Determination of fluoxetine and its metabolite norfluoxetine in human liver microsomes by reversed-phase HPLC in vitro

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KEY WORDS fluoxetine; high pressure liquid chromatography; liver microsomes

ABSTRACT

AIM: A high-performance liquid chromatography (HPLC) method was developed for the determination of fluoxetine (FLU) and its metabolite norfluoxetine (N-FLU) in human liver microsomes in vitro. METHODS: An incubation buffer containing human liver microsomes, NADPH-generating system, and FLU, after termination of enzyme reaction and addition of nortriptiline (NOR) as internal standard (IS), was extracted with n-hexane/acetonitrile, and separated on a reversed-phase ODS column. Detection was achieved at 226 nm by ultraviolet detector (UV). RESULTS: The limit of detection was 5 μg/L for both FLU and N-FLU. No potential interference was found. The method provides recoveries of up to 94% - 104% and acceptable coefficients of variation were found for both within-run (< 7.8%) and day to day (<9.1%) assays. CONCLUSION: This method is rapid, sensitive, and simple for studying the metabolism of FLU and N-FLU.

INTRODUCTION

Fluoxetine (FLU) is an antidepressant drug that enhances serotonergic neurotransmission through the selective inhibition of neuronal reuptake of serotonin[1]. Norfluoxetine (N-FLU), FLU N-demethylated metabolite, also inhibits serotonin reuptake.

FLU and N-FLU were quantified by gas chromatography with electron capture detection (GC-ECD) according to Nash et al.[2]. More recently, a GC-mass spectrometric (GC-MS) method that allowed the simultaneous determination of the enantiomers of FLU and N-FLU was developed[3]. However, high-performance liquid chromatography (HPLC) with ultraviolet[4-8] or fluorescence detection[9,10] is the most widely used analytical method. These chromatographic methods allow quantification of FLU and N-FLU after liquid[4-8] or solid-phase extraction[9,10]. Several of these previously published studies involve lengthy, multiple step extraction taking a long time, and with relatively low recovery. In addition, most HPLC methods reported previously were suitable for the quantification of FLU and N-FLU in vivo, but a rapid, sensitive, simple and reproducible analytic method was required for the purpose of investigating the metabolism of FLU or N-FLU in vitro.

This report described a reversed-phase HPLC method for simultaneous quantification of FLU and N-FLU in human liver microsomes in vitro.

MATERIALS AND METHODS

Chemicals FLU and N-FLU, both as hydrochloride salts, were purchased from Research Biochemicals International (Natick, USA). Nortriptylne, β-nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co (St Louis, USA). Acetonitrile (Tedia Company Inc, USA) of HPLC grade and doubly distilled water were used for HPLC with UV detector. All other chemicals were of AR grade.

Chromatography The chromatography consisted of HP 1050 series, a reversed-phase Hypersil-ODS C18 column (250 mm × 4 mm ID, 5 μm particle size) and ultraviolet detector. The UV detection was set at 226 nm. The mobile phase was a mixture of potassium dihydrogen phosphate buffer 0.05 mol/L and acetonitrile (40/60, vol/vol). The pH of the mobile phase was finally adjusted to 3.3 by orthophosphoric acid. The flow rate was 1.3 mL/min and the column temperature was maintained at 50 °C.
Sample preparation Six human liver specimens with normal histology from patients undergoing partial hepatectomy were used to prepare microsomes by differential centrifugation. Microsomal protein concentration was determined colorimetrically. The incubation buffer contained microsomes 1.0 g/L, potassium phosphate buffer 0.1 mol/L (pH 7.4), NADPH-generating system, and various concentrations of FLU with or without CYP-selective inhibitors in a final volume of 500 μL. The reactions were initiated by adding 115 μL NADPH-generating system consisting of NADP 1 mmol/L, glucose-6-phosphate 10 mmol/L, glucose-6-phosphate dehydrogenase 2 kU/L, MgCl₂ 10 mmol/L. After 75 min of shaking in a water bath at 37 °C, reaction was stopped by cooling on ice and addition of 100 μL acetonitrile. A previous study showed that the rate of formation of N-FLU was linear with respect to time (through 50 min) and protein concentration (through 2.0 g/L). The present study also confirms this and observes linearity with respect to time (up to 75 min) and protein concentration (up to 2.0 g/L).

