DEVELOPMENT AND VALIDATION OF A HPTLC METHOD FOR THE ESTIMATION OF FLUOXETINE AND ITS N-DEMETHYLATED METABOLITE (NORFLUOXETINE)

Dr Manish S. Bhatia 1*, Smita T. Kumbhar 2

1Department of Pharmaceutical Chemistry, Bharati Vidyapeeth College of Pharmacy, Kolhapur-416013, India.

2Department of Pharmaceutical Chemistry, D.S.T.S. Mandal’s College of Pharmacy, Solapur-413004, Maharashtra, India.

ABSTRACT

An HPTLC Method for the estimation of Fluoxetine and its N-demethylated metabolite (Norfluoxetine) has been developed. It employs aluminium backed silica gel 60 F 254 TLC plates, (20 cm × 10 cm, layer thickness 0.2 mm) prewashed with methanol and mobile phase comprising of toluene: 2-propanol: ammonia 2:2:0.4 (v/v/v). The developing solvent was run upto 80 mm in camag chamber previously saturated with 10.0 mL of solvent mixture for 30 min. Densitometric scanning was then performed with Camag TLC scanner-3 equipped with winCATS software Version 1.3.0 at λ max 227 nm. The R f values were found to be 0.14 and 0.74 for Norfluoxetine and Fluoxetine respectively. The proposed method can also be used to accurately determine Fluoxetine and Norfluoxetine in plasma, the R f values were found to be the same for plasma and standard and there was no interference from the biological matrices. The limit of detection and limit of quantitation were found to be 43.55 ng/spot and 131.99 ng/spot for Fluoxetine and 170.68 ng/spot, 571.23 ng/spot for Norfluoxetine. The % RSD of intra-day variation and inter day variation were 0.54 and 0.41 respectively for Fluoxetine and 0.46 and 0.39 for Norfluoxetine respectively.

Key-words: HPTLC, Fluoxetine (FLX), Norfluoxetine (N-FLX), plasma, analytical method development.
INTRODUCTION

Fluoxetine (FLX), N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine, it exhibits selective inhibition of serotonin uptake in presynaptic neurons [1]. It is prescribed for a variety of psychopathological conditions including mood and eating disorders, obsessive-compulsive disorders, depression in the elderly and dysthymia. It is official in United States Pharmacopoeia [2]. FLX is extensively metabolized by cytochrome P450 (CYP) isoenzymes in the liver to form an active N-demethylated metabolite Norfluoxetine (N-FLX) which has similar potency and selectivity with regard to the serotonin reuptake inhibiting effect of parent drug [1]. The degree of serotonin reuptake inhibition is correlated with the FLX plasma concentration. FLX is well absorbed after oral administration and disappears from plasma with half-time of 1-3 days; and its metabolite N-FLX has a plasma half-time of 7-15 days. After administration of FLX, approximately 65% of the administered dose of this drug is recovered in urine and about 15% in faces. The therapeutic dosage for fluoxetine is 20 mg/day which is metabolized in the liver to N-FLX and other unidentified metabolites. Overdoses of FLX have been reported to cause death. The plasma concentrations of the drug in these fatalities are 1.93-4.57 µg/ml.

The literature survey reveals that FLX and N-FLX have been simultaneously estimated by spectrophotometric [3], HPLC [4-6], GC [7-8], thermal [9], capillary electrophoresis [10], LC-MS [11,12], HPLC-DAD [13], TLC with derivatization [14].

The aim of present study were to develop an accurate, precise, specific and sensitive HPLTC method for quantitation of such metabolites in presence of the parent drug and other interfering components of the body fluids which could be useful in a clinical laboratory for therapeutic drug monitoring, metabolic and bioequivalence studies.

MATERIALS AND METHODS

Instrument

CAMAG (Muttenz, Switzerland) HPTLC system including a linomat V applicator, Camag TLC scanner-3 and WinCATS (version 1.3.0) data processor was used.

Chemical and materials

FLX and N-FLX were kindly supplied by Cadila Pharmaceuticals Ltd., Ahemdabad. Toluene, isopropanol and ammonia used were of analytical grade from E-Merck Ltd.
Procedure

Preparation of standard stock solution for Fluoxetine
Accurately weighed quantity of about 40 mg FLX was transferred to 100 mL volumetric flask. It was dissolved in methanol and the volume was made up to mark with the same solvent to get concentration of 400 µg/mL of FLX.

