# Determination of fluoxetine and its metabolite norfluoxetine in human liver microsomes by reversed-phase HPLC *in vitro*[1]

LIU Zhao-Qian, CHENG Ze-Neng, WANG Wei, TAN Zhi-Rong, OU-YANG Dong-Sheng, ZHOU Hong-Hao[2]
( *Pharmacogenetics Research Institute, Hunan Medical University, Changsha 410078, China* )

**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 nortriptyline (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 $\mu$g/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 serotoninergic 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 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). Nortriptyline, $\beta$-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 $\mu$m 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 ℃.

**Sample preparation**   Six human liver specimens with normal histology from patients undergoing partial hepatectomy were used to prepare microsomes by differential centrifugation[11]. Microsomal protein concentration was determined colorimetrically[12]. 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 ℃, reaction was stopped by cooling on ice and addition of 100 μL acetonitrile. A previous study[13] 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 ℃. 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.
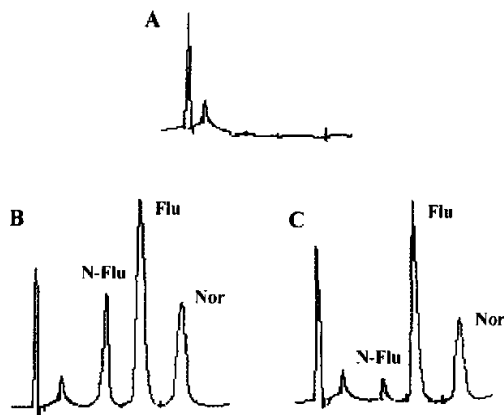


Fig 1. Chromatogram of (A) extracted blank incubation buffer of human liver microsomes and (B) spiked with N-FLU, FLU, and NOR, and (C) extracted incubation buffer of human liver microsomes with FLU 5 μmol/L.

**Validation study**   The RSD for intra- and interday 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, *ie*, 10 – 800 $\mu$g/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 $\mu$g/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).**

| Concentration ($\mu$g/L) | | Intra-assay ($n = 5$) (%) | Inter-assay ($n = 10$) (%) | Recovery ($n = 10$) (%) $\bar{x} \pm s$ |
|---|---|---|---|---|
| FLU | 10 | 6.4 | 8.4 | 98.2 ± 5.6 |
| | 200 | 4.8 | 7.8 | 97.1 ± 4.7 |
| | 800 | 2.6 | 2.9 | 102.0 ± 3.0 |
| N-FLU | 10 | 7.8 | 9.1 | 94.8 ± 2.4 |
| | 200 | 3.0 | 5.6 | 103.9 ± 3.8 |
| | 800 | 4.0 | 4.2 | 100.4 ± 4.2 |

**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.**

| Concentration ($\mu$g/L) | Area ratio ($A_{FLU}/A_{IS}$) | Area ratio ($A_{N-FLU}/A_{IS}$) |
|---|---|---|
| 10 | 0.090 | 0.0022 |
| 25 | 0.134 | 0.0035 |
| 50 | 0.198 | 0.0061 |
| 100 | 0.354 | 0.0105 |
| 200 | 0.685 | 0.0195 |
| 400 | 1.405 | 0.0365 |
| 800 | 2.775 | 0.0691 |



Fig 2. Calibration curves for norfluoxetine (A) and fluoxetine (B). Norfluoxetine and fluoxetine ranged from 10 – 800 $\mu$g/L, the concentration of IS was 400 $\mu$g/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 $\mu$mol/L as a substrate are shown in Fig 1C. Six cytochromes P-450 selective inhibitors, including furafylline, sulfaphenazole, quinidine, diethyldithiolcarbamate, triacetyloleandomycin, and ketoconazole were proved to give no chromatographic peak interfering with those of FLU, N-FLU, and NOR.

## DISCUSSION

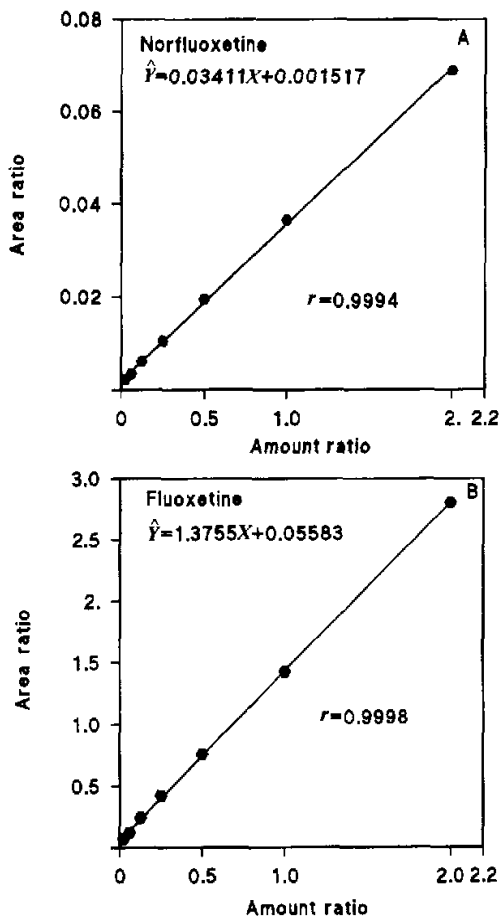This HPLC-UV method was used to study the metabolism of FLU in human liver microsomes *in vitro*. Unlike plasma and/or urine, the ingredients of incubation buffer for *in vitro* studies of drug metabolism are uncomplicated and definite. Thus, a single-step extraction was made in this method and good precision was achieved regarding recovery. It is noted that similar extraction procedures reported[6,7] 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)[7], (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[14,15], 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 isoforms responsible for FLU N-demethylation and in our *in vivo* studies on quantification of FLU and N-FLU in human serum (unpublished observations).

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## 反相高效液相色谱法测定体外人肝微粒体中氟西汀及其代谢产物去甲氟西汀[1]

刘昭前, 程泽能, 王　伟, 谭志荣, 欧阳冬生, 周宏灏[2]　(湖南医科大学遗传药理研究所, 长沙 410078, 中国)

**关键词**　氟西汀; 高压液相色谱法; 肝微粒体

**目的**: 建立同时测定体外肝微粒体中氟西汀及其代谢产物去甲氟西汀的反相高效液相色谱紫外检测法. **方法**: 含微粒体蛋白和 NADPH 发生系统及氟西汀的孵育液加入冰乙腈酸终止反应后, 再加入去甲替林作为内标并以 n-正己烷和乙腈的混合液进行萃取, 然后以反相 ODS 柱分离, 在 226 nm 处以紫外检测器进行检测. **结果**: 孵育体系中没有明显的干扰峰出现, 氟西汀和去甲氟西汀洗脱快, 分离好, 线性范围均为 $10–800$ μg/L, 最低检测限均为 5 μg/L, 相对回收率为 94 % – 104 %, 变异系数少于 9.1 %. **结论**: 本法快速, 灵敏, 回收率高, 且萃取过程简单, 可用于体外氟西汀的代谢研究.

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