

## Method of Test for *N*-Nitroso Fluoxetine in Fluoxetine Drug Substance

### 1. Scope

This method is applicable to the determination of *N*-nitroso fluoxetine in fluoxetine drug substances.

### 2. Method

After extraction, *N*-nitroso fluoxetine is determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

#### 2.1. Equipment

##### 2.1.1. Liquid chromatograph/tandem mass spectrometer

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: Symmetry C18, 3.5  $\mu\text{m}$ , 4.6 mm i.d.  $\times$  15 cm, or an equivalent product.

2.1.2. Ultrasonicator.

2.1.3. Vortex mixer.

2.1.4. Centrifuge: centrifugal force  $\geq 3000 \times g$ .

#### 2.2. Chemicals

Methanol, HPLC grade;

Acetonitrile, HPLC grade;

Ammonium formate, reagent grade;

Deionized water, resistivity  $\geq 18 \text{ M}\Omega \cdot \text{cm}$  at (25°C);

*N*-nitroso fluoxetine, reference standard;

*N*-nitroso fluoxetine- $\text{d}_5$  isotope-labeled internal standard.

#### 2.3. Apparatus

2.3.1. Volumetric flask: 10 mL, amber flask.

2.3.2. Centrifuge tube: 15 mL, PP.

2.3.3. Membrane filter: 0.22  $\mu\text{m}$ , PVDF.

#### 2.4. Mobile phase

##### 2.4.1. Solvent A:

Accurately weigh 0.63 g of ammonium formate and transfer into a 1 L volumetric flask and dilute to volume with deionized water. Filter with a membrane filter.

##### 2.4.2. Solvent B: Acetonitrile

#### 2.5. Internal standard solution preparation

Transfer about 2.5 mg of *N*-nitroso fluoxetine-d<sub>5</sub> internal reference standard accurately weighed into a 10 mL volumetric flask, dissolve and dilute to volume with methanol as the internal standard stock solution. Store in a refrigerator. Upon use, dilute the internal standard stock solution with methanol to 10 ng/mL as the internal standard solution.

## 2.6. Standard solution preparation

Transfer about 5 mg of *N*-nitroso fluoxetine reference standard accurately weighed into a 10 mL volumetric flask, dissolve and dilute to volume with methanol as the standard stock solution. Store in a refrigerator. Upon use, mix appropriate volume of the standard stock solution and the internal standard solution, and dilute with methanol to 1-40 ng/mL (containing 1 ng/mL internal standard) as the standard solutions.

## 2.7. Standard calibration curve establishment

Accurately inject 5 µL of the standard solution into LC-MS/MS separately, and operate according to the following conditions. Establish the standard calibration curve of *N*-nitroso fluoxetine by the ratios of the peak area of *N*-nitroso fluoxetine to that of the internal standard vs. the concentrations of *N*-nitroso fluoxetine.

LC-MS/MS operating conditions<sup>(note)</sup>:

Column: Symmetry C18, 3.5 µm, 4.6 mm i.d. × 15 cm.

Column temperature: 40°C.

Mobile phase: a gradient program of solvent A and solvent B is as follows.

Time (min)	A (%)	B (%)
0.0 → 2.0	35 → 35	65 → 65
2.0 → 7.0	35 → 5	65 → 95
7.0 → 9.0	5 → 5	95 → 95
9.0 → 9.1	5 → 35	95 → 65
9.1 → 12.0	35 → 35	65 → 65

Flow rate: 0.8 mL/min.

Inject volume: 5 µL.

Ion spray voltage: 5.5 kV.

Ionization mode: ESI<sup>+</sup>.

Ion source temperature: 500°C.

Nebulizer gas, Gas 1: 50 psi.

Heated gas, Gas 2: 60 psi.

Curtain gas: 35 psi.

Collision gas: high.

Detection mode: multiple reaction monitoring (MRM). Selected ion pair, declustering potential and collision energy are as follows.

Analyte	Ion pair		Declustering potential (V)	Collision energy (eV)
	Precursor ion ( $m/z$ )	Product ion ( $m/z$ )		
<i>N</i> -nitroso fluoxetine	339	177*	60	14
	339	117	60	26
<i>N</i> -nitroso fluoxetine- $d_5$ (I.S.)	344	182	80	13

\* Quantitative ion pair

Note: 1. If a divert valve is available, the direction of the mobile phase can be diverted as follows.

Time (min)	Position
0.0 → 5.0	Waste
5.0 → 6.2	MS
6.2 → 12.0	Waste

2. All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

## 2.8. Sample solution preparation

Transfer about 0.1 g of sample accurately weighed to a centrifuge tube, and add 1 mL of the internal standard solution and 9 mL of methanol. Mix well, sonicate for 5 min and centrifuge at 3000 ×g for 5 min. Filter the supernatant with a membrane filter, and take the filtrate as the sample solution.