Preparation of standard stock solution for Norfluoxetine
Accurately weighed quantity of about 40 mg (N-FLX) was transferred to 10 mL volumetric flask. It was dissolved in methanol and the volume was made up to mark with the same solvent to get concentration of 4000 µg/mL of (N-FLX).

Chromatographic conditions
The experiment was performed on aluuminium backed silica gel 60 F$_{254}$ TLC plates, (20 cm × 10 cm, layer thickness 0.2 mm) prewashed with methanol and mobile phase comprising of toluene: 2-propanol: ammonia 2:2:0.4 (v/v/v). The developing solvent was run upto 80 mm in camag chamber previously saturated with 10.0 mL of solvent mixture for 30 min. Samples were applied as 6 mm wide bands and the distance between the bands was 11.6 mm. The developing solvent was run upto 80 mm, (distance to the lower edge was 10 mm) and the development was performed at 25 ± 2 °C. After development, the plate was dried at 50 °C in an oven for 5 minutes. Densitometric scanning was then performed with camag TLC scanner 3 equipped with winCATS software Version 1.3.0 at $\lambda_{\text{max}}$ 227 nm, using deuterium light source and the slit dimensions were 6.00 × 0.45 mm.

Linearity study for FLX
Standard solution 1-10 µl (400-4000 ng/spot) was applied on TLC plate with the help of microlitre syringe, using linomat V sample applicator. The plate was developed using mobile phase comprising toluene: 2-propanol: ammonia 2:2:0.4 (v/v/v) in twin trough chamber to a distance of 8 cm and scanned in the above established chromatographic conditions. Each concentration was spotted six times on the plate. Peak area was recorded for each concentration of drug; the observations are reported and calibration curve was obtained by plotting peak areas against concentration of FLX. A typical HPTLC chromatogram is shown in Figure 1. 3D Linearity spectra of FLX standard solution is shown in Figure 2.
Linearity study for N-FLX
Standard solution 1-10 µl (4-40 µg/spot) was applied on TLC plate with the help of microlitre syringe, using linomat V sample applicator. The plate was developed using mobile phase comprising toluene: 2-propanol: ammonia 2:2:0.4 (v/v/v) in twin trough chamber to a distance of 8 cm and scanned in the above established chromatographic conditions. Each concentration was spotted six times on the plate. Peak area was recorded for each concentration of drug; the observations are reported and calibration curve was obtained by plotting peak areas against concentration of N-FLX. A typical HPTLC chromatogram is shown in Figure 3. 3D Linearity spectra of N-FLX standard solution is shown in Figure 4. Calibration curve for FLX and N-FLX are shown in Figure 5 and 6.

Simultaneous estimation of Fluoxetine and Norfluoxetine
Accurately weighed quantity 40 mg (FLX) and 40 mg (N-FLX) was transferred to 100 mL volumetric flask. It was dissolved in methanol and volume was adjusted to mark. The solution (10 µL, containing 4000 ng) was spotted. Typical overlain chromatogram of N-FLX and FLX were shown in Figure 7. 3D Reproducibility spectra of N-FLX and FLX is shown in Figure 8.

Simultaneous estimation of Fluoxetine and Norfluoxetine from plasma
Accurately weighed quantity 40 mg (FLX) and 40 mg (N-FLX) was transferred to 10 mL of plasma. Then volume was made by methanol upto 100 mL and volume was adjusted to mark. The solution (10 µL, containing 4000 ng) was spotted. Typical overlain chromatogram of N-FLX and FLX were shown in Figure 9. 3D Reproducibility spectra of N-FLX and FLX in plasma is shown in Figure 10.

Method validation
The proposed method was validated according to ICHQ2B guidelines for validation of analytical procedures in order to determine accuracy, precision, repeatability, robustness, linearity, limit of detection and limit of quantitation. Results are shown in Table 1 to 3.

