

Development and Validation of Newer High Performance Thin Layer Chromatographic Method for Quantification of Eflornithine Hydrochloride in Pharmaceutical Formulations

Amit Kumar¹, Vijender Singh², Praveen Kumar^{3,*}

¹Department of Pharmaceutical analysis, NKBR College of Pharmacy & Research Centre, Meerut, India

²Department of Pharmaceutical analysis, BBS Institute of Pharmaceutical & Allied Sciences, Greater Noida, India

³Department of Pharmaceutical Chemistry, S. D. College of Pharmacy and Vocational Studies, Muzaffarnagar, India

*Corresponding author: praveensha77@gmail.com

Received Mayh 05, 2013; Revised May 19, 2013; Accepted May 20, 2013

Abstract A new, simple, sensitive, precise and robust high performance thin layer chromatography (HPTLC) method was developed for the estimation of eflornithine hydrochloride (DFMO) in pharmaceutical dosage forms. Estimation was performed on TLC aluminum plates precoated with silica gel 60F₂₅₄ as stationary phase. Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase consisting of methanol: chloroform: acetic acid: 1% triethylamine (4:6:0.1:0.5 v/v/v) at room temperature (25±2°C). Analysis of the plate in absorbance mode at 220nm was carried out. The system was found to give compact spots for DFMO with R_f (Retardation factor) value of 0.55 respectively. The data for calibration plots showed good linear relationship with correlation coefficient of 0.999 in the concentration range of 300–800ng mL⁻¹ for DFMO respectively. The values of limit of detection (LOD) were 0.6238ng mL⁻¹ and limit of quantification (LOQ) were 1.8903ng mL⁻¹ for DFMO respectively. The accuracy of the method was 100.44%. The precision demonstrated a relative standard deviation of less than 2.5%. The results were satisfactory when compared with the literature. This new method was validated according to the International Conference on Harmonization (ICH) guidelines which include linearity, precision, accuracy, specificity, robustness, detection and quantitation limits. The developed methods found to be sufficiently precise and reproducible for established conditions and after validation may be used for routine analysis of eflornithine hydrochloride in pharmaceutical formulations.

Keywords: eflornithine hydrochloride (DFMO), quantification, validation, ICH guidelines, HPTLC

1. Introduction

Eflornithine (2-fluoromethyl-DL-ornithine; MDL 71782A; DFMO) is a selective, irreversible inhibitor of ornithine decarboxylase enzyme, one of the key enzymes in the polyamine biosynthetic pathway [1,2]. The drug was originally developed for use in cancer, and is in phase III clinical trials for its use in preventing recurrence of superficial bladder cancer. It has been used as antiprotozoal agent in the treatment of meningoencephalic stage of trypanosomiasis caused by *Trypanosoma brucei gambiense* (African trypanosomiasis) [3,4,5]. It is now licensed for use in sleeping sickness in the USA, Europe and twelve African countries [6]. The drug was originally developed for use in cancer, and is in phase III clinical trials for its use in preventing recurrence of superficial bladder cancer. It has been used as antiprotozoal agent in the treatment of meningoencephalic stage of trypanosomiasis caused by *Trypanosoma brucei gambiense* (African trypanosomiasis) [7,8]. DFMO currently is in development and testing for its anti inflammatory activity [3]. DFMO 13.9%

cream is used to inhibit growth and reduce the amount of facial hair in women. The drug development process of DFMO in these diseases is currently at a relatively early stage, and therefore the full pharmacokinetic characterization in patients, in conjunction with pharmacodynamics (clinical efficacy/safety) is essential for optimization of drug therapy [9].

A number of analytical methods have been reported for measuring DFMO in biological fluids and tissue extracts. These methods involved HPLC techniques [10,11,12]. The HPLC techniques currently available for the quantification of DFMO in biological fluids involve either pre or post column derivatization with UV or fluorescence detection [13,14] and LC carried out by evaporative light scattering detection [15]. Few methods have been reported in the literature for the analysis of DFMO including spectrophotometry [16,17].

