

## Development and Validation of Reverse Phase HPLC Method for Determination of *ortho* and *meta* isomers in *N*-Methyl fluoxetine oxalate

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### Abstract

The present work deals with the development and validation of a stability indicating reverse phase HPLC method for the determination of *ortho* and *meta* isomers in *N*-Methyl fluoxetine oxalate on Zorbax Eclipse XDB C-18 column (150 mm x 4.6 mm, 5  $\mu$ m). A mobile phase consisting of solution-A (0.05M Disodium hydrogen ortho phosphate with Triethylamine and the resulting mixture pH adjusted to 3.0) and solution-B [Methanol : Tetrahydrofuran /80:20 (v/v)] in the ratio of 60:40 (v/v). The flow rate was 1.5 ml/min. The separation was performed at 40 °C and detection was carried out at  $\lambda_{215}$  nm. The developed method was statistically validated for the linearity, accuracy, specificity, robustness, LOD and LOQ. The specificity of the method was ascertained by forced degradation studies with acid, alkali, oxidation, thermal and photolysis. The degraded products were well separated from the main peak with significant differences in their retention time.

### Keywords:

*N*-Methyl fluoxetine oxalate; RP-HPLC; Fluoxetine impurities; Forced degradation studies; *N*-Methyl Prozac

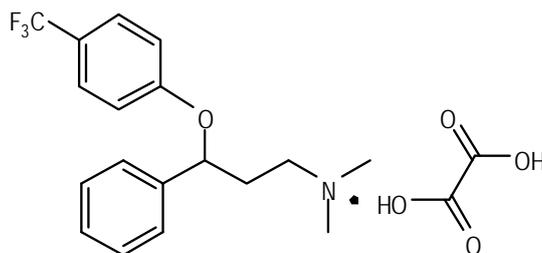
### 1. Introduction

*N*-Methyl fluoxetine oxalate (NMF) (Fig. 1), is the precursor [1-3] and a key intermediate in the synthesis of Fluoxetine hydrochloride. The two positional isomers (*ortho*-NMF and *meta*-NMF) generated during the manufacturing of *N*-Methyl fluoxetine oxalate play a vital role in the quality determination of Fluoxetine hydrochloride. It is very important to identify and control these isomeric impurities in *N*-Methyl fluoxetine oxalate.

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**Fig. 1:** Chemical structure of *N*-Methyl fluoxetine oxalate

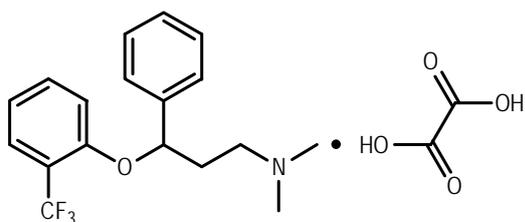
Some literatures are available for the determination of fluoxetine using LC, LC/MS, Capillary electrophoresis, HPTLC, spectrophotometry in pharmaceutical preparations [4-14]. Fluoxetine and its simultaneous determination with other substances are also present in the literature [15-25].

Till date no method is available for the determination of the impurities (*ortho*-NMF and *meta*-NMF) in *N*-methyl fluoxetine oxalate and the present study describes a novel method for the determination of structural isomers in *N*-methyl fluoxetine oxalate. A novel methodology has been contributed through this article for the separation and determination of two isomers (*ortho*-NMF and *meta*-NMF). The accuracy, linearity, precision, limit of detection (LOD), limit of quantification (LOQ), robustness and ruggedness of the method was determined in accordance with ICH guidelines [26].

