

## Bioanalytical methods for determination of fluoxetine, an antidepressant, in biological fluids

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### Abstract

*Fluoxetine, a selective serotonin reuptake inhibitor, is a first-line drug in the treatment of depression, with fewer side effects and better tolerated by the patient.*

*Fluoxetine is a good candidate for therapeutic drug monitoring due to the fact that it has a very extensive pharmacokinetic interindividual variability and it is bio-transformed to a pharmacological active metabolite, norfluoxetine.*

*No data that provides a good correlation between fluoxetine and active metabolite levels and therapeutic effect exist. Still, plasma concentrations that correspond to therapeutic doses are found near the value of 0.4 µg/mL, requiring very sensitive methods.*

*Methods for fluoxetine determination most commonly found in literature are chromatographic methods (especially HPLC and GC) coupled with various detectors. In terms of equipment and costs, UV detection is the most accessible but its sensitivity and selectivity are greatly surpassed by fluorescence and mass spectrometry detection methods.*

*Plasma and urine are the most common samples used for drug determination, with liquid-liquid extraction or solid phase extraction as preparation methods, often using an internal standard.*

**Keywords:** *Fluoxetine, Therapeutic drug monitoring, Bioanalytical, Antidepressant, Validation.*

## I. INTRODUCTION

Depression is a mental disorder characterized by extreme sadness, anxiety, pessimism accompanied by cognitive impairment, altered affective state and a marked reduction of psychomotor activity and initiative. It is an illness that has both a physical and a psychological

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component and has the following effects: decreased appetite, shorter sleeps period, and the distorted perception of oneself and of others (Sartorius N, 2007).

From a pharmacological point of view, depression is caused by adrenergic and serotonergic hypofunction, with noradrenaline, adrenaline and serotonin as the neuromediators involved (Sartorius N, 2007).

The first antidepressants were discovered in the late 1950s. Two groups of compounds were defined: tricyclic antidepressants (TACs) and monoamine oxidase inhibitors (MAOIs), representing the basic treatment for major depression between 1960 - 1980 (Sartorius, 2007).

Later on, because patients with depression presented low cerebral serotonin levels (Cristea, 2009), the selective serotonin reuptake inhibitors (SSRIs) were discovered, which proved safer than TACs in terms of toxicological profile and patient tolerance. Thus the SSRIs became the first choice for treatment of major depression replacing, although not completely, the tricyclic antidepressants (Sartorius, 2007).

The SSRIs are potently and selectively inhibit serotonin reuptake, with no effect on catecholamine reuptake. They do not present affinity for cholinergic, muscarinic, histaminergic or  $\alpha$ -adrenergic receptors, thus they cause no adverse effect associated with the respective receptors (Cristea, 2009).

The SSRIs are first line drugs in the treatment of depression, obsessive-compulsive disorders (OCDs) and panic attacks. They have also been used with success in the treatment of bipolar depression, atypical depression, nervous bulimia, personality disorders, anxiety postpartum depression and alcoholism (Lemke & Williams, 2007).

Almost all antidepressants, SSRIs included, meet the requirements and are subject to therapeutic drug monitoring (TDM).

TDM may be defined as the use of drug or metabolite monitoring in body fluids as an aid to the management of therapy (the term therapeutic drug management is now also employed as an alternative description). Different patients need different doses of drug to produce optimum pharmacological effect because individuals vary widely regarding drug absorption and elimination (pharmacokinetics) and also drug effect (pharmacodynamics) (Hallworth, 2011). The phases of a drug from prescription by the physician to obtaining the pharmacological effect are best illustrated in Figure 3.