RESULT AND DISCUSSION
The peak area was observed to be dependent on the amount of the standard, FLX and N-FLX and a linear relationship (r = 0.9992, 0.9998 respectively) was found between the peak areas of FLX and N-FLX at various concentrations over the range 400-4000 ng, 4000-40000 ng respectively. The solvent system used for development of the plates produced no interfering
peaks in the area under the curve. The R_f values of FLX and N-FLX under the conditions used were found to be 0.74±0.02 and 0.14±0.02 and spots were quantified at a wavelength of 227 nm. The proposed method can also be used to accurately determine FLX and N-FLX in plasma, the R_f values were found to be the same for plasma and standard and there was no interference from the biological matrices. The limit of detection and limit of quantitation were found to be 43.55ng/spot and 131.99 ng/spot for FLX and 68.27 ng/spot, 228.49 ng/spot for N-FLX. The % RSD of intra-day variation and inter day variation were 0.54 and 0.41 respectively for FLX and 0.46 and 0.39 for N-FLX respectively.

Figure 1: Densitogram of standard FLX (1000 ng/spot) (R_f 0.74± 0.02), measured at 227 nm, Mobile phase: Toluene: 2-Propanol: Ammonia 2:2:0.4 (v/v/v).

Figure 2: 3D Linearity spectra of FLX standard solution.
Figure 3: Densitogram of standard N-FLX (Rf 0.14 ± 0.02), measured at 227 nm, mobile phase Toluene: 2-Propanol: Ammonia 2:2:0.4 (v/v/v).

Figure 4: 3D Linearity spectra of Norfluoxetine standard solution.

Figure 5: Calibration curve for Fluoxetine.
Figure 6: Calibration curve for Norfluroxetine

Figure 7: Typical overlain chromatogram of 1. N-FLX and 2.FLX.

Figure 8: 3D Reproducibility spectra of N-FLX and FLX.
Figure 9: Typical overlain chromatogram of 1. N-FLX and 2. FLX from plasma.

Figure 10: 3D Reproducibility spectra of FLX and N-FLX from plasma

Figure 11: Typical overlain spectra of 1. FLX and 2. N-FLX
Table 1: Assay of FLX and N-FLX from physical mixture

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% Amount found* (Mean ± SD)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLX</td>
<td>101.21 ± 1.26</td>
<td>1.25</td>
</tr>
<tr>
<td>N-FLX</td>
<td>100.29 ± 1.64</td>
<td>1.64</td>
</tr>
</tbody>
</table>

*Average of nine determinations.

Table 2: Assay of FLX and N-FLX from plasma:

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% Amount found* (Mean ± SD)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLX</td>
<td>99.89 ± 0.86</td>
<td>0.85</td>
</tr>
<tr>
<td>N-FLX</td>
<td>101.45 ± 0.92</td>
<td>0.91</td>
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</tbody>
</table>

*Average of nine determinations.

Table 3: Method Validation Parameters:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fluoxetine</th>
<th>Norfluoxetine</th>
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<tbody>
<tr>
<td>Linearity range</td>
<td>400-4000 ng/spot</td>
<td>4000-40000 ng/spot</td>
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<tr>
<td>Correlation coefficient (r²)</td>
<td>0.9992</td>
<td>0.9998</td>
</tr>
<tr>
<td>Precision (n = 9) (%RSD)</td>
<td>Intraday-0.54</td>
<td>Intraday-0.46</td>
</tr>
<tr>
<td></td>
<td>Interday-0.41</td>
<td>Interday-0.39</td>
</tr>
<tr>
<td>Specificity</td>
<td>Specific</td>
<td>Specific</td>
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<tr>
<td>Repeatability (n = 7) (%RSD)</td>
<td>1.71</td>
<td>0.93</td>
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<tr>
<td>Limit of detection (LOD)</td>
<td>43.55 ng/spot</td>
<td>68.27 ng/spot</td>
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<tr>
<td>Limit of quantitation (LOQ)</td>
<td>131.99 ng/spot</td>
<td>228.49 ng/spot</td>
</tr>
<tr>
<td>Ruggedness studies (n = 6) (%RSD)</td>
<td>Analyst I- 0.18, Analyst II- 0.50</td>
<td>Analyst I- 0.95, Analyst II- 0.58</td>
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<tr>
<td>Robustness studies (n = 6) (%RSD)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

RSD stands for Relative Standard Deviation.
CONCLUSION

The developed HPTLC technique is simple, precise, specific and accurate for simultaneous estimation of FLX and N-FLX in plasma. The result of analysis clearly indicates absence of interference from the biological matrices. The statistical analysis proves that method is reproducible and selective for the analysis of FLX and N-FLX in plasma. This method does not require an internal standard. The proposed method could be applied for routine analysis in a clinical laboratory for therapeutic drug monitoring, metabolic and bioequivalence studies.

REFERENCES


