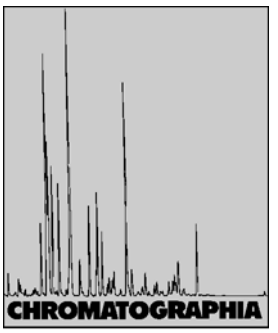
Determination of Fluoxetine and its Major Active Metabolite Norfluoxetine in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry



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R. Green* / R. Houghton / J. Scarth / C. Gregory

HFL, Newmarket Road, Fordham, Cambridgeshire, CB7 5WW, UK

Key Words

Column liquid chromatography-tandem mass spectrometry
Human plasma
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Summary

A sensitive and specific bioanalytical method for the determination of fluoxetine and norfluoxetine in human plasma has been developed. Automated solid phase extraction on Oasis HLB cartridges was used to extract the analytes from human plasma. Analysis was by reverse phase liquid chromatography on a Xterra MS C18 column using a fast gradient. Fluoxetine, norfluoxetine and fluvoxamine (internal standard) were ionised using the Turbolonspray interface operating in positive ion mode. Detection was via multiple reaction monitoring (MRM) of the characteristic ion dissociation transitions *m/z* 310.3 → 44.1, 296.2 → 134.3 and 319.2 → 71.1 for fluoxetine, norfluoxetine and fluvoxamine respectively. The method is linear over the range 0.5 – 50 ng mL^{-1} (using a sample volume of 0.5 mL). The method is accurate and precise with intra-batch and inter-batch precision (%CV) of < 15% and accuracy (%RE) of < ± 15% for both analytes. A run time of 4 minutes means a high throughput of samples can be achieved. The method has been be used to support a clinical study.

Introduction

Fluoxetine is a potent and selective inhibitor of serotonin reuptake. It is extensively metabolised in the liver to its primary active metabolite, norfluoxetine. Fluoxetine is used in the treatment of depression, bulimia nervosa and obsessive-compulsive disorder.

A method was required to support a clinical study to evaluate the pharmacokinetic characteristics and bioavailability of fluoxetine, comparing two formulations. In order to gain sufficient data to show bioequivalence, it was necessary for the method to measure levels of both fluoxetine and norfluoxetine down to 1 ng mL^{-1} or below (from 0.5 mL human plasma). To meet the timelines of the clinical study, rapid sample turnaround was required.

Several high performance liquid chromatography (HPLC) and gas chromatography (GC) methods have been described for the determination of fluoxetine and norfluoxetine in biological matrices. Many of these have been reviewed by Eap and Baumann [1]. Of those reviewed, none was capable of achieving the sensitivity required here. In addition, the majority of the methods used liquid-liquid extraction which was considered too time-consuming.

More recently described by Addison et al. [2] was a GC-MS method with a lower limit of quantitation (LLOQ) of 1 ng mL^{-1}. This method employed solid phase extraction followed by derivatisation with trifluoroacetic anhydride. This method of sample preparation was again considered too lengthy. A GC run time of ~15 minutes also prolonged the sample turnaround time.

It was our aim therefore to develop and validate a liquid chromatography tandem mass spectrometry (LC-MS-MS) method that had the required sensitivity, with an automated extraction procedure and a short chromatographic run time.
The structures of fluoxetine, norfluoxetine and fluvoxamine, the internal standard used for the analysis of both compounds, are presented in Figure 1.

Experimental

Chemicals and Reagents

Fluoxetine and norfluoxetine were supplied by Sigma (Poole, UK). Fluvoxamine (internal standard) was supplied by Promochem Ltd. (Welwyn Garden City, UK).

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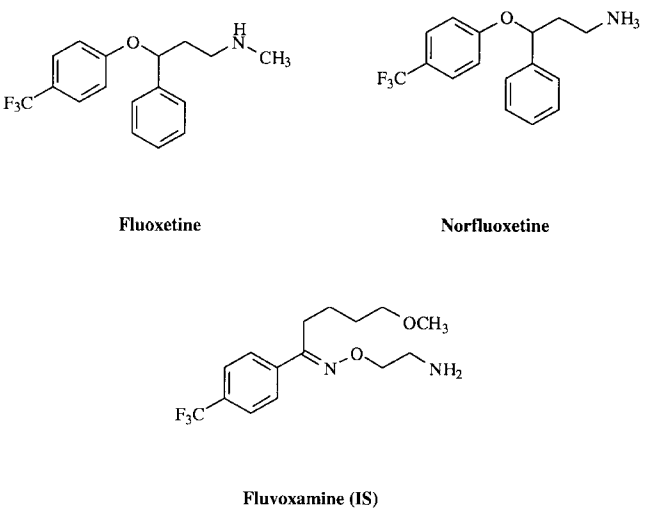

Figure 1. Structures of fluoxetine, norfluoxetine and the internal standard.