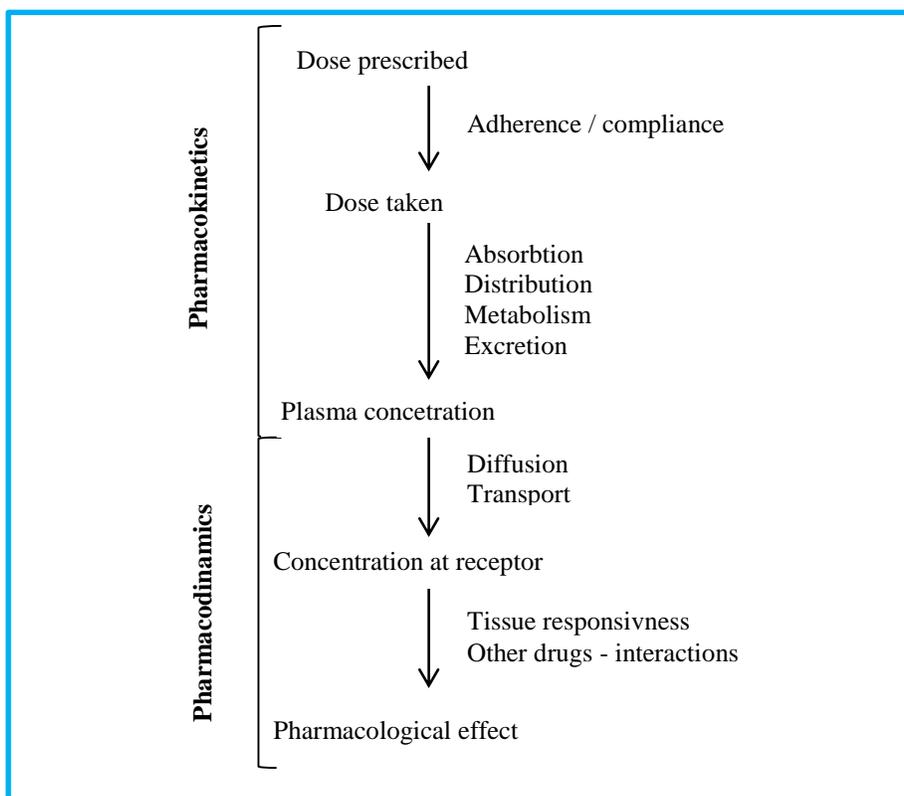


Figure 1. Processes involved in drug action (Hallworth, 2011)

## II. FLUOXETINE

### 1. Pharmacokinetics

Fluoxetine ((RS)-N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy]propan-1-amine – Figure 1) is a selective serotonin reuptake inhibitor, used primarily in the treatment of major depression, bulimia nervosa and obsessive-compulsive disorder.

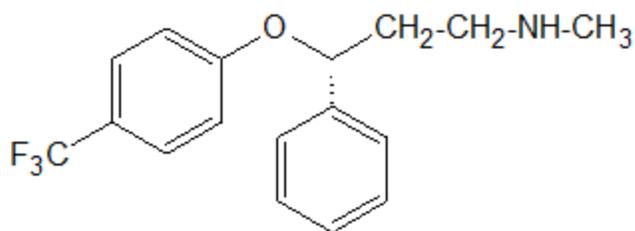
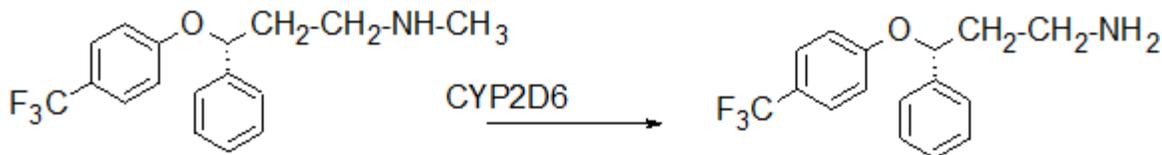


Figure 2. Fluoxetine

Fluoxetine usually exists as a racemic mix of R and S-fluoxetine, S being the more active enantiomer (Cristea, 2009).

The substance is readily absorbed in the gastrointestinal tract, with a plasmatic peak at 6 to 8 hours after administering an oral dose. The presence of food does not influence bioavailability and protein binding is estimated at 95% (Sweetman, 2009).

