 9.0.
4. IARCMonographs on the Evaluation of Carcinogenic Risks to Humans: M-Phenylenediamine, 1978; 86(16): 111; 1987; 7: 70.
5. Guideline on the Limits of Genotoxic Impurities, Committee for medicinal products for human use (CHMP), European Medicines Agency (EMEA), London, 28 June 2006 (CPMP/SWP/5199/02, EMEA/CHMP/QWP/251344/2006).
6. US Food and Drug Administration CDER Guidance for industry, http://www.fda.gov/cder/guidance/7834dft.pdf (accessed 14 January 2009).
7. ICH Validation of analytical procedures: text and methodology Q2 (R1), International Conference on Harmonization, 2005.
8. Wang PG, Krynitsky AJ. Rapid determination of paraphenylenediamine by gas chromatography-mass spectrometry with selected ion monitoring in henna containing cosmetic products. J Chromatograph B., 2011; 879: 1795-801.
9. Akyuz M, Ata S. Determination of aromatic amines in hair dye and henna samples by ion-pair extraction and gas chromatography mass spectrometry. J Pharm Biomed Anal, 2008; 47: 68-80.
10. Stambouli A, Bellimam MA, El Karni N, Bouayoun T, El Bouri A. Optimization of an analytical method for detecting paraphenylenediamine (PPD) by GC/MS-ion trap in biological liquids. 146 Suppl Forensic Sci Int., 2004; 146: S87-92.
11. Wang SP, Huang T-H. Separation and determination of aminophenols and phenylenediamines by liquid chromatography and micellar electrokinetic capillary chromatography. Anal Chim Acta, 2005; 534: 207-14.
12. Wang LH, Tsai SJ. Simultaneous determination of oxidative hair dye p-phenylenediamine and its metabolites in human and rabbit biological fluids. Anal Biochem, 2003; 312: 201-7.
13. Lin CE, Chen YT, Wang TZ. Separation of benzenediamines, benzenediols and aminophenols in oxidative hair dyes by micellar electrokinetic chromatography using cationic surfactants. J Chromatography A., 1999; 837: 241-52.
14. Hung-Yu Ko, Yi-Hui Lin, Chi-Jen Shih, Yen-Ling Chen, Determination of phenylenediamines in hair colors derivatizated with 5-(4, 6-dichlorotriazinyl) amino fluorescein via micellar electrokinetic chromatography. Journal of food and drug analysis, 2019; 27: 825-831.