Methanol and acetonitrile (HPLC grade) were from Rathburn Chemicals Ltd. (Walkerburn, UK). Formic acid and acetic acid (AnalaR grade) were obtained from BDH (Lutterworth, UK). Triethylamine was from Fisher Scientific Ltd. (Loughborough, UK). Drug-free human plasma (with heparin as anticoagulant) was supplied by Charterhouse Clinical Research (London, UK).

Apparatus

Solid phase extraction was performed on an ASPEC XL4 (Gilson, Villiers-le-Bel, France). HPLC was carried out on an Agilent 1100 series pump (Agilent Technologies, Waldbronn, Germany) with a HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland). An API 3000 triple quadrupole mass spectrometer (PE Sciex, Toronto, Canada) was used with the TurboIonspray interface. All data collection, processing and storage was performed using Analyst software (V1.0, PE Sciex).

Preparation of Standard Solutions, Calibration Standards and Quality Control Samples

Stock solutions of fluoxetine and norfluoxetine were prepared by dissolving approximately 5 mg of the hydrochloride salt in the appropriate volume of methanol to give a free base concentration of 1 mg mL^{-1}. The stock solutions were then diluted with methanol: water (50:50 *v/v*) to give mixed solutions at concentrations 500, 200, 100, 50, 20, 10 and 5 ng mL^{-1}. These solutions were used to prepare calibration standards. 50 µL of the appropriate standard solution was added to ~0.5 mL of drug-free human plasma.

QC standard solutions at 400, 50, 10 and 5 ng mL^{-1} were prepared from separate stock solutions. QC samples were prepared in bulk by addition of 100 µL of standard solution to 9.9 mL of drug-free human plasma. Samples were then divided into 0.5 mL aliquots.

All solutions were stable for at least one month when stored at *ca* 4 °C.

Solid Phase Extraction

Prior to extraction, fluvoxamine solution (50 ng mL^{-1}, 50 µL) was added to each plasma sample, followed by 1% triethylamine aqueous solution (1 mL) with vortex mixing. The samples were placed on the ASPEC XL4 to extract. Oasis HLB 30 mg/1 cc cartridges were first conditioned with water (1 mL) followed by methanol (1 mL). The plasma samples were then loaded onto the cartridges. The cartridges were washed with 1% triethylamine in methanol:water, 1:9 (1 mL). The compounds were then eluted from the sorbent with 2% acetic acid in methanol:-water, 9:1 (2 × 1 mL). The eluent was evaporated to dryness under a stream of nitrogen at 40 °C. The eluates were reconstituted in 0.1% formic acid in methanol : water, 1:1 (100 µL) and transferred to autosampler vials.

Table I. Mobile phase gradient.

Time (min)	% A	% B
0	40	60
2.0	80	20
2.1	40	60
4.0	40	60

A = 0.05% formic acid in methanol; B = 0.05% formic acid.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS)

HPLC was carried out on an Xterra MS C_{18} 50 × 2.1 mm 3.5 µm column maintained at 50 °C. The analytes were eluted by a fast gradient method, with a mobile phase comprised of 0.05% formic acid in methanol: 0.05% formic acid pumped at a flow rate of 0.6 mL/min. The mobile phase gradient is shown in Table I. The column effluent was split at a ratio of 3:1 before entering the MS.

Mass spectrometric detection was performed on a Sciex API3000 triple quadrupole instrument. Ionisation was via the TurboIonSpray interface operating in positive ion mode with a voltage of 2000 V and a source temperature of 375 °C. The analytes were detected by multiple reaction monitoring (MRM) of the transitions *m/z* 310.3 → 44.1 for fluoxetine, *m/z* 296.2 → 134.3 for norfluoxetine, and *m/z* 319.2 → 71.1 for the internal standard. Collision energies were 43, 11 and 33 eV for fluoxetine, norfluoxetine and the internal standard respectively. Nitrogen was used as the collision gas.