Fluoxetine is metabolized by N-demethylation by the isoform CYP2D6 (Figure 2) to a pharmacological active metabolite, norfluoxetine and, to a lesser extent by O-alkylation to p-trifluoromethylphenol, an inactive metabolite (Wong DT, 1993).



**Figure 3. Fluoxetine and norfluoxetine**

It is highly likely that fluoxetine metabolism is influenced by genetic polymorphism as different levels of fluoxetine and metabolites are observed in low metabolizers. This does not seem to affect the pharmacological activity (Sweetman, 2009).

The two norfluoxetine enantiomers are weaker inhibitors of noradrenaline reuptake in comparison with fluoxetine enantiomers. Serotonin reuptake in the cerebral cortex (rats) lasts for more than 24 hours after administering S-norfluoxetine (Wong DT, 1993). Therefore the activity of S-norfluoxetine is comparable to that of the parent compound, while R-norfluoxetine is much less potent (Sweetman, 2009).

Fluoxetine is widely distributed throughout the body and is renally excreted. The process is slow and subject to interindividual variability (Cristea, 2009). Fluoxetine and norfluoxetine have long half-lives (Borsen & Rasmussen, 1996; Preskorn, 2010), between 1 and 3 days after immediate use or 4 to 6 days after chronic administration (Sweetman, 2009), up to 10 days (Cristea, 2009). S-fluoxetine elimination is slower however (Sweetman, 2009) and the half-life of norfluoxetine is 3 to 30 days (Cristea, 2009) or 4 to 16 days (Sweetman, 2009), depending on the source cited.

Fluoxetine's and norfluoxetine's long half-lives have clinical implications. Steady state concentrations are reached after several weeks (Cristea, 2009). Also they persist for a considerable time after treatment, so precautions must be taken when starting the patient on other serotonergic drugs (Sweetman, 2009).

## **2. Pharmacodynamics**

It is an antidepressant, 3-phenoxy-3-phenylpropylamine derivative, used in therapy as a racemic mix which mainly inhibits serotonin uptake in the brain. The presence of trifluor-methyl functional group is responsible for the selective pharmacological activity on CNS serotonin reuptake (Lemke & Williams, 2007).

The mechanism of action consists in blocking 5-HT<sub>1A</sub> and 5-HT<sub>2C</sub> receptors. It is also a weak noradrenaline reuptake inhibitor.

### **3. Toxicology**

Fluoxetine does not cause adverse effects common in TACs: arterial hypotension, cholinergic effects or cardiotoxicity (Cristea, 2009) and is less sedating, like most SSRIs, when compared to TACs and it is ranked as a high therapeutic index drug (Sweetman, 2009). It does cause serotonergic effects like anxiety, insomnia, nausea, diarrhea, anorexia, sexual dysfunction, cephalalgia, dizziness, tremor, seizures, rashes and vasculitis (Sweetman, 2009; Cristea, 2009).

### **4. Therapy and posology**

Preparations with fluoxetine are orally administered. The usual dose is 20-60 mg/day and can be increased up to 80 mg/day. The treatment starts at low dose (20 mg/day), which is gradually increased (Sweetman, 2009).

The therapeutic windows found in literature vary slightly but maintain in the range of 0.15-0.8 µg/mL (fluoxetine + norfluoxetine), fluoxetine levels being slightly higher than norfluoxetine.

The relationship between blood concentrations of racemic fluoxetine and norfluoxetine and clinical outcome or adverse events have been studied but no solid correlation between clinical outcome and plasma concentrations of either fluoxetine or norfluoxetine or the sum of both could be found. This is because the enantiomers of both active compound and metabolite differ significantly and clinical results are dependent of the ratio of enantiomers. Chiral analysis performed in conjunction with TDM studies might help clarify this problem (Hiemke & Härter, 2000).