Regarding all the above mentioned, we decided to develop a newer HPTLC method suitable for determination of DFMO. The chemical structure of DFMO is presented in (Figure 1).

Since there is no HPTLC analytical method for the determination of DFMO in the pharmaceutical formulations described in the literature. Therefore, the aim

of this work was to develop and validate such a method that was newer, simple, precise, sensitive, selective, economic, rapid and accurate.

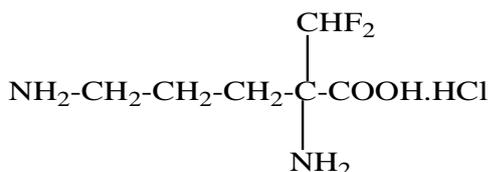


Figure 1. Chemical structure of eflornithine hydrochloride (MDL 71782A)

In pharmaceutical industry the analysis of pharmaceuticals is an integral and increasingly important part of an overall drug development process. Therefore, rapid and simple methods for routine analysis and quality control of commercial formulations are very desirable. Hence, in the present study, new, simple and selective high performance thin layer chromatography (HPTLC) method for the determination of DFMO drug in commercially available pharmaceutical preparations were developed.

2. Materials and Methods

2.1. Equipment

The instrument used in the present study was Camag Linnomat V- automatic sample applicator, Camag microliter syringe (100 μ l), Camag TLC scanner 3, Camag Twin trough chamber of appropriate size, Analytical weighing balance (Shimadzu AX 200), Sonicator (model SONICA 2200MH) were used throughout the experiment. Camag Wincats software was used for acquisition, evaluation and storage of chromatographic data.

2.2. Reagents and Chemicals

Reference standard of Eflornithine certified to contain 99.96% purity and Pharmaceutical preparation: Ornidyil Vial (SVP) 200mg/ml of DFMO was received as a gift samples from WINTAC Limited, Bangalore, India. Silica gel (TLC plates) 60F₂₅₄ (10cm \times 10cm with 0.2mm thickness) were used as stationary phase. All chemicals and reagents used were of analytical grade and obtained from Qualigens. Methanol was from Sigma-Aldrich. Silica gel HPTLC plates (10x10cm) were from E. Merck, Germany using a Camag Linomat automatic TLC sampler 5 (Switzerland).

2.3. Preparation of Solutions

2.3.1. Stock and Working Standard Solutions

A standard stock solution containing 1000ng mL⁻¹ of DFMO was prepared by dissolving in methanol separately. A working standard solution was prepared separately from the standard stock and further diluted with methanol to obtain the different working standard solutions ranging from 300-800ng mL⁻¹.

2.3.2. Sample Solutions

The sample vial (SVP) containing 200mg mL⁻¹. Pipetted out 1ml and diluted to 100ml with methanol.

Now further dilutions were done to get the final concentration of 1000ng mL⁻¹. The solutions were sonicated for 30 min and filtered through 0.45 μ nylon membrane filter. From the filtrate 10mL of solution was taken and used for further reading. The proposed HPTLC method was applied and the concentration of each component in both the formulations was determined.

2.4. Preparation of Calibration Curves

A methanolic solution of DFMO (1mg/ml) was prepared. This solution was further diluted with methanol to yield a solution containing 1 μ g mL⁻¹. Different concentrations of DFMO in a concentration range of 300-800ng/ml were applied on plates as 8mm bands, 8mm apart and 1cm from edge of the plate, by means of Camag Linomat V automatic sample applicator fitted with 100 μ l Hamilton syringe. A methanol blank was applied to parallel track. The mobile phase consisted of methanol: chloroform: acetic acid: 1% triethylamine (4:6:0.1:0.5 v/v/v). Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature. The length of chromatogram run was approximately 80mm. Subsequent to the development; TLC plate was dried in a current of air with the help of an air-dryer. Densitometric scanning was performed on Camag TLC scanner 3 with winCATS software (slit-micro, 6x0.30mm) in the absorbance mode at 220nm for the estimation of DFMO. The source of radiation utilized was deuterium lamp (D₂ Lamp) and then the calibration curves were set up by plotting peak area against the drug quantity per spot. The equations of calibration curves were estimated using linear and non-linear regression analysis and correlation coefficients (r) were calculated. The linear regression data for the calibration plots illustrate a good linear relationship over a concentration range of 300-800ng spot⁻¹ for DFMO with respect to the peak area. Each concentration was repeated six times. Area is plotted graphically as a function of analyte concentration (Figure 2). Chromatogram is shown in (Figure 3).