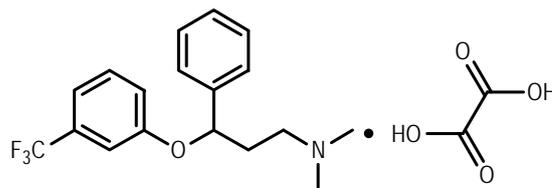
## 2. Experimental

### 2.1. Standards and Reagents

*N*-Methyl fluoxetine oxalate and its isomeric impurities namely *ortho*-NMF and *meta*-NMF (Fig. 2) were synthesized in Research Division of Vindhya Organics Pvt. Ltd. All reagents used were of Analytical Reagent grade. Milli Q water, HPLC-grade Acetonitrile, Methanol, Tetrahydrofuran, AR-grade Disodium hydrogen ortho phosphate, Triethylamine and Ortho phosphoric acid were procured from Merck.



Dimethyl-[3-phenyl-3-(2-trifluoromethyl-phenoxy)-propyl]-amine oxalic acid salt (*ortho*-NMF)



Dimethyl-[3-phenyl-3-(3-trifluoromethyl-phenoxy)-propyl]-amine oxalic acid salt (*meta*-NMF)

**Fig.2:** Impurities of *N*-Methyl Fluoxetine Oxalate

### 2.2. Instruments

The HPLC system used was equipped with quaternary gradient pumps with auto injector (Shimadzu LC 2010, Japan) and controlled with LC solutions software. Metler analytical balance was used for all weighings and Polmon pH meter was used for pH adjustments of buffer solutions.

### 2.3. Preparation of system suitability solution

Accurately weighed 5mg of NMF and 5mg of *meta*-NMF were transferred into a 25 mL volumetric flask, added about 5 mL of diluent, sonicated to dissolve and diluted up to the mark with diluent.

### 2.4. Preparation of sample solution (100% concentration)

Accurately weighed 50 mg of NMF sample was transferred into a 50 mL volumetric flask, added about 25 mL of diluent, sonicated to dissolve and diluted up to the mark with diluent. During the whole process this concentration shall be treated as 100% concentration.

### 2.5. Preparation of diluent:

Water and acetonitrile in the ratio of 50:50 (v/v) was used as a diluent.

### 2.6. Optimization of Chromatographic conditions

The study was initiated using Phosphate buffer and Acetonitrile mixture and observed that product and impurity peak are co-eluting. Then we used the mixture of Disodium buffer and Acetonitrile mixture but the peak shape and resolution was not good, then we replaced the Acetonitrile with Methanol and Triethylamine added to buffer to improve the peak shape and resolution, as a result little better chromatogram observed. Then pH optimized to 3.0 and addition of Tetrahydrofuran to improve the peak shape and resolution. With this mobile phase composition we observed good resolution and peak shape.

Final method optimization is achieved on a Zorbax Eclipse XDB C-18 150 x 4.6 mm, 5  $\mu$ m particle size column. The Isocratic LC method employs with the composition of solution A and B as mobile phase in the ration of 60:40 (v/v), respectively. Solution A contains 0.05M Disodium hydrogen ortho phosphate, 3 ml of Triethylamine (for 1000 ml) and pH was adjusted to 3.0 with dilute Ortho phosphoric acid solution. Solution B contains HPLC grade Methanol and Tetrahydrofuran in the ratio of 80:20 (v/v). The flow rate of the mobile phase was 1.5 mL min<sup>-1</sup>. The detection was performed by UV detector at 215 nm. The injection volume was 20  $\mu$ L.

## 3. Validation of the Method

### 3.1. Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the developed HPLC method for NMF was carried out in the presence of its impurities namely, *ortho*-NMF and *meta*-NMF. Stress studies were performed for N-Methyl fluoxetine oxalate to provide an indication of the stability indicating property and specificity of the proposed method.

### 3.2. Forced degradation studies

The N-Methyl fluoxetine oxalate was subjected to various forced degradation conditions to effect partial degradation of the NMF. The forced degradation studies provide information about the conditions in which the NMF is unstable, so that measures can be taken during the manufacturing to avoid any potential instability. The stability samples were prepared by dissolving the N-Methyl fluoxetine oxalate in diluent, aqueous hydrochloric acid, aqueous sodium hydroxide or aqueous hydrogen peroxide solution at a concentration of 1000  $\mu$ g mL<sup>-1</sup>, separately. After the degradation, these samples were collected, filtered using syringe filters and injected in to HPLC system.

