

Original Research Article

Spectrophotometric Determination of Eflornithine Hydrochloride using Vanillin as Derivative Chromogenic Reagent

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Abstract

Purpose: To develop and validate a spectrophotometric method for the quantitative determination of eflornithine hydrochloride as a pure compound and in pharmaceutical formulations.

Methods: The method involved the reaction of the target compound with vanillin reagent at specific pH 5.6 to produce a green reddish color chromogen. The derivative chromogen exhibits absorption maxima at 578 nm. At the pH of the reaction, there was no degradation. The developed method was validated as per International Conference of Harmonization (ICH) guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness.

Results: Under the indicated conditions, the proposed method was linear over the concentration range of 5 - 25 µg/ml and the correlation coefficient (r^2) was > 0.998 ($n = 6$) with a relative standard deviation (RSD) of 1.003 % ($n = 6$). Mean recovery of the target compound was 100.58 % with a limit of quantification (LOQ) of 4.3 µg/ml and limit of detection (LOD) of 1.2 µg/ml.

Conclusion: The results demonstrate the stability-indicating power of the proposed method which is precise, accurate, simple and economic. Thus, the method can be applied to the routine analysis of eflornithine hydrochloride in bulk and pharmaceutical formulations.

Keywords: Eflornithine hydrochloride, Vanillin, Spectrophotometric assay, Validation, ICH guidelines

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INTRODUCTION

Eflornithine hydrochloride (difluoromethylornithine, DFMO) is a selective, irreversible inhibitor of ornithine decarboxylase enzyme, one of the key enzymes in the polyamine biosynthetic pathway [1]. Eflornithine hydrochloride (DFMO) has the chemical name (2-fluoromethyl- -ornithine).

DFMO is an antiprotozoal agent which is used for meningoencephalic stage of trypanosomiasis. It

is caused by *Trypanosoma brucei gambiense*, African trypanosomiasis [1,2]. DFMO is effective in the treatment of facial hirsutism [3] as well as in African trypanosomiasis, also known as sleeping sickness [2].

The methods available for the determination of DFMO include high pressure liquid chromatography techniques [3-5]. The high pressure liquid chromatography methods available for the determination of eflornithine involve either pre- or post-column derivatization

with UV or fluorescence detection [6,7] and LC carried out by evaporative light scattering detection [8]. Few analytical developed methods have been proposed for the determination of DFMO [9,10].

The aim of this work was to develop a sensitive and simple spectrophotometric method for the quantification of DFMO using a specific derivative chromogenic reagent (vanillin) for the analysis of the target compound in pharmaceutical preparations without the requirement of further separation.

The developed techniques were validated for parameters including linearity, accuracy, precision, specificity and selectivity, detection and quantification limits.

EXPERIMENTAL

Materials and reagents

All chemicals and reagents used were of analytical grade purity, taken in an accurate amount and was not further standardized. Eflornithine hydrochloride is marketed under the trade name Ornidyl. Each sample vial (SVP) is containing 200 mg/ml. The pure drug (DFMO) was a gift from Wintac Limited, Bangalore, India. Reagents used included vanillin, potassium hydrogen phthalate, sodium hydroxide, hydrochloric acid potassium dihydrogen phosphate were supplied from Merk Germany. Anhydrous glacial acetic acid (GAA) was purchased from Rankam group Ltd, Hong Kong. Double-distilled water was used through this study.

Preparation of solutions

Universal buffer solution of different pH values ranging from 2 to 6 were prepared by adjusting 100 ml solution of the acid mixture to the desired pH value using 0.1 N NaOH solutions. Acid phthalate buffer and neutralised phthalate buffer solutions were prepared using recommended method by Indian Pharmacopoeia [11].

Potassium hydrogen phthalate and potassium dihydrogen phosphate (each 0.2 M) solutions were prepared by dissolving the accurately weighed (40.86 and 27.218 g) of potassium hydrogen phthalate in double distilled water and was made up to the mark in a 1000 ml volumetric flask respectively.

Vanillin solution (1 % w/v) was prepared by dissolving the accurately weighed 1 g of vanillin

in double distilled water and was made up to the mark in a 100 ml volumetric flask.

Apparatus

Spectrophotometric measurements were carried out on a UV-1700 Shimadzu double beam spectrophotometer (Japan) with a fixed slit width of 2 nm using a pair of 1cm matched quartz cells. All pH measurements were made with Digisun D1-707 digital pH meter.