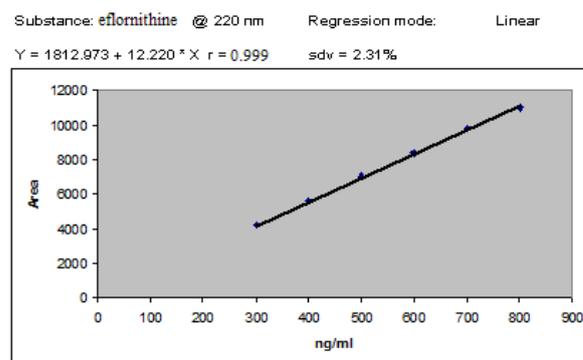


Figure 2. Calibration curve of DFMO at 220nm

2.5. Method Validation

2.5.1. Linearity and Range

The linear regression data for the calibration plots illustrate a good linear relationship over a concentration range of 300-800ng spot⁻¹ for DFMO with respect to the peak area. From the statistical treatment of the linearity

data of DFMO, it is clear that the response of DFMO is linear between lower to higher level. Each concentration was repeated six times and obtained information on the

variation in peak area response. Area is plotted graphically as a function of analyte concentration (Figure 2). Chromatogram is shown in (Figure 3).

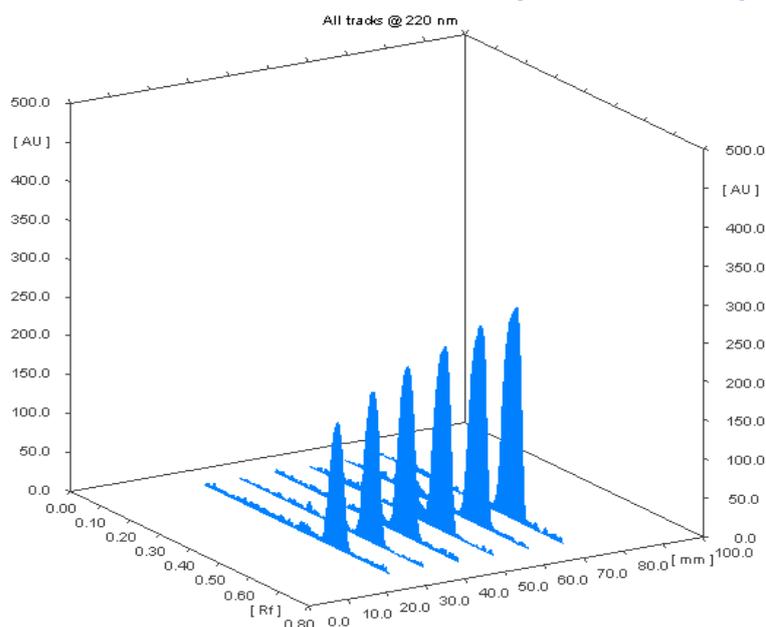


Figure 3. Chromatograms showing linearity of DFMO

2.5.2. Accuracy

Accuracy was found out by recovery study from prepared mixture at three levels of standard addition 50%, 100% and 150% by considering 800ng mL⁻¹ concentrations as 100% for DFMO respectively. Accuracy is often expressed as percentage recovery by the assay of known added amounts of analyte. The accuracy is calculated as the percentage of the analyte response of sample compared to that of a solution containing the analyte at a concentration corresponding to 100.44% recovery (Table 1).