### 3.2.1 Neutral hydrolysis

Solutions for neutral degradation studies were prepared in diluent (50:50 v/v) at a concentration of  $1000 \mu\text{g mL}^{-1}$  by taking 200 mg N-Methyl fluoxetine oxalate in 200 mL of diluent, at room temperature and heated in a water bath at  $80^\circ\text{C}$  for 2h. The mixture was then allowed to cool to room temperature, filtered using syringe filters and analyzed.

### 3.2.2 Acid hydrolysis

Solutions for acid degradation studies were prepared in 1N hydrochloric acid at a concentration of  $1000 \mu\text{g mL}^{-1}$ , at room temperature by dissolving 200 mg of N-Methyl fluoxetine oxalate and 5 mL of 1N hydrochloric acid solution in diluent to get a concentration of  $1000 \mu\text{g mL}^{-1}$ . The resultant solution was heated in a water bath at  $80^\circ\text{C}$  for 2h. The mixture was then allowed to cool to room temperature, filtered using syringe filters and analyzed.

### 3.2.3 Base hydrolysis

Solutions for base degradation studies were prepared in 1N sodium hydroxide at a concentration of  $1000 \mu\text{g mL}^{-1}$ , at room temperature by dissolving 200 mg of N-Methyl fluoxetine oxalate and 5 mL of 1N sodium hydroxide solution in diluent to get a concentration of  $1000 \mu\text{g mL}^{-1}$ . The resultant solution was heated in a water bath at  $80^\circ\text{C}$  for 2h. The mixture was then allowed to cool to room temperature, filtered using syringe filters and analyzed.

### 3.2.4 Oxidation studies

Solutions for oxidation studies were prepared in 30% hydrogen peroxide solution at a concentration of  $1000 \mu\text{g mL}^{-1}$ , at room temperature by dissolving 200 mg of N-Methyl fluoxetine oxalate and 2 mL of 30% hydrogen peroxide solution in diluent to get a concentration of  $1000 \mu\text{g mL}^{-1}$ . The resultant solution was heated in a water bath at  $80^\circ\text{C}$  for 2h. The mixture was then allowed to cool to room temperature, filtered using syringe filters and analyzed.

### 3.2.5 Photo-stability studies

N-Methyl fluoxetine oxalate in powder form was directly exposed to short wavelength light (254 nm) for 4 days. The degraded sample were withdrawn at appropriate time and subjected to analysis.

### 3.2.6 Temperature stress studies

N-Methyl fluoxetine oxalate in powder form was exposed to dry heat ( $80^\circ\text{C}$ ) in an oven for 4 days. The degraded sample were withdrawn at appropriate time and subjected to analysis.

All the impurities and degradation products were separated with appropriate resolution by the developed method.

## 3.3. Precision

The precision of the related substance method was checked by injecting six individual preparations of NMF spiked with 0.5 % of each impurity with respect to the NMF analyte concentration of  $1.0 \text{ mg mL}^{-1}$ . The % RSD of the area of each impurity was calculated. The intermediate precision of the method was also evaluated using different analyst and instrument in the same laboratory.

### 3.4. LOD and LOQ

The LOD and LOQ were determined by measuring the standard deviation of the response and slope. 0.02%, 0.04% and 0.06% solutions of NMF, *ortho*-NMF and *meta*-NMF were injected and calculated the slope and standard deviation of the response. LOD was calculated as  $3.3 \times \text{standard deviation/slope}$  and LOQ was calculated as  $10 \times \text{standard deviation/response}$ . The precision study was also carried out at the LOQ level by injecting six injections of NMF, *ortho*-NMF and *meta*-NMF, calculating % RSD for the areas of each impurity.

### 3.5. Accuracy

The accuracy of the method for all the related substances was determined by analyzing NMF sample solutions spiked with all the related substances at four different concentration levels of LOQ, 50, 100 and 150 % of each in triplicate. 50%, 100% & 150% of impurities solutions were prepared considering 0.5% (impurity limit) as 100%. LOQ is the concentration obtained in LOQ study. The percentage of recoveries for the impurities was calculated by injecting the standard solution for each level.