## **III. Therapeutic drug monitoring (TDM)**

Pharmacological effects can be measured with the use of markers – the response of the patient to the treatment. In practice this is easy when the response is readily measurable. The response can be of clinical nature (e.g. blood pressure in the case of antihypertensive drugs) or with an appropriate laboratory marker (e.g. glucose determination for hypoglycemic agents or cholesterol/triglycerides levels for lipid-lowering drugs) (Hallworth, 2011).

Dose adjustment is much more difficult (but no less necessary) when drug response cannot be rapidly assessed clinically, which is the case for antidepressants. With certain conditions accomplished and suitable analytical methods the plasma concentration of a drug and/or metabolite can be determined and may serve as an effective and clinically useful surrogate marker of response. However TDM is not just about the analytical result. It implies that a several determinations at different moments must be made and the analytical results interpreted in order to make the best decision for the patient's benefit (Hallworth, 2011).

The AGNP (Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie) members provided a consensus guideline regarding optimal use of TDM in clinical context for psychotropic drugs.

They state that the development of new, sensitive analytical techniques showed that drug concentrations vary widely due to a very high interindividual variability in the pharmacokinetics and pharmacodynamics of these drugs by correlating plasmatic concentration with dosage and adverse effects (Baumann, et al., 2004). This encouraged clinicians to use TDM in combination with pharmacogenetic testing in order to demonstrate genetically determined metabolism.

If plasma drug concentrations are to be a useful surrogate marker of response, two premises must be satisfied:

- The drug concentration in plasma accurately reflects the concentration at the site of action (the receptor),
- The drug concentration at the receptor should provide an accurate indication of pharmacological response.

According to “Clarke’s Analysis of Drugs and Poisons” and the AGNP the essential criteria for TDM to be clinically useful may be summarized as follows:

- Poor correlation between the dose given and the plasma concentration obtained in different patients (wide inter-individual pharmacokinetic variability).
- Good correlation between plasma concentration and pharmacological effect in different patients (low inter-individual pharmacodynamic variability); this also implies an established therapeutic range of plasma concentrations or a good relationship between plasma concentration and effect.
- TDM is clinically relevant only for drugs that show significant toxic or undesirable effects at plasma concentrations only slightly above those required for useful effect (low therapeutic index).
- TDM is redundant for drugs where there is a better clinical marker of both the desired effect and any associated adverse effects.
- Long-term treatment.

Five levels of recommendation for TDM were proposed by the AGNP (Baumann et al., 2004; Baumann et al., 2005):

1. Standard of care (strongly recommended): established therapeutic window.
2. Recommended: presumed therapeutic window determined from fixed dose studies.
3. Useful: suggested therapeutic ranges obtained from steady-state pharmacokinetic studies at therapeutically effective doses with clinical data backup.
4. Unclear (probably useful): therapeutic ranges obtained from steady-state pharmacokinetic studies at therapeutically effective doses with little or no valid clinical data.
5. Not recommended: unique pharmacology of the drug (blockade of an enzyme) or flexible dosing according to clinical symptoms.

However if suspicion of non-compliance exists, TDM is indicated in all cases.

Considering all of the above, the AGNP have classified fluoxetine as a class 3 drug regarding TDM (Baumann et al., 2005), while another source states that monitoring has not shown to be applicable (Hallworth, 2011).

For a good TDM practice sensitive analytical methods are necessary to correctly evaluate the low level concentrations in body fluids (ng/mL), often presented by antipsychotics and antidepressants. Selectivity is also an issue due to comedication (drugs administered simultaneously may interfere in the assay). The fact that most antidepressants are metabolized to an active compound, the challenge of developing a suitable method is even greater. In order to have all the information to assess pharmacological and toxicological effects, both the parent compound and the metabolite must be correctly assayed in order to make good decisions regarding patient therapy.

Plasma or serum samples are generally used for TDM. Chromatography is the most used method, high performance liquid chromatography (HPLC) or gas-chromatography (GC), coupled with various detectors: UV (although not very sensitive), fluorescence, MS, MS-MS or nitrogen-phosphorous detectors for GC. Also there is need for sample clean-up, which is a very time consuming step, in order to remove matrix effect.