Table 1. Recovery Study from Synthetic Mixture

DFMO		
Excess drug added to analyte (%)	Conc. (ng mL ⁻¹)	Percentage Recovery ±RSD
0	400	98.9049±0.3226
50%	600	101.654±0.4956
100%	800	99.5157±1.64
150%	1000	101.6944±1.592

2.5.3. Precision

The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample.

2.5.3.1. Method Reproducibility

Method reproducibility was demonstrated by intraday and inter-day precision measurements of peak area for each title ingredient.

The intraday (within-day in three replicates) and inter-day precision (for three days) was carried out using three different concentrations (300, 400, 500ng spot⁻¹ of DFMO) in five times. The result expressed in terms of percent relative standard deviation (%R.S.D.). The obtained results within and between days were in acceptable range indicating good precision of the proposed method (Table 2 and Table 3).

Table 2. Precision Study Results (Intraday)

Intraday	Conc. (ng spot ⁻¹)	Mean area	%RSD	%recovery
DFMO	300	4195.333	0.947087	97.66
	400	5453.233	0.815223	99.166
	500	6426.367	0.23344	100.542

Table 3. Precision Study Results (Inter-day)

Inter-day	Conc. (ng spot ⁻¹)	Mean area	%RSD	%recovery
DFMO	300	4391.333	0.383665	99.60
	400	5503.1	0.373295	98.89
	500	6570.567	1.240529	99.23

2.5.4. Robustness

By introducing small changes in the mobile phase composition, duration of mobile phase saturation and activation of pre-washed TLC plates with methanol, the effects on the results were examined. Robustness of the method was done in triplicate at concentration level of 800ng spot⁻¹ and the %R.S.D. was calculated. The low values of %R.S.D. indicated the robustness of the method (Table 4).

Table 4. Robustness of the HPTLC Method (n = 800 ng spot⁻¹ of DFMO)

Robustness	Wavelength(nm)			Mobile Phase	
	208	220	248	2:6:0.05:1(v/v/v)::1(v/v/v)	1:4:0.05:0.5(v/v/v)
area	6456.5	4629.6	3256.2	4394.0	5501.1
%RSD	1.371	0.4493	0.2161	0.446914	0.98816

2.5.5. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Calibration curve was repeated for 6 times and the standard deviation (SD) of the intercepts was calculated. The limit of detection was calculated by LOD=3.3σ_S,

where σ is the standard deviation of the response of the blank or the standard deviation of intercepts of regression lines and S is the slope of the calibration curve. The limit of quantification was calculated by $LOQ=10\sigma/S$ under the ICH guidelines [18]. The values of LOD, LOQ and all analytical parameter for the determination of eflornithine hydrochloride are given in (Table 5).

Table 5. Analytical Parameters for the Determination of DFMO Using the Proposed Method

Parameter	DFMO
λ_{max} (nm)	220
Beer's Law Limit (ng mL ⁻¹)	300-800
Linear regression equation $A = mC + b$	
Slope (m)	1812.973
Intercept (b)	12.220
Correlation coefficient (r)	0.999
LOD (ng mL ⁻¹)	0.6238
LOQ (ng mL ⁻¹)	1.8903
Standard deviation (%)	2.31
Percentage Recovery	100.44

* A is the absorbance and b concentration in ng mL⁻¹.

2.6. Analytical Procedure for the Assay of DFMO in Pharmaceutical Formulations

Applicability of the proposed method was tested by analyzing the commercially available Ornidyl the sample vial (SVP) to containing 200mg mL⁻¹ of DFMO. The pharmaceutical sample solutions were prepared as described in experimental section. The analysis was repeated in triplicate. Active component was analyzed separately to study the interference of pharmaceutical excipients during estimation.

