Clinical Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Simultaneous determination of fluoxetine and norfluoxetine in dried blood spots using high-performance liquid chromatography-tandem mass spectrometry

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ARTICLE INFO

Keywords:
Fluoxetine
Norfluoxetine
LC-MS/MS
Therapeutic monitoring
DBS

ABSTRACT

Background: Therapeutic drug monitoring (TDM) of the widely prescribed antidepressant fluoxetine (FLU) is recommended in certain situations, such as occurrence of toxicity, inadequate response or suspect of poor adherence. Dried blood spot (DBS) sampling is an increasingly studied alternative for TDM, particularly for outpatients, due to its ease of collection and inherent stability.

Objectives: The aim of this study was to develop and validate an LC-MS/MS assay for the simultaneous quantification of FLU and norfluoxetine (NFLU) in DBS.

Design and methods: The assay is based on a liquid extraction of single DBS with 8 mm of diameter, using FLU-D6 as the internal standard, followed by reversed phase separation in an Accucore® C18 column (100 × 2.1 mm, 2.6 μm). Mobile phase was composed of water and acetonitrile (gradient from 80:20 to 50:50, v/v), both containing formic acid 0.1%. The assay was validated and applied to 30 patients under FLU pharmacotherapy.

Results: The assay was linear in the range 10–750 ng mL^{-1}. Precision assays presented CV% of 3.13–9.61 and 3.54–7.99 for FLU and NFLU, respectively, and accuracy in the range of 97.98–110.44% and 100.25–105.8%. FLU and NFLU were stable at 25 and 45 °C for 7 days. The assay was evaluated in 30 patients under FLU treatment. Concentrations of both compounds were higher in DBS than in plasma, and the use of the multiplying factors 0.71 and 0.68 for FLU and NFLU, respectively, allowed acceptable estimation of plasma concentrations, with median prediction bias of − 0.55 to 0.55% and mean differences of 0.4 to 2.2 ng mL^{-1}.

Conclusions: The presented data support the clinical use of DBS for therapeutic drug monitoring of FLU.

1. Introduction

Fluoxetine (FLU) is a first line drug for treating depression, a condition with reported prevalence ranging from 6.5 to 21% in different low, middle and high-income populations throughout the World [1]. FLU is classified as a selective serotonin reuptake inhibitor (SSRI), and its antidepressant action is explained by its capacity for desensitization of somatodendritic serotonin 1A autoreceptors in the midbrain raphe [2].

The main metabolite of FLU is norfluoxetine (NFLU), which is formed mainly by demethylation catalyzed by CYP2D6. NFLU has similar potency and selectivity of 5-HT uptake inhibition compared with the parent compound [3,4]. The formation rate of NFLU from FLU is affected both by genetic and environmental factors, with the concentration ratio [NFLU]/[FLU] being a phenotypic index of CYP2D6 activity [4,5]. About 30 to 40% of patients do not present an adequate therapeutic response to FLU, which can be partially attributed to interindividual variations in the formation of the active metabolite NFLU [6].

Therapeutic drug monitoring (TDM) of FLU is classified as recommended by the Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie (AGNP), once plasma concentrations are related to clinical effects, and adverse effects are more frequent at concentrations above the suggested therapeutic range [7]. Moreover, the measurement of plasma concentrations of FLU and NFLU could help to identify non-adherence, as well as the metabolizer phenotype of CYP2D6 [7]. The AGNP Consensus Guideline for TDM in Psychiatry recommends a therapeutic reference range for the sum of though plasma

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http://dx.doi.org/10.1016/j.clinbiochem.2017.10.002
Received 7 August 2017; Received in revised form 12 September 2017; Accepted 3 October 2017

concentrations of FLU and NFLU of 120–500 ng mL^{-1}, with an expected ratio of concentrations NFLU/FLU from 0.7 to 1.9 [7].

The classical matrix for TDM laboratory testing is plasma or serum, obtained from a venous blood collection. Several reports were published on the application of dried blood spots (DBS), usually obtained after finger pricks, for TDM in the field of neuropsychiatry. These reports include drugs such as amitriptyline, nortriptyline, imipramine, clomipramine, and their active metabolites [8], carbamazepine [9], topiramate [10], valproic acid [11] venlafaxine and *O*-desmethylvenlafaxine [12]. The potential advantages and limitations of the use of DBS for TDM have been recently reviewed [13,14]. The main advantage of DBS sampling is the simplicity of sample collection, which is minimally invasive, associated with compound stabilization due to sample drying, allowing remote collection for outpatients. On the other hand, conversion of measurements in the whole blood present in the DBS to plasma levels, the matrix in which the current reference levels were established, is highly affected by the blood hematocrit, particularly when the drug has preferential bonding to blood cells [13].

Despite the clinical relevance of FLU, there is only one previous report on the measurement of FLU and NFLU in DBS, in which the analysis was performed using gas chromatography couples to tandem mass spectrometry, with negative-ion chemical ionization (GC-NICI-MS-MS) [15], an instrumental set-up rarely available in clinical laboratories. This previous study evaluated FLU and NFLU concentrations in only one volunteer, without evaluation of the correlation between plasma and DBS concentrations, which is critical to the clinical use of the assay.

The present study presents a novel and simple DBS assay for simultaneous measurement of FLU and NFLU concentrations using an entry-level, high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS), also investigating strategies for translation of DBS measurements to clinically useful plasma levels.