### 3.6. Linearity

The Linearity of the method for all the related substances was determined by analyzing dilute solution of NMF and its related substances at five different concentration levels of LOQ, 25, 50, 100 and 150 % of each in triplicate. The correlation coefficient was calculated for each substance.

### 3.7. Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between the NMF, *ortho*-NMF and *meta*-NMF was recorded. The parameters selected were mobile phase composition ( $\pm 10\%$ ), pH of the mobile phase ( $\pm 0.2$  units), column oven temperature ( $\pm 2$  °C) and flow rate ( $\pm 0.2$  ml/min).

### 3.8. Solution and mobile phase stability

To determine the stability of sample solution, NMF samples spiked with related substances at specified level were prepared and analyzed after 48 h. The results of these studies indicated the stability of sample solution at room temperature for 48 h. The mobile phase prepared and kept at room temperature for 48 h. After 48 h NMF sample spiked with impurities at specified level were prepared and analyzed. The results were statistically evaluated and meeting system suitability and precision requirement which indicates the mobile phase is stable for 48 h at room temperature.

## 4. Results and Discussion

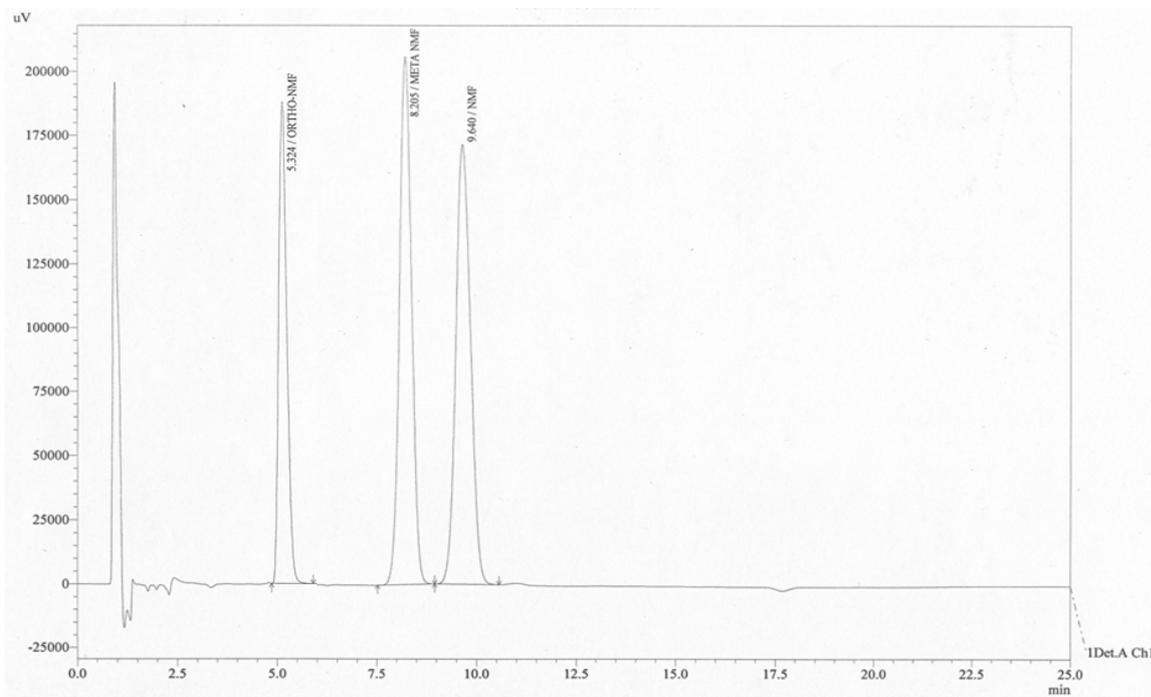
### 4.1. Optimization of chromatographic conditions