A single spot at $R_f=0.55$ was observed when scanned at 220nm in the chromatogram of the commercial formulation which was compared with standard mixture. There was no interference from the excipients and the other active components present in the formulation (Figure 4). The values of % recovery from formulation as shown in the (Table 6) was found to be very close to each other as well as to the label value of commercial pharmaceutical formulation, and is within the limit (label claim $\pm 5\%$) which shows that the method is applicable for determination of DFMO.

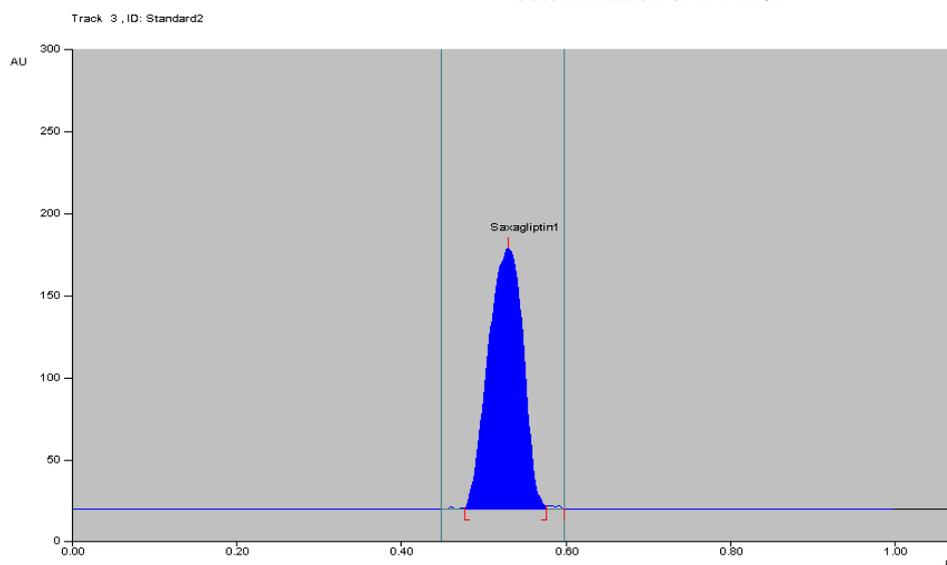


Figure 4. Chromatogram of assay of DFMO

Table 6. Analysis of Commercial Formulation (Ornidyl)

DFMO		
Conc. Spotted (ng mL ⁻¹)	Conc. Found (ng μ L ⁻¹)	Percentage recovery \pm %RSD
1000	991.919	99.1919 \pm 0.67

3. Results and Discussion

The objective of the proposed work was to develop simultaneous methods for the determination of DFMO, and to validate the methods according to USP and ICH guidelines and applying the same for its estimation in marketed formulations. There is no official method for the estimation of DFMO.

In stability indicating HPTLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to separate title ingredients. Mobile phase

selection was based on R_f (retardation factor) values and peak purity parameters (peak start, peak apex, peak end position of the spot). The solvent system with methanol: chloroform: acetic acid: 1% triethylamine (4:6:0.1:0.5:v/v/v) was quite robust.

The optimum wavelength for detection was 220nm at which better detector response for both the drugs was obtained. The average retardation factor for DFMO was found to be 0.55 ± 0.002 min, respectively. The data for calibration plots showed good linear relationship with $r^2=0.996$ with concentration range of 300-800ng mL⁻¹ respectively. The low values of % R.S.D indicate the method is precise and accurate. The mean recoveries were found in the range of 98-102 %.

Sample to sample precision and accuracy were evaluated. Day to day variability was also assessed over a period of three days. These results showed the accuracy and reproducibility of the assay.

Robustness of the proposed method was determined by analysis of aliquots by varying saturation time and ratio of the mobile phase conditions; the % R.S.D. reported was

found to be less than 2%. The proposed method was validated in accordance with ICH parameters and the applied for analysis of the same in marketed formulations.