2. Experimental

2.1. Standards, solvents and materials

FLU and deuterated FLU (FLU-D6) solutions were acquired from Cerilliant (Round Rock, USA) and NFU was from Toronto Research Chemicals (NorthYork, Canada). Acetonitrile, methyl-*tert*-butyl ether (MTBE), n-hexane, methanol and formic acid were bought from Merck (Darmstadt, Germany) and tris(hydroxymethyl)aminomethane (TRIS) was acquired from Sigma Aldrich (Saint Louis, USA). Whatman 903® paper was obtained from GE Healthcare (Westborough, USA).

2.2. Solutions

NFLU stock solution, at the concentration of 2 mg mL^{-1}, was prepared by dissolution in methanol. FLU and FLU-D6 stocks were purchased at the concentrations of 1000 and 100 μg mL^{-1}, respectively. FLU, FLU-D6 and NFLU intermediate solutions, at the concentration of 10 μg mL^{-1}, were prepared by dilution with methanol. Combined working solutions of FLU and NFLU were prepared at concentrations 20 times higher than calibration and control levels, also by dilution with methanol. TRIS buffer pH 10.0 was prepared by dissolution of TRIS in water to obtain a 10 mM solution, with pH adjustment with a sodium hydroxide solution. DBS extraction solution consisted of a mixture of methanol and acetonitrile (3:1, v/v), containing FLU-D6 at 0.4 ng mL^{-1}. The internal standard solution for plasma analysis was FLU-D6 at 320 μg mL^{-1}, in methanol. Mobile phase A was purified water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid.

2.3. Chromatographic and mass spectrometric conditions

Samples were analyzed using an Ultimate 3000 XRS UHPLC coupled to a TSQ Quantum access triple quadrupole mass spectrometer, purchased from Thermo Scientific (San Jose, USA). Separation was performed in an Accucore C18 (100 × 2.1 mm, p. d. 2.6 μm) column, also from Thermo Scientific. The column temperature was 40 °C, and eluent flow rate was fixed at 0.4 mL min^{-1}. Initial eluent composition was 80% A, maintained for 1.0 min, followed by a linear 5.0 min ramp to 50% A, which was held for 1.0 min, returning to the initial composition at 7.5 min. Column equilibration time was 2 min. The MS conditions were as follows: ESI positive mode, capillary voltage of 4.5 kV; sheath gas, nitrogen, 40 arb; auxiliary gas, nitrogen, 15 arb; collision gas, argon, 1.5 mTor; vaporizer temperature, 380 °C; ion transfer temperature 210 °C. MRM transitions were: FLU m/z 310 → 44 (quantification); 310 → 42 and 310 → 117 (qualification); NFLU m/z 296 → 134 (quantification); 296 → 105 and 296 → 30 (qualification); FLU-D6 m/z 316 → 44 (quantification); 316 → 187 and 316 → 42 (qualification). Collision energies were 13, 79 e 34 eV for FLU; 5, 16 e 12 eV for NFLU and 13, 52 e 70 eV for FLU-D6.

2.4. Preparation of DBS

DBS calibration and quality control (QC) samples were prepared by pipetting 50 μL of blood in Whatman 903® paper, followed for a minimum drying time of 3 h before extraction. Calibrators and QC samples were prepared diluting working solutions of FLU and NFLU with venous blood in the proportion 1:20 (v/v). Blood used for the preparation of validation DBS samples had a Hct% of 40 unless otherwise stated. DBS samples from finger-pricks were obtained by application of one drop of blood to the paper, directly from the patient finger, without touching the surface of the collection area. As well as calibration and QC samples, patient DBS samples were allowed to dry at room temperature for 3 h after collection and then stored in plastic bags with desiccants at room temperature. Analysis were performed within 72 h of collection.

2.5. DBS sample preparation

One DBS disk with 8 mm diameter was cut in 4 pieces and transferred to a 2 mL polypropylene microtube, followed by the addition of 400 μL of the DBS extraction solution. The tube was agitated at 500 rpm for 30 min in a ThermoMixer® (Eppendorf), while maintaining the temperature at 45 °C. After centrifugation for 10 min at 10.000*g*, an aliquot of 350 μL supernatant was evaporated in a vacuum centrifuge at 45 °C. The resulting dried extract was recovered with 100 μL of initial mobile phase, followed by 10 min of centrifugation at 10.000*g* and 20 μL of the resulting supernatant was injected into the LC-MS/MS system.

2.6. Linearity

Calibration samples had concentrations of 10, 20, 50, 100, 250, 500 and 750 ng mL^{-1}, processed in sextuplicate. Calibration curves were obtained relating the area ratios from FLU and NFLU to FLU-D6 peaks. Homoscedasticity of calibration data was evaluated with F-test at the confidence level of 95%. Weighted least-squares linear regression was used to generate calibration models, which were evaluated through their coefficients of correlation (r) and cumulative percentage relative error (Σ%RE) [16].

2.7. Precision and accuracy

QC samples were prepared at the concentration levels of 40 (quality control at low concentration, QCL), 200 (quality control at medium concentration, QCM) and 600 ng mL^{-1} (quality control at high concentration, QCH). QC samples were processed and analyzed in triplicate, in each of 5 days. Within-assay precision and between-day precision were calculated by one-way analysis of variance and expressed as CV%. Accuracy was evaluated as the percentage of the nominal