Results and Discussion

Mass Spectrometry

Using the TurboIonSpray interface in positive ion mode, fluoxetine and norfluoxetine formed quasi-molecular ions at *m/z* 310.3 and 296.2 respectively. The parent ions were fragmented using collision activated dissociation (CAD) producing strong product ions at 44.4 and 134.3. Monitoring the transitions using MRM gave excellent selectivity and sensitivity.

Chromatography

Due to the selective nature of the mass spectrometric detection, fluoxetine and norfluoxetine were able to be chromatographed in under 3 minutes, using a fast

gradient. The run time was kept to 4 minutes. The mobile phase conditions gave sharp, symmetrical peaks for both analytes and the internal standard.

Precision and Accuracy

The method was validated for both fluoxetine and norfluoxetine in human plasma over the concentration range 0.5–50 ng mL^{-1}. The accuracy and precision of the method was assessed on three separate occasions by analysing six replicate samples at each of four concentrations of each analyte. These were 0.5, 1, 5 and 40 ng mL^{-1}. Accuracy was calculated as the percentage relative error between the theoretical and measured concentrations. Precision was calculated as the coefficient of variation. The results for intra-batch and inter-batch precision and accuracy are presented in Tables II and III. They show the precision and accuracy are well within the acceptance criteria of 15%.

Table II. Precision data.

	Concentration (ng mL^{-1})	Batch 1	Batch 2	Batch 3	Inter-batch
Fluoxetine	0.5	4.0	2.1	4.1	6.6
	1	3.6	2.6	4.1	5.5
	5	2.4	2.9	1.2	5.9
	40	1.4	2.1	1.7	4.7
Norfluoxetine	0.5	2.8	3.2	2.3	4.9
	1	4.8	2.1	3.6	4.0
	5	1.6	1.6	1.8	4.5
	40	2.1	2.8	1.7	5.4

Table III. Accuracy data.

	Concentration (ng mL^{-1})	Batch 1	Batch 2	Batch 3	Inter-batch
Fluoxetine	0.5	−2.6	5.3	− 8.0	−1.8
	1	5.0	2.0	− 5.3	0.6
	5	5.6	− 2.3	− 7.0	−1.2
	40	−0.5	− 4.2	−10.4	−5.0
Norfluoxetine	0.5	2.0	10.2	11.9	8.0
	1	2.8	4.2	7.8	4.9
	5	2.3	− 5.8	3.8	0.4
	40	0.3	− 9.7	0.2	−3.1

Linearity

Calibration lines were constructed by plotting peak area ratios against concentration using a weighted (1/y) least squares regression. The method showed good linearity over the range 0.5–50 ng mL^{-1} for both analytes. The mean slopes over three batches were 0.173 for fluoxetine and 0.229 for norfluoxetine with CVs of 8.9% and 11.3% respectively. Intercepts were negligible.

Selectivity

Several different batches of blank plasma were evaluated for interference from endogenous compounds present in the extracts. No interference was observed in any of the MRM chromatograms. This included the pre-dose samples from the clinical study. A chromatogram from blank plasma containing internal standard is shown in Figure 2.

Lower Limit of Quantitation (LLOQ)

The LLOQ was defined as the lowest calibration point with precision and accuracy values within 20%. The data in Tables II and III show that at 0.5 ng mL^{-1} the intra-batch values were $\leq \pm 11.9\%$ for accuracy and $\leq 4.1\%$ for precision for both analytes. The inter-batch precision values at 0.5 ng mL^{-1} were 6.6% for fluoxetine and 4.9% for norfluoxetine. Inter-batch accuracy values were −1.8% for fluoxetine and 8.0% for norfluoxetine. A chromatogram of a 0.5 ng mL^{-1} drug standard is included in Figure 3.

Stability

Plasma samples stored frozen at *ca* −20 °C were found to be stable for at least 1 month, and after three freeze/thaw cycles. Sample extracts were stable for at least 48 hours at *ca* 4 °C and at least 24 hours at room temperature.