## 2.9. Identification and quantification

Accurately inject 5 µL of sample solution and standard solution into LC-MS/MS separately, and operate according to the conditions in section 2.7. Identify *N*-nitroso fluoxetine based on the retention time and the relative ion intensities<sup>(note)</sup>. Calculate the amount of *N*-nitroso fluoxetine in the sample by the following formula:

The amount of *N*-nitroso fluoxetine in the sample ( $\mu\text{g/g}$ ) =  $\frac{C \times V}{M} \times 10^{-3}$

Where,

C: the concentration of *N*-nitroso fluoxetine in the sample solution calculated by the standard calibration curve (ng/mL)

V: the final make-up volume of the sample (mL)

M: the weight of the sample (g)

Note: Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitative ions ( $\leq 100\%$ ). Maximum permitted tolerances of relative ion intensities by LC-MS/MS are as follows.

Relative ion intensity (%)	Tolerance (%)
> 50	$\pm 20$
> 20-50	$\pm 25$
> 10-20	$\pm 30$
$\leq 10$	$\pm 50$

#### Remark

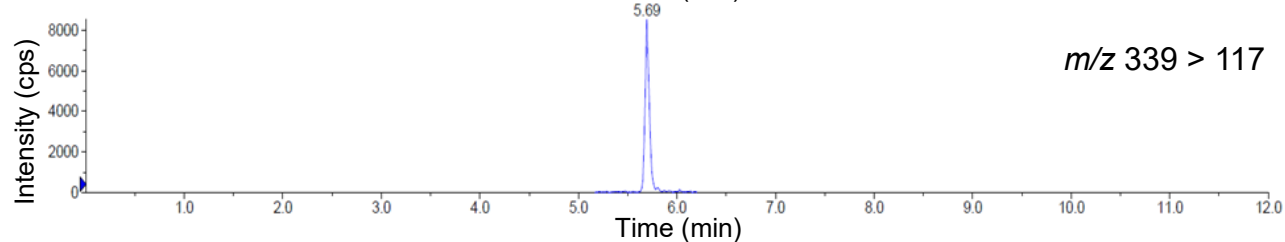
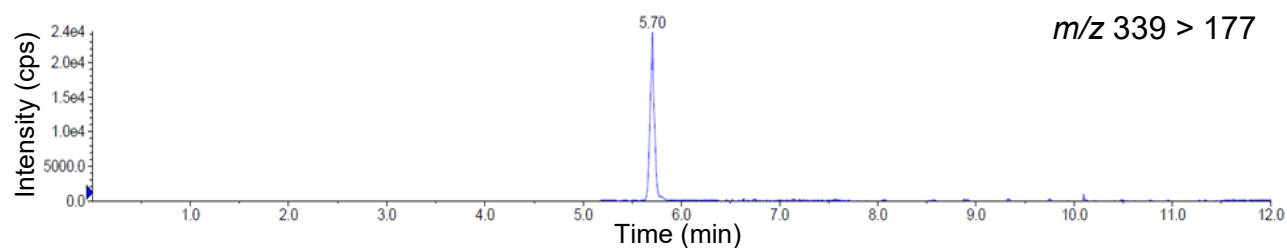
1. Limit of quantification (LOQ) for *N*-nitroso fluoxetine is 0.1  $\mu\text{g/g}$ .
2. Further validation should be performed when interference compounds appear in samples.

#### Reference

1. Ahmad, I., Ullah, Z., Khan, M. I., Alahmari, A. K. and Khan, M. F. 2021. Development and validation of an automated solid-phase extraction-LC-MS/MS method for the bioanalysis of fluoxetine in human plasma. J. Adv. Pharm. Technol. Res. 12: 267-273.
2. Qiu, X., Wang, H. W., Yuan, Y., Wang, Y. F., Sun, M. and Huang, X. S. 2015. An UPLC-MS/MS method for the analysis of glimepiride and fluoxetine in human plasma. J. Chromatogr. B 980: 16-19.

## Reference chromatogram

### (A) *N*-nitroso fluoxetine



### (B) *N*-nitroso fluoxetine- $d_5$ (I.S.)

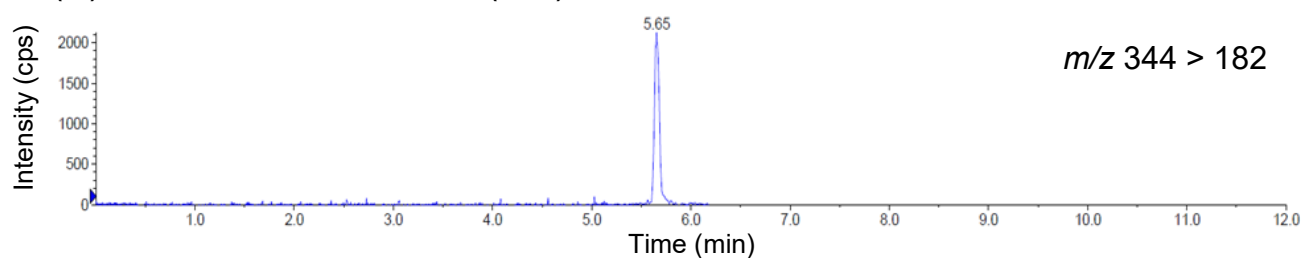


Figure. The MRM chromatograms of *N*-nitroso fluoxetine standard (A) and *N*-nitroso fluoxetine- $d_5$  internal standard (B) analyzed by LC-MS/MS.