The major advantage of HPTLC was that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis. Suspensions, dirty or turbid samples can be directly applied. It facilitates automated application and scanning in situ. HPTLC facilitates repeated detection (scanning) of the chromatogram with the same or different

parameters. The aim of this work was to develop an accurate, specific, repeatable method for the determination of DFMO per ICH guidelines. Initially methanol: chloroform: acetic acid: triethylamine in varying ratios were tried. Finally, the mobile phase consisting of methanol: chloroform: acetic acid: 1% triethylamine (4:6:0.1:0.5 v/v/v) gave a sharp and well-defined peak at R_f value of 0.55 for DFMO (Figure 5). Well-defined spots were obtained when the chamber was saturated with mobile phase for 20 min at room temperature.

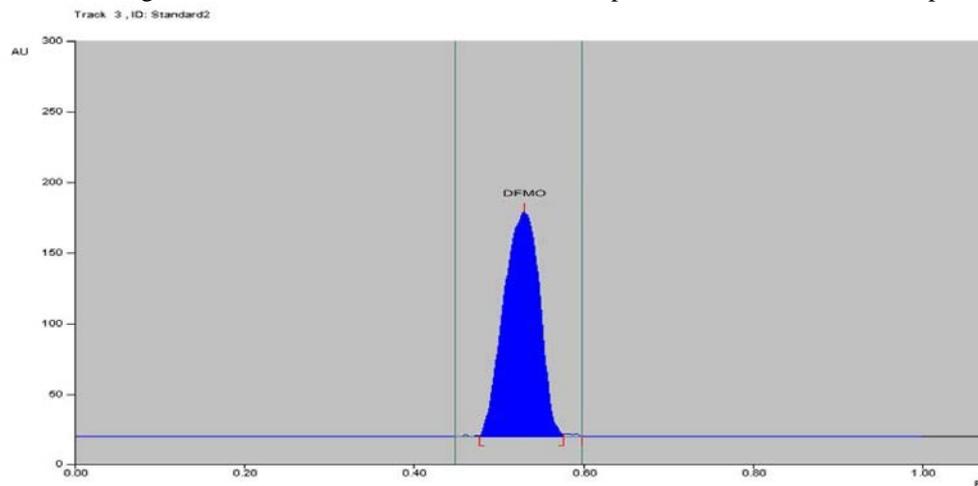


Figure 5. Representative densitogram of DFMO R_f 0.55 respectively

4. Conclusion

The developed HPTLC technique was found a precise, specific, accurate and robust for the simultaneous determination of DFMO. Statistical analysis proves that the method was reproducible and selective for the analysis of title ingredients. Since the proposed mobile phase effectively resolves DFMO the method can be used for qualitative as well as quantitative analysis of DFMO in commercial formulations as well as in laboratory prepared mixtures.

Acknowledgements

This study is a part of Ph.D. research work of Mr. Amit Kumar, registered in IFTM University, Moradabad 244102 India, which is done at NKBR College of Pharmacy & Research Centre, Meerut, Uttar Pradesh, India. We thank Mr. S.P.Venkatesh Prasad, PEC College of Pharmacy, Bangalore, India, for his support and Wintac Ltd, Bangalore, India, for providing a sample of DFMO as a gift.

Competing Interests

NONE

Abbreviations

UV, ultra-violet; HPTLC, high-performance thin layer chromatography.