Preparation of stock solutions of DFMO

Eflornithine hydrochloride (100 mg) was dissolved in anhydrous glacial acetic acid (GAA) into 100 ml volumetric flask and was made up to the mark in a 100 ml volumetric flask. Now pipette 50 ml of this solution and diluted up to 100 ml with (GAA) to get 500 µg/ml.

This solution was prepared daily. Working standard solutions were prepared immediately before use by a suitable dilution of the corresponding stock solution to appropriate concentration levels by using anhydrous GAA as the diluent.

Preparation of sample solution

Twenty vials of Ornidyl 200 mg/ml were used. The sample vial (SVP) containing 200 mg ml⁻¹. Pipetted out 1 ml and diluted to 100 ml with anhydrous GAA. Now pipette 50 ml of this solution and diluted up to 100 ml with GAA to get 500 µg/ml.

Determination of absorption maxima of eflornithine (DFMO)

An aliquot (0.5 ml) of standard eflornithine hydrochloride (DFMO) solution of 500 µg/ml was pipetted into a 25 ml volumetric flask. To this 10 ml of Phthalate buffer (pH 5.6) and 3.5 ml of 1 % w/v reagent solution of vanillin were added and heated on a water bath for 1 h at 90 °C. After one hour, solution was cooled to room temperature. The final volume was made up to 25 ml with double-distilled water and resultants green reddish color solution was then scanned 400-800 nm.

Determination of eflornithine (DFMO) using vanillin

The linearity of the method was demonstrated over the concentration range of 5-25 µg/ml for DFMO. Aliquots of 0.25 ml, 0.5 ml, 0.75 ml, 1 ml, 1.25 ml 3.5 ml of 500 µg ml⁻¹ solution of standard eflornithine (DFMO) was pipetted into five 25 ml

volumetric flasks respectively. 10 ml of phthalate buffer solution (pH 5.6) and 3.5 ml of 1 % w/v reagent solution of vanillin were added and heated on a water bath for 1 h at 90 °C. The volumetric flasks were cooled to room temperature and volume was made up to 25 ml with double-distilled water. The absorbance of solutions was measured at 578 nm against blank.

Procedure for the parenteral formulations

Twenty vials of Ornidyl 200 mg/ml were used. The sample vial (SVP) containing 200 mg/ml was pipetted out and diluted to 100 ml with anhydrous glacial acetic acid (GAA). Now further dilutions were done with GAA to get the final concentration of 500 µg/ml. The procedure mentioned above was followed where different concentrations of Ornidyl in the range of 5-25 µg/ml were added respectively. The drug concentration was calculated from the standard calibration graph prepared under identical conditions.

Effect of pH on DFMO-vanillin derivative chromogenic reaction

An aliquot (1 ml) of eflornithine hydrochloride solution of 500 µg/ml was pipetted into each of eight 25 ml volumetric flasks. To this 10 ml of phthalate buffer solution of various pH 2.6, 3, 3.4, 4, 4.6, 5, 5.6 and 6 was added to each 25 ml volumetric flasks, respectively. This was followed by 3.5 ml of 1 % w/v solution of vanillin to all volumetric flasks and heated on a water bath at 90 °C for 1 h and cooled to room temperature. The final volume of each volumetric flask was made up to 25 ml with double-distilled water. The absorbance was measured at 578 nm against reagent blank.

Effect of volume of buffer solution on DFMO-VANILLIN derivative chromogenic reaction

An aliquot of 1 ml eflornithine hydrochloride (DFMO) solution of 500 µg/ml was pipetted into each of five 25 ml volumetric flasks respectively. To this various volumes of phthalate buffer solutions of pH 5.6, viz, 2.5, 5, 7.5, 10 and 12.5 ml were added to each series of volumetric flasks respectively. This was followed by addition of 3.5 ml of 1 % w/v solution of vanillin to all volumetric flasks and heated on a water bath for 1 h at 90 °C and cooled to room temperature. The final volume was made up to 25 ml with double-distilled water. The absorbance was measured at 578 nm against reagent blank.