Regarding the methods used in bioanalysis the FDA (Food and Drug Administration) has issued a guideline (Guidance for Industry – Bioanalytical method validation) in which general recommendations regarding method validation are presented.

The information in the guidance applies to bioanalytical procedures and immunological and microbiological procedures that are performed for the quantitative determination of drugs and/or metabolites, and therapeutic proteins in biological matrices, such as blood, serum, plasma, urine, tissue, and skin (FDA, 2013).

Validating bioanalytical methods includes performing all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix (e.g., blood, plasma, serum, or urine) is reliable and reproducible for the intended use. Fundamental parameters for this validation include the following: accuracy, precision, selectivity, sensitivity, reproducibility, stability (FDA, 2013).

#### **IV. BIOANALYTICAL METHODS FOR DETERMINATION OF FLUOXETINE AND METHABOLITE**

In Table 1 the acceptance criteria for validation parameters of bioanalytical methods are presented. These criteria are according to Guidance for Industry – Bioanalytical method validation Draft Guidance from 2013 provided by the FDA/CDER.

**Table 1. Acceptance criteria for method validation parameters according to FDA/CDER Guidance for Industry (DRAFT) (FDA, 2013)**

Parameter	Acceptance criteria
Accuracy (recovery %)	Recovery of 100%±15% (±20% for LLoQ)
Precision (coefficient of variation CV %)	Relative standard deviation of 15% (20% for LLoQ)
Linearity and range	Calibration curve with a correlation factor (R) NLT 0.999
Selectivity	No interference from biological matrix, metabolites and concomitant medication
Limit of quantitation	LLoQ an ULoQ (consistent with accuracy, precision and linearity)
Stability	Freeze and Thaw stability / Long-term stability

Most methods used for fluoxetine determination (with or without norfluoxetine) are HPLC methods, more precisely RP-HPLC (reversed-phase high performance liquid chromatography). Most common type of chromatographic columns had octadecylsilyl based stationary phase (C18) and, to a lesser extent, octylsilyl (C8) stationary phase. Other types of columns (chiral, CN, etc) were also used. In some cases the chromatographic column was used together with a guard column to increase lifetime.

Mobile phases were for the most part polar with a aqueous buffer solution with an acidic pH (1,9-5,5) mixed in different proportions with an organic solvent. Generally the elution system was isocratic, but gradient elution methods were also used.

Detection methods used vary between methods. Fluorescence detection was used for some methods as it provides suitable sensibility and selectivity for fluoxetine and norfluoxetine determination (which are normally found at plasmatic concentrations less than 1 µg/mL). The excitation wavelength was in the range 227-230 nm and the emission wavelength had a wider range, from 290 to 312 nm.

More details about the RP-HPLC methods with fluorescence detection method are presented in Table 2.

In two cases the focus was on separating fluoxetine and norfluoxetine enantiomers (Bueno, Silva, & Queiroz, 2011; Unceta, et al., 2007). The columns used are special chiral columns. In one study the analyst employed a RP-HPLC method, using a cellulose derivative stationary phase

(Bueno, Silva, & Queiroz, 2011). The other method used a RP-HPLC separation system for parent compound and metabolite and a NP-HPLC method (normal-phase high performance liquid chromatography) for determination of enantiomers (Unceta et al., 2007).

These methods can allow for a better correlation between therapeutic effect, fluoxetine and norfluoxetine content and enantiomer proportions respectively.

A derivatization step was added in one of the methods with no visible improvement in method sensitivity or selectivity (Higashi, Gao, & Fujii, 2009).

Most methods studied selectivity for drugs that can be administered simultaneous with fluoxetine or regular drugs to assess their influence on the analysis.