After termination of enzyme reaction, 500 μL of incubation buffer was mixed with NOR 100 μL (4 mg/L stock solution), and 200 μL sodium carbonate solution (1.0 mol/L, pH 12). Samples were then vigorously shaken for 1 min and consequently extracted into 6 mL n-hexane/acetonitrile (98/2, vol/vol) on a reciprocating shaker for 3 min. After centrifugation for 5 min (1000 × g), the upper organic layer was pipetted into another clean centrifuge tube. Samples were eventually evaporated till dry under a gentle stream of nitrogen at 37 °C. The residue was dissolved in 100 μL mobile phase and 20 μL aliquot was used for HPLC.

Calibration curves To prepare standard curves, appropriate amounts of FLU and N-FLU were added to 500 μL of blank incubation buffer to yield the following concentration of each: 10, 25, 50, 100, 200, 400, and 800 μg/L. These samples were then prepared according to the procedure described above. Quantification was performed by calculating the peak-height ratio of each compound to the IS.

Validation study Standard incubation buffer was prepared by spiking an aliquot of each standard solution (FLU, N-FLU, NOR) into the blank incubation buffer which was incubated with human liver microsomal preparations and NADPH-generating system but without FLU (substrate) as described in sample preparation above. Standard curves were produced by plotting the amount of FLU or N-FLU against the peak-height ratio of the amount of FLU or N-FLU to that of the IS with FLU or N-FLU at 10, 25, 50, 100, 200, 400, and 800 μg/L and IS in the blank incubation buffer. Three standard solutions containing, respectively, 10, 200, and 800 μg/L of both FLU and N-FLU were prepared to test the accuracy and precision of the assay. The lower limit of detection was defined as a signal-to-noise ratio of 3:1. Concentrations of FLU and N-FLU in the unknown samples were determined from the peak-height ratio of the calibration samples at 3 different levels within the linear range.

RESULTS

Chromatographic separation The chromatographic separations obtained from extracted blank incubation buffer of human liver microsomes and spiked with N-FLU, FLU, and NOR, and extracted incubation buffer of human liver microsomes with FLU and NOR, were shown in Fig 1. Retention times for N-FLU, FLU, and NOR are 3.3, 4.1, and 5.2 min, respectively. The three peaks are completely resolved without any interference.

![Fig 1. Chromatogram of (A) extracted blank incubation buffer of human liver microsomes and (B) spiked with N-FLU, FLU, and NOR, and (C) extracted incubation buffer of human liver microsomes with FLU 5 μmol/L.](image)

Validation study The RSD for intra- and inter-day reproducibility ranged from 2.6 % - 7.8 % and 2.9 % - 9.1 % for both FLU and N-FLU, respectively. The average recoveries of FLU and N-FLU ranged from 94 % - 104 % (Tab 1). A good linear relationship was
obtained in the range assay, i.e., 10 – 800 µg/L for both FLU (r = 0.9998) and N-FLU (r = 0.9994). The linear regression equations are \( \hat{Y} = 1.3755X + 0.05583 \) for FLU and \( \hat{Y} = 0.03411X + 0.001517 \) for N-FLU. The limit of detection under the described conditions was 5.0 µg/L for both compounds (Tab 2, Fig 2).