Effect of the vanillin concentration on the vanillin-DFMO reaction

The optimum vanillin-DFMO ratio for the DFMO-Vanillin reaction was determined by adding varying volumes of vanillin solutions to a known constant concentration of DFMO. An aliquot of 1 ml eflornithine hydrochloride (DFMO) solution of 500 µg/ml was pipetted into each of eight 25 ml volumetric flasks respectively. 10 ml of phthalate buffer solution (pH 5.6) was added to each volumetric flask respectively and followed by 0.5, 1, 1.5, 2, 3 ml, 3.5, 4 and 5 ml of 1 % w/v reagent solution of vanillin to each volumetric flask respectively. The volumetric flasks were heated on a water bath for 1 h at 90 °C and cooled to room temperature. The final volume was made up to 25 ml with double distilled water. The absorbance was measured at λ_{\max} 578 nm against reagent blank.

Effect of heating time on the DFMO-vanillin reaction

An aliquot of 2 ml and 3 ml of 500 µg/ml of eflornithine hydrochloride solution was pipetted into each of six 25 ml volumetric flasks respectively. 10 ml phthalate buffer (pH 5.6) and 3.5 ml of 1 % w/v solution of reagent vanillin were added to each of volumetric flasks respectively and heated on a water bath at different times i.e., 00 min, 15 min, 30 min, 45 min, 60 min and 75 min at 90 °C. The volumetric flasks were cooled to room temperature and the volume was made up to 25 ml with double distilled water. The absorbance of each solution was measured at 578 nm against blank.

Statistical analysis

The data obtained are presented as mean \pm standard deviation (SD). Statistical analysis was performed using one-way ANOVA with the aid of Microsoft Excel 2003 software. Differences between formulations were considered significant at $p \leq 0.05$.

RESULTS

Reliability

The results of reliability studies showed that the proposed derivative chromogenic spectrophotometric method is highly reproducible during one run and between different runs. The low values of % RSD for reliability studies (Table 1) indicate that there was no statistically significant difference ($p \leq 0.05$) between the two different

UV spectrophotometer instruments used in similar settings.

Validation results

The proposed method was validated according to ICH guidelines (12) in terms of linearity, accuracy, precision LOD and LOQ. The validation results indicate good linearity parameters of the proposed method (Table 2).

Accuracy

The accuracy of the proposed method was established by measuring the content of

eflornithine hydrochloride in pure form at different concentration levels. The percentage recovery of eflornithine hydrochloride was found to be in the range of 99.17 to 100.58 % (Table 3).

Precision

The precision of the proposed method was determined by measuring the content of eflornithine hydrochloride in pure form at different concentration levels. Inter- and intra-day precision of the proposed method is performed by carrying out three replicate experiments at each concentration level within four days and shown in Table 4.

Table 1: Results of reliability studies

UV spectrophotometer	Amount taken ($\mu\text{g/ml}$)	Amount found ($\mu\text{g/ml}$)	% RSD*
Equipment 1	15	14.98 \pm 0.01	0.082
Equipment 2	15	14.96 \pm 0.02	0.173

*RSD = relative standard deviation

Table 2: Linearity parameters of the proposed method

Linearity parameter	DFMO
λ_{max} (nm)	578
Drug's linearity ranges ($\mu\text{g/ml}$)	5-25
Molar extinction coefficient ($\text{Lmole}^{-1}\text{cm}^{-1}$)	1.35×10^2
Sandall's sensitivity (μgcm^{-2})	0.783
Intercept	0.07
Slope	0.0073
Correlation coefficient(r^2)	0.9987
Percentage recovery (%)	99.17-100.58
SD	0.62-0.64
RSD (%)	0.997-1.003
LOD ($\mu\text{g/ml}$)	1.2
LOQ ($\mu\text{g/ml}$)	4.3

Table 3: Accuracy of the proposed method

Analyte	Amount taken ($\mu\text{g/ml}$)	Amount added ($\mu\text{g/ml}$)	Amount found ($\mu\text{g/ml}$)	Recovery \pm RSD (%)*
DFMO	10	5	14.87	99.17 \pm 0.091
	10	10	20.02	100.11 \pm 0.85
	10	15	25.14	100.58 \pm 0.31

* $n = 6$

Table 4: Intra- and inter-day precision

DFMO Conc. ($\mu\text{g/ml}$)	% recovery*			
	Intra-day (%RSD)	Inter-day (%RSD)	Intra-day	Inter-day
5	1.0094	1.0222	100.2	99.60
15	0.9925	0.9765	99.56	98.89
25	0.9896	1.0091	98.75	101.23

*Average of six readings

Recovery experiment of commercial formulation

Applicability of the method was tested by analyzing the commercially available vials formulation containing DFMO (ornidyl, label claim, 200 mg/ml/vial). The assay of commercial formulation (ornidyl) was done with 5 µg/ml. the concentration was found 5.05 µg/ml. Recovery from formulation was 100.5 ± 0.98 . Data were very close to each other as well as to the label strength of the reference commercial pharmaceutical formulation, which shows that the method can be applicable to the determination of DFMO in formulations.