Extraction Recovery

Recovery was evaluated using six replicates at three concentrations over the calibration range. Responses from extracted samples were compared with those from replicate (n = 6) blank samples spiked at equivalent concentrations after extraction. The mean recoveries were 87.1% and 82.6% for fluoxetine and norfluoxetine, respectively. The internal standard recovery at the spiked concentration was 87.6%.

Application to Clinical Samples

The method was used to determine concentrations of fluoxetine and norfluoxetine in human plasma samples from a clinical study to evaluate the pharmacokinetic characteristics and bioavailability of fluoxetine, comparing two 40 mg formulations. The two formulations were administered using a randomised single dose, two way cross over design to 26 healthy male volunteers. Blood samples were collected in each period pre-dose and at 1.5, 3, 4, 5, 5.5, 6, 7, 8, 9, 10, 12, 24, 48, 72, 120, 264, 552 and 840 hours after dosing. Figure 4 shows the mean plasma concentration of fluoxetine and norfluoxetine in samples from period 1. The mean time to peak plasma concentration was 5.5 h for fluoxetine and 72 h for norfluoxetine. The mean plasma terminal half-life for fluoxetine was 4.1 days while for norfluoxetine it was 3.7 days. The lower limit of quantitation of the method (0.5 ng mL^{-1}) was sufficient to allow calculation of the necessary pharmacokinetic parameters to show bioequivalence.

Conclusion

A method for the determination of fluoxetine and norfluoxetine in human plasma has been developed and validated. The method is specific and sensitive, with a lower limit of quantitation of 0.5 ng mL^{-1}

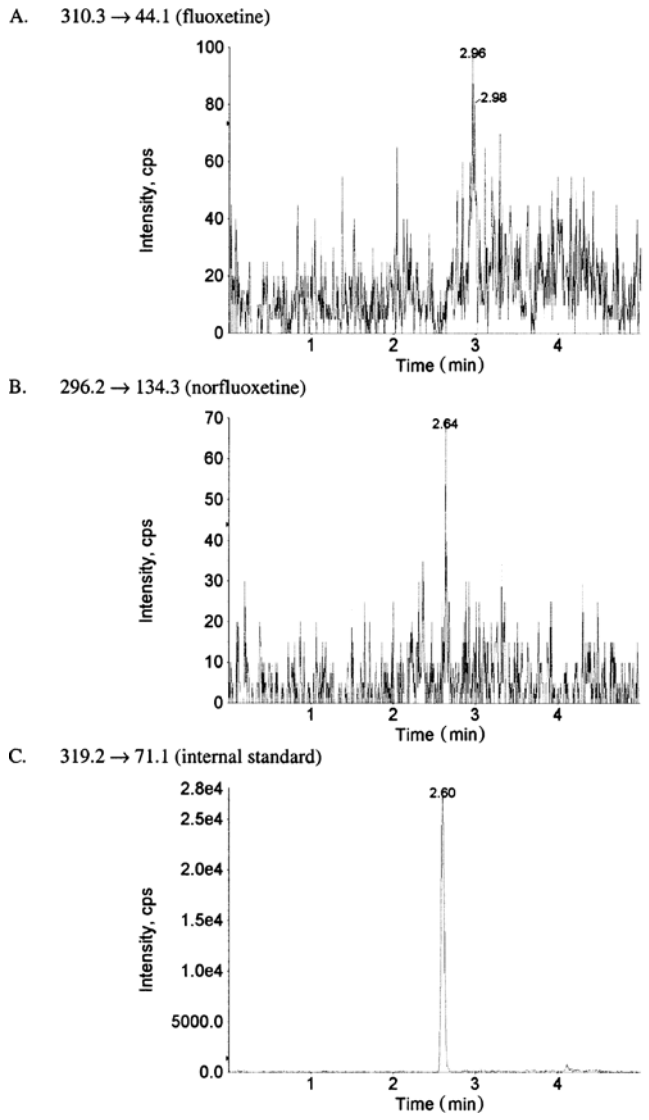

Figure 2. MRM chromatograms of a blank plasma sample (containing no analytes) spiked with internal standard only at 5 ng mL^{-1}. In **A.** the y-axis is displayed up to 100 cps, and in **B.** up to 70 cps; this 'zooming in' manifests that there would be negligible interference in the MRM transitions of the two analytes.