**Tab 1. Intra- and inter-assay validation and relative recovery of fluoxetine (FLU) and norfluoxetine (N-FLU).**

<table>
<thead>
<tr>
<th>Concentration (µg/L)</th>
<th>Intra-assay (n = 5) (%)</th>
<th>Inter-assay (n = 10) (%)</th>
<th>Recovery (n = 10) (%) x ± s</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLU</td>
<td>6.4, 8.4</td>
<td>2.6, 2.9</td>
<td>98.2±5.4, 97.1±4.7</td>
</tr>
<tr>
<td>200</td>
<td>4.8, 7.8</td>
<td>3.0, 5.6</td>
<td>102.0±3.0, 103.9±3.8</td>
</tr>
<tr>
<td>N-FLU</td>
<td>7.8, 9.1</td>
<td>4.0, 4.2</td>
<td>94.8±2.4, 100.4±4.2</td>
</tr>
</tbody>
</table>

**Tab 2. Area ratio (area for FLU or N-FLU/area for IS) of different concentration of FLU and N-FLU in blank incubation buffer of human liver microsomes.**

<table>
<thead>
<tr>
<th>Concentration (µg/L)</th>
<th>Area ratio ( (A_{FLU}/A_{IS}) )</th>
<th>Area ratio ( (A_{N-FLU}/A_{IS}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.090</td>
<td>0.0022</td>
</tr>
<tr>
<td>25</td>
<td>0.134</td>
<td>0.0035</td>
</tr>
<tr>
<td>50</td>
<td>0.198</td>
<td>0.0061</td>
</tr>
<tr>
<td>100</td>
<td>0.354</td>
<td>0.0106</td>
</tr>
<tr>
<td>200</td>
<td>0.685</td>
<td>0.0195</td>
</tr>
<tr>
<td>400</td>
<td>1.405</td>
<td>0.0385</td>
</tr>
<tr>
<td>800</td>
<td>2.775</td>
<td>0.0691</td>
</tr>
</tbody>
</table>

**Fig 2. Calibration curves for norfluoxetine (A) and fluoxetine (B).** Norfluoxetine and fluoxetine ranged from 10 – 800 µg/L, the concentration of IS was 400 µg/L.

**Application** This method was found to be reproducible, as indicated by low coefficients of variation (CV) (Tab 1). Representative chromatograms from incubation buffer using FLU 5 µmol/L as a substrate are shown in Fig 1C. Six cytochromes P-450 selective inhibitors, including furafylline, sulfaphenazole, quinidine, diethylidithiocarbamate, triacetyloleandomycin, and ketoconazole were proved to give no chromatographic peak interfering with those of FLU, N-FLU, and NOR.

**DISCUSSION**

This HPLC-UV method was used to study the metabolism of FLU in human liver microsomes in vitro. Unlike plasma and/or urine, the ingredients of incubation buffer for in vitro studies of drug metabolism are uncomplicated and definite. Thus, a single-step extraction was made in this method and good precision was achieved regarding recovery. It is noted that similar extraction procedures reported6,7 could make good recovery from plasma, urine, frontal cortex and caudate nucleus, and was further proved by clinically monitoring antidepressants with this method.

Nortriptyline was chosen as an internal standard because (1) it is less frequently requested for TDM than any other of the tricyclic antidepressants (TCA), (2) it is well separated from FLU and N-FLU, (3) the percentage recovery of it from samples was similar to that of FLU and N-FLU under the conditions used, and (4) no interfering peaks were detectable in blank incubation buffer of
human liver microsomes.

Usually, a great range of substrate concentration is used in in vitro studies, and due to the limited formation of metabolite in vitro, the ratio of metabolite to a parent drug in incubation buffer is relatively lower than that in plasma and urine. Therefore, an HPLC method for studying in vitro metabolism of drugs must have a wide linear range and must be more sensitive than for determining in vivo drug concentration. The results of validation study indicate that our method meets this demand.

This HPLC-UV method described here enables the determination of FLU and its metabolite N-FLU in human liver microsomes in vitro, with the same validation performance as that of previously published in vivo study methods and improved extraction recoveries. This method is simple, sensitive and yields a good recovery and thereafter has been successfully utilized in our in vitro studies on identification of CYP isozymes responsible for FLU N-demethylation and in our in vivo studies on quantification of FLU and N-FLU in human serum (unpublished observations).

REFERENCES