DISCUSSION

Many researchers have studied eflornithine hydrochloride separately in dosages forms [8-10]. The good regression coefficient indicates that Beer's was obeyed. The experimental factors affecting the development and stability of the colored chromogens produced were carefully studied. The optimum pH of potassium hydrogen phthalate buffer required to produce stable chromogen (green reddish color chromogen) having maximum absorbance was found to be 5.6. The colored chromogens were stable for at least 1 h which permits the convenient application of the proposed method. At pH 6 the green reddish color chromogen disappears and the solution changes to brownish-black indicating the degradation of the chromogen, and the degradant exhibit an absorbance of 0.1023 at 578 nm.

Different experimental conditions, especially pH and vanillin concentration, were carefully selected as they could greatly affect the quantification of the target compound. As reported, the reactivity of compounds possessing a primary amino group is pH specific. Attempts were made to improve pH control in the target reaction. It has been carried out by several assays of solutions containing 5, 15 and 20 µg/ml of DFMO and 10 ml of different buffer solutions that covered a wide pH range. As a result of this experience, it is necessary to maintain a pH of 5.6 as optimum to obtain the derivative chromogen. Several buffers of different compositions could be used but the best results were obtained with an acid phthalate buffer solution of 0.2 M of pH 5.6. The volume of buffer necessary to obtain the highest and most stable absorbance was determined and established as 10 ml.

The optimum volume of 3.5 ml of 1 % w/v of vanillin reagent was required to produce a stable

chromogen with a maximum absorbance at 578 nm. Eflornithine hydrochloride (DFMO) derivative chromogen showed maximum absorbance after heating for 60 min at 90 °C. The reaction between DFMO and vanillin (Figure 1) was spontaneous and produced a coloured complex which was quite stable for over one hour, giving enough time for the analysis. Recovery experiment showed high quantitative recoveries with low standard deviation by the proposed assay method.

The effect of vanillin concentration on the derivative chromogen formation was observed by measuring the absorbance at different vanillin-DFMO concentration ratios, while all other experimental conditions were kept constant at the optimum values. In subsequent work, vanillin-DFMO ratio of 600:1 was employed as the minimum for the determination of DFMO throughout the rest of the experimental work. The order of mixing of the analyte, buffer, NQS and water to obtain the derivative chromogen was examined. No appreciative changes were observed, hence the choice of analyte, buffer, vanillin for the present study.

In order to obtain optimum derivative chromogen with the highest and most stable absorbance, the effect of the reaction time and heating temperature on the absorbance of the reaction product was studied. The reaction was carried out at different temperatures using thermostated water. Maximum and constant absorbance was obtained at 90 °C after 60 min.

Reaction of the proposed method

Vanillin could react with the amino group of primary amino derivative. Alpha amino group of DFMO displays nucleophilicity due to the fact that its lone pairs of electrons of nitrogen can attack the electron deficient center. Hence DFMO can react with vanillin in a condensation reaction. Vanillin to form Schiff's base, the possible reaction equation is shown in Fig. 1.

CONCLUSION

The data obtained show that the proposed method is simple, accurate and sensitive with good precision and accuracy. Also, the reagent utilized in the proposed method is low-cost, readily available and the procedures do not involve any or tedious sample preparation. The short analysis time and low cost reagents used are also benefits of this method. Thus, the method can be applied for the determination of eflornithine hydrochloride in pure form and in pharmaceutical preparations.