References

- [1] Bacchi, C. J., Goldberg, B., Carofalo-Hannan, J., Rattendi, D., Lyte, P. and Yarlett, N, "In vivo effects of α -DL-Difluoromethylornithine on the metabolism and morphology of *Trypanosoma brucei brucei*," *J. Biochem.*, 309. 737-40. 1995.
- [2] Bitonti, A. J., Bacchi, C. J., McCann, P. P. and Sipertsma, A, "Intestinal changes caused by DL- α -difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase," *Biochem. Pharmacol.*, 34. 1773-78. 1985.
- [3] McCann, P. P., Bbacchi, C. J. and Clarkson, A. B, "In vivo effects of α -DL-Difluoromethyl ornithine on the metabolism and morphology of *Trypanosoma brucei*," *Med. Biol.*, 59. 434-37. 1981.
- [4] Sjoerdsma, A, "Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL- α -difluoromethylornithine), an inhibitor of ornithine decarboxylase," *Clin. Pharmacol. Ther.*, 30. 3-8. 1981.
- [5] Sjoerdsma, A. and Schechter, P, "Difluoromethylornithine in the treatment of African trypanosomiasis," *Clin. Pharmacol. Ther.*, 35. 287-90. 1984.
- [6] Available: http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.SearchAction&SearchType=BasicSearch&searchTerm=eflornithine&Search_Button=Submit. [April 18, 1986].
- [7] Pepin, J., Guern, C., Milord, F. and Schechter, P. J, "Difluoromethylornithine for arseno-resistant *trypanosoma brucei* gambiense sleeping sickness," *The Lancet*, 330. 1431-1433. 1987.
- [8] Merali, S. and Clarkson, A. B. Jr, "Polyamine content of *Pneumocystis carinii* and response to the ornithine decarboxylase inhibitor DL-alpha-difluoromethylornithine," *Antimicrob. Agents Chemother.*, 40. 973-978. 1996.
- [9] Balfour, J. A. and McClellan, K, "Topical eflornithine," *Am. J. Clin. Dermatol.*, 2(3). 197-201.
- [10] Cohen, J. L., Ko, R. J., Lo, A. T., Shields, M. D. and Gilman, T. M, "High-pressure liquid chromatographic analysis of eflornithine in serum," *J. Pharm. Sci.*, 78(2). 114-6. Feb. 1989.
- [11] Huebert, N. D., Schwartz, J. J. and Haegele, K. D, "Analysis of 2-difluoromethyl-DL-ornithine in human plasma, cerebrospinal fluid

- and urine by cation-exchange high-performance liquid chromatography," *J. Chromatogr. A.*, 762(1-2). 293-8. Feb. 1997.
- [12] Saravanan, C., Kumudhavalli, M. V., Kumar, M. and Jayakar, B, "A new validated RP-HPLC method for estimation of eflornithine hydrochloride in tablet dosage form," *J. Phar. Res.*, 2. 1730-1731. 2009.
- [13] Kilkenney, M. L., Slavik, M., Christopher, M. R. and Stobaugh, J. F, "Plasma analysis of alpha-difluoromethylornithine using pre-column derivatization with naphthalene-2,3-dicarboxaldehyde/CN and multidimensional chromatography," *J. Pharm. Biomed. Anal.*, 17. 1205-1213. 1998.
- [14] Jansson-Löfmark, R., Römsing, S., Albers, E. and Ashton, M, "Determination of eflornithine enantiomers in plasma by precolumn derivatization with *o*-phthalaldehyde-*N*-acetyl-L-cysteine and liquid chromatography with UV detection," *Biomed. Chromatogr.*, 24(7). 768-773. July 2010.
- [15] Malm, M. and Bergqvist, Y, "Determination of eflornithine enantiomers in plasma, by solid-phase extraction and liquid chromatography with evaporative light-scattering detection," *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, 846. 98-104. 2007.
- [16] Kumar, A., Venkatesh, Prasad, S. P., Mohan, S. and Kumar, P, "Spectrophotometric determination of eflornithine hydrochloride as active pharmaceutical ingredient using sodium 1,2-naphthoquinone-4-sulfonate as the derivative chromogenic reagent," *Trade Sci. Inc.*, 7, 2008.
- [17] Kumar, A., Venkatesh, Prasad, S. P., Mohan, S. and Singh, A. K, "Estimation of eflornithine hydrochloride by UV spectroscopy," *Trade Sci. Inc.*, 8. 2009.
- [18] Validation of Analytical Procedures, Methodology ICH Harmonised Tripartite Guideline, Having Reached Step 4 of the ICH Process at the ICH Steering Committee meeting on November 6, 1996.