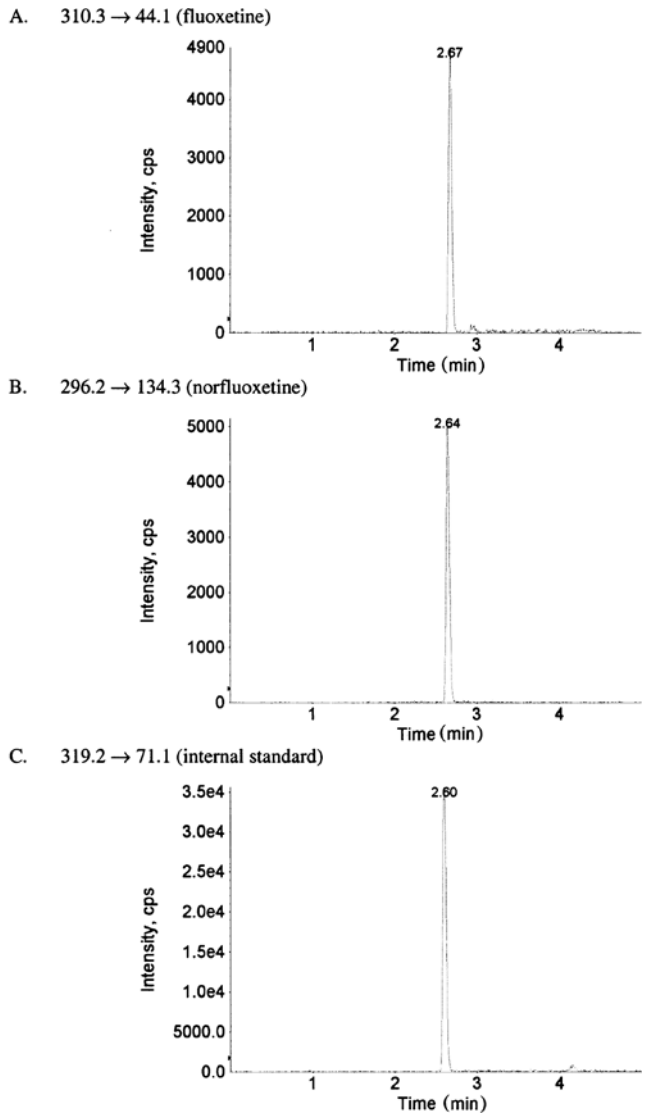

Figure 3. MRM chromatogram from a single injection of a plasma calibration standard spiked with the two drug analytes as indicated, at 0.5 ng mL^{-1} (LLOQ), and with i.s. at 5 ng mL^{-1}.

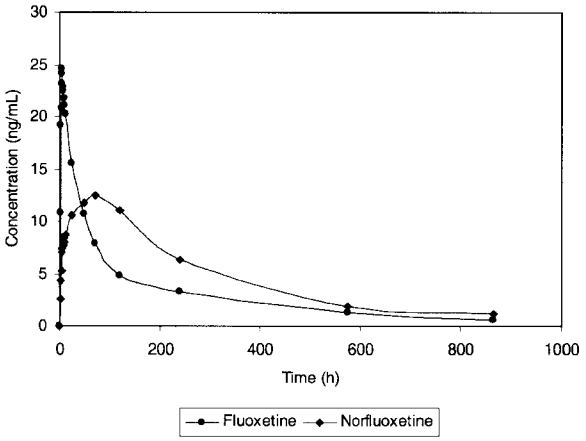

Figure 4. Mean plasma concentrations (26 volunteers).

for each analyte, using 0.5 mL of plasma. The method has a linear dynamic range of 0.5–50 ng mL^{-1}, which is appropriate for supporting clinical pharmacokinetic studies following single oral doses of 40–60 mg of fluoxetine. The precision and accuracy over this range were within acceptable limits. The use of automated solid phase extraction minimises the manual sample preparation time, and a LC-MS-MS run-time of 4 minutes means rapid turnaround of samples is achievable. The method has been successfully used for the analysis of samples from a clinical study.

References

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