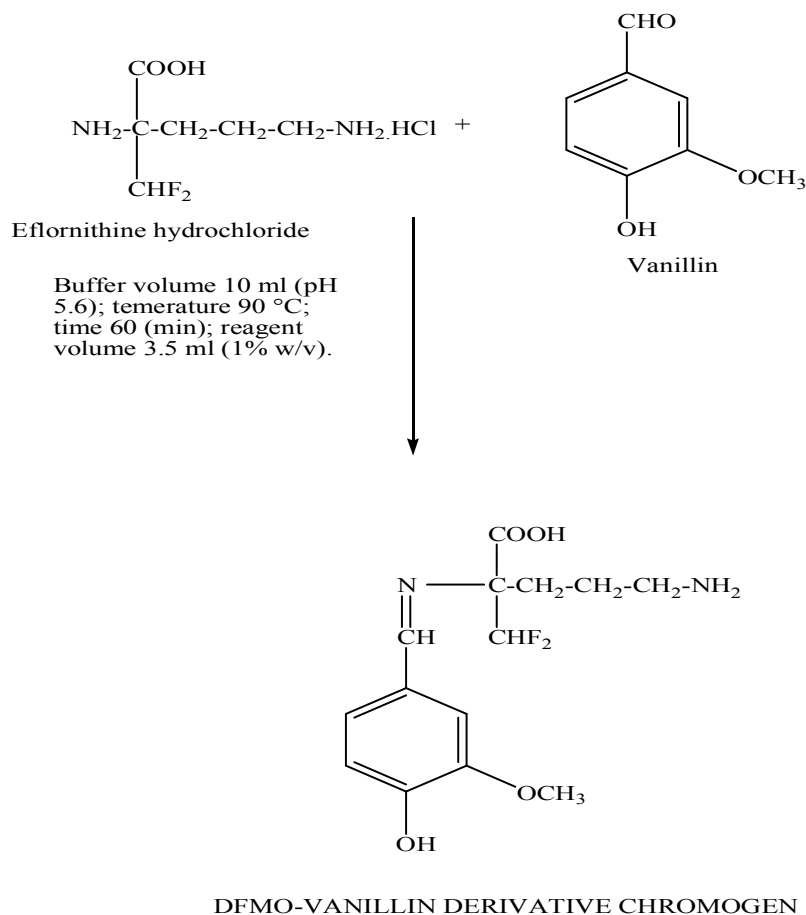


Figure 1: Reaction of the proposed method

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REFERENCES

- Merali S, Clarkson AB (Jr). Polyamine content of *Pneumocystis carinii* and response to the ornithine decarboxylase inhibitor DL-alpha-difluoromethylornithine". *Antimicrob Agents Chemother* 1996; 40: 973-978.
- Pepin J, Guern C, Milord F, Schechter PJ. "Difluoromethylornithine for arseno-resistant trypanosoma brucei gambiense sleeping sickness". *Lancet* 1987; 330: 1431-1433.
- Cohen JL, Ko RJ, Lo AT, Shields MD, Gilman TM. "High-pressure liquid chromatographic analysis of DFMO in serum". *J Pharm Sci.* 1989; 78(2): 114-116.
- Huebert ND, Schwartz JJ, Haegele KD. "Analysis of 2-difluoromethyl-DL-ornithine in human plasma, cerebrospinal fluid and urine by cation-exchange high-performance liquid chromatography". *J Chromatogr A.* 1997; 21; 762(1-2): 293-298.
- Saravanan C, Kumudhavalli MV, Kumar M, Jayakar B. A new validated RP-HPLC method for estimation of DFMO hydrochloride in tablet dosage form. *J Pharm Res.*2009; 2: 1730-1731.
- Kilkenny ML, Slavik M, Christopher MR, Stobaugh JF. "Plasma analysis of alpha-difluoromethylornithine using pre-column derivatization with naphthalene-2,3-dicarboxaldehyde/CN and multidimensional chromatography". *J. Pharm. Biomed. Anal.* 1998; 17: 1205-1213.
- Jansson-Löfmark R, Römsing S, Albers E, 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.* 2010; 24: 768-773.
- Malm M, Bergqvist Y. "Determination of DFMO enantiomers in plasma, by solid-phase extraction and liquid chromatography with evaporative light-scattering detection". *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* 2007; 846: 98-104.
- Kumar A, Venkatesh Prasad SP, Mohan S, 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.* 2008; 7(2): 225.
10. Kumar A, Venkatesh Prasad SP, Mohan S, Singh AK. Estimation of eflornithine hydrochloride by uv Spectroscopy". *Trade Sci. Inc.* 2009; 8: 117-124.
 11. The Controller of publication. *Indian Pharmacopoeia*, 2007; Vol 2; pp 144-145.
 12. ICH Harmonised Tripartite Guideline 2005. *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Validation of Analytical Procedures: Text and Methodology Q2 (R1), Complementary Guideline on Methodology*, London, pp 11-12.