The main objective of the chromatographic method was to separate NMF from their related impurities *ortho*-NMF and *meta*-NMF. Impurities were co-eluted using different stationary phases such as C-8, cyano column with different mobile phases. During evaluation of different column chemistry, Agilent make Zorbax Eclipse XDB C-18 column was observed to give better resolution with the buffer pH of 3.00. A good resolution and peak shape was optimized as mentioned under section "Chromatographic conditions". In optimized chromatographic conditions NMF, *ortho*-NMF and *meta*-NMF were separated with a good resolution of greater than 2, typical relative retention times for *ortho*-NMF and *meta*-NMF were approximately 0.552 and 0.851, respectively (Fig. 3). Specificity details are mentioned in the Table 1.

**Table 1.** Specificity details

Parameter	NMF	<i>ortho</i> -NMF	<i>meta</i> -NMF
RT	9.640	5.324	8.205
RRT	1.000	0.552	0.851
N	4209	4698	4410
TF	1.67	1.73	1.63

RT = Retention time, RRT = Relative retention time, N = Theoretical plates and TF = Tailing factor.

**Fig.3:** *N*-Methyl Fluoxetine Oxalate spiked with impurities

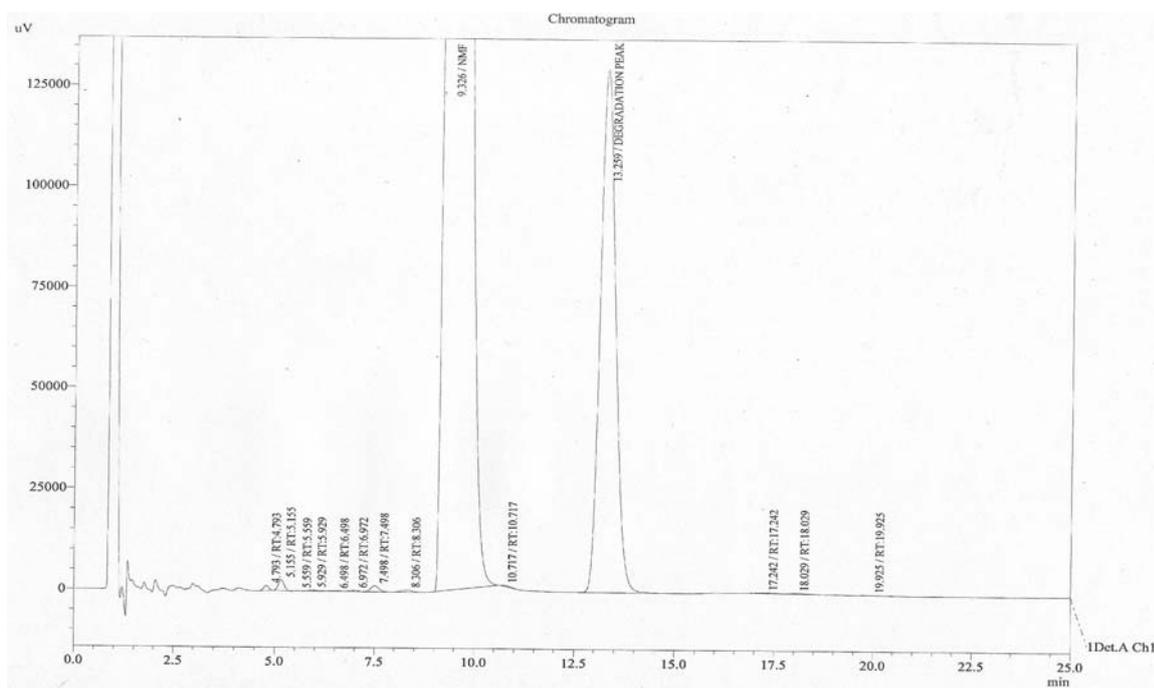
## 4.2. Validation of the method

### 4.2.1. Forced degradation

Degradation was not observed in NMF sample when subjected to neutral, thermal and photolytic stress conditions. NMF was degraded under oxidation condition (Fig.4). The summary of the forced degradation study is mentioned in Table 2. No degraded peaks were co-eluted with the *N*-methyl fluoxetine oxalate and impurities peaks and no interference is observed. This shows the specificity of the method.