UV detection methods have also been used, but with poorer sensitivity when compared to MS and fluorescence detection (Orsulak, Kenney, Crowley, & Wittman, 1988; El Maanni, Combourieu, Bonini, & Creppy, 1993; Samanidou & Kourti, 2009).

MS and MS-MS detection methods coupled with HPLC or gas-chromatographs provided similar sensitivity and selectivity with fluorescence detectors, showing no advantage over the latter, as the instrument is far more costly and requires special training (Açikkol & Salkim, 2010; Deglon, Lauer, Thomas, Mangin, & Staub, 2010; El-Rjoob, Tahtamouni, & Tahboub, 2010; Green, Houghton, Scarth, & Gregory, 2002).

Regardless of the detection method used, sample preparation is a very important step. Sample cleanup is essential to lower the noise obtained in the chromatogram and also to reduce potential damage done to various parts of the equipment. Samples were prepared by liquid-liquid extraction, solid-phase extraction and protein precipitation.

## **V. CONCLUSIONS**

The methods described, fluorescence and MS in particular, are selective and sensitive methods capable of detecting fluoxetine and norfluoxetine at levels much lower than the therapeutic range and have the following applications:

- Pharmacokinetic studies
- Bioequivalence studies
- Therapeutic drug monitoring for patients under fluoxetine treatment
- Pharmacogenetic studies

Fluoxetine is a drug for which some data exists, but more analytical results correlated with pharmacological data can help improve treatment schemes and patient care.

**Table 2. Fluoxetine and norfluoxetine determination by HPLC coupled with fluorescence detection**

Parameter	HPLC method with fluorescence detection					
	(Raggi et al., 1999)	(Vlase, Imre, & Leucuta, 2005)	(Bueno, Silva, & Queiroz, 2011)	(Unceta et al., 2007)	(Freitas, Dobrovolskin-Porto, Pizzamiglio Vieira, & Bastos de Siqueira, 2010)	(Higashi, Gao, & Fujii, 2009)*
Stationary phase – chromatographic column	C8	C18	Tris-(3,5-dimethylphenyl carbamate) cellulose column	CN column and silicagel column	C18	C18
Mobile phase	Acetonitrile: Buffer solution pH 1.9 = 55:45 (v/v)	Acetonitril: Buffer solution pH 2.3 = 31:69 (v/v)	Buffer solution pH 3.0:Acetonitrile = 75:25 (v/v)	Buffer solution: Acetonitrile = 75:25 (v/v) Tetrahydrofuran: isoocctan = 40:60 (v/v)	Buffer solution pH 4.5: Acetonitrile = 50:50 (v/v)	Acetonitrile: Ethanol: Buffer solution = 45:20:45 (v/v/v)
Detection Ex/Em	230/294 nm	298/340 nm 230/312 nm	230/290 nm	227/305 nm	230/290 nm	470/540 nm
Internal standard	Paroxetine	Paroxetine	-	-	-	Protriptyline
Limit of detection	1 ng/mL	0.4 ng/mL	5 ng/mL	3.2 ng/mL	-	5 ng/mL
Limit of quantitation	2.5 ng/mL	1 ng/mL	30 ng/mL	5 ng/mL	5 ng/mL	10 ng/mL
Linearity and range	2.5-500 ng/mL	1-39 ng/mL	30-1000 ng/mL	5-500 ng/mL	25-800 ng/mL	10-500 ng/mL
Precision (coefficient of variation CV %)	<4%	<15%	<15%	<9%	<13%	<15%

Accuracy (recovery %)	88,5 – 92,0 %	92,6 – 109%	91,8 – 117,8%	95.5%-108.3%	86,7% - 116,2%	88,0 – 110,0%
Sample preparation	Liquid-liquid extraction	Liquid-liquid extraction	Protein precipitation	Solid phase extraction	Liquid-liquid extraction	Liquid-liquid extraction with derivatization step
Sample type	Plasma	Plasma	Plasma	Plasma and rat brain	Plasma	Serum Urine

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\*Validation results presented for serum sample. In the method validation urine samples were also used.

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