**Table 2.** Forced degradation study result of NMF

Degradation condition	Purity, %	RRT of major degradation peaks
Control sample	99.62	-
Neutral condition (80 °C, 2hr only diluent)	99.53	-
Acid hydrolysis (80 °C, 2hr, 5 ml, 1 N HCl)	99.32	0.49, 0.51, 0.82, 1.86
Base hydrolysis (80 °C, 2hr, 5 ml, 1 N NaOH)	99.51	0.47, 0.51, 0.64, 0.71
Oxidation (80 °C, 1hr, 2 ml, 30 % Hydrogen peroxide)	84.71	0.52, 0.64, 0.75, 1.43, 1.86, 1.95
Photolytic degradation (UV $\lambda_{254}$ , 4 days)	99.57	No major degradation peak was observed
Thermal degradation (105 °C, 4 days )	99.56	No major degradation peak was observed



**Fig.4:** NMF peroxide degradation sample 2 ml 30 % Hydrogen peroxide (1 hr at 80 °C)- Major degradation product observed at 13.259 min. with 14.87% degradation.

#### 4.2.2. Precision

The Precision was determined at the LOQ to 150% concentration for NMF, *ortho*-NMF and *meta*-NMF and the % RSD was found to be below 5.0 for all impurities.

#### 4.2.3. Limit of detection and limit of quantification

The Values of LOD and LOQ for NMF, *ortho*-NMF and *meta*-NMF are mentioned in the Table 3.

**Table 3.** Validation results summary

Parameter	NMF	<i>ortho</i> -NMF	<i>meta</i> -NMF
R <sup>2</sup> value	0.9972	0.9981	0.9930
Response Factor	1.00	0.99	0.98
LOD in µg per ml	0.070	0.035	0.065
LOQ in µg per ml	0.210	0.11	0.20
% RSD at LOQ	1.887	4.527	4.233
Precision	0.667	1.025	1.757
Accuracy at LOQ	--	101.06	96.20
Accuracy at 50 %	--	96.16	101.09
Accuracy at 100 %	--	94.49	97.63
Accuracy at 150 %	--	102.64	104.56

#### 4.2.4. Linearity

Linearity calibration plot for the related substances method was obtained over the calibration ranges tested *i.e.* LOQ, 50 %, 100 % and 150 % of the NMF concentration 1.0 mg/ml and impurity limit of 0.5%. The correlation co-efficient obtained was greater than 0.99. The above results show that an excellent correlation existed between the peak area and the concentration of two impurities.

#### 4.2.5. Accuracy

The two impurities were spiked with N-Methyl fluoxetine oxalate and calculated the percentage recovery by injecting the impurity standard solutions, test solutions and spiked solutions. The Accuracy of all these related substances was found to be in between the predefined acceptance criteria of 80 to 120 %. The results of accuracy is given in Table III.

#### 4.2.6. Robustness

When the chromatographic conditions like flow rate, mobile phase composition, column oven temperature and pH were deliberately varied, the resolution, %RSD for area and retention time was calculated and the results are well within the acceptance criteria as per the ICH, which illustrates the robustness of the method.

#### 4.2.7. Solution stability

There were no significant changes in the amounts of the impurities during solution stability experiment performed using the related substances method. The results from the studies indicated, the sample solution was stable at room temperature for 48 hours.

### 5. Conclusion

A novel, accurate and selective isocratic HPLC method was developed for the determination of isomeric impurities in N-Methyl fluoxetine oxalate and validated as per the ICH guidelines. The method was found to be simple, selective, precise, accurate and robust. Therefore, this method can be used for routine testing as well as stability analysis of NMF. All statistical results were within the acceptance criteria.

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