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Sensitive liquid chromatographic–tandem mass spectrometric method for the determination of fluoxetine and its primary active metabolite norfluoxetine in human plasma

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Abstract

A sensitive method for the simultaneous determination of fluoxetine and its major active metabolite norfluoxetine in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The samples were extracted from alkalised plasma with hexane–isoamyl alcohol (98:2, v/v) followed by back-extraction into formic acid (2%). Chromatography was performed on a Phenomenex® Luna C_{18} (2) 5 μm, 150×2 mm column with a mobile phase consisting of acetonitrile–0.02% formic acid (340:660, v/v) at a flow-rate of 0.35 ml/min. Detection was achieved by a Perkin-Elmer Sciex API 2000 mass spectrometer (LC–MS–MS) set at unit resolution in the multiple reaction monitoring mode. TurboIonSpray ionisation was used for ion production. The mean recoveries for fluoxetine and norfluoxetine were 98 and 97%, respectively, with a lower limit of quantification set at 0.15 ng/ml for the analyte and its metabolite. This assay method makes use of the increased sensitivity and selectivity of mass spectrometric (MS–MS) detection to allow for a more rapid (extraction and chromatography) and sensitive method for the simultaneous determination of fluoxetine and norfluoxetine in human plasma than has previously been described.

Keywords: Kinetic studies; Validation; Pharmaceutical analysis; Fluoxetine; Norfluoxetine; Doxepin

1. Introduction

Fluoxetine, *N*-methyl-γ-[4-(trifluoromethyl)-phenoxy]benzenepropanamine is an antidepressant which enhances serotoninergic neurotransmission through potent and selective inhibition of neuronal reuptake of serotonin [1]. Fluoxetine is extensively metabolised, by demethylation, in the liver to its primary active metabolite, norfluoxetine. The elimination half-life of fluoxetine is 2–3 days, and that of norfluoxetine 7–16 days [2]. An oral dose of fluoxetine (40 mg) leads to maximum plasma concentrations of around 39 ng/ml for fluoxetine [3]. In this study we determined the pharmacokinetics of fluoxetine and norfluoxetine after a single oral dose of 40 mg fluoxetine hydrochloride. The low dosage and long elimination half-life of the drug and metabolite stresses the need to develop a sensitive assay method for the determination of fluoxetine and norfluoxetine in plasma, to be used in pharmacokinetic studies.

Several methods have been described for the determination of fluoxetine and norfluoxetine in

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plasma. The most widely used methods involve high-performance liquid chromatography (HPLC) with ultraviolet detection achieving lower limits of quantification (LLOQs) of around 3–5 ng/ml [4,5]. Other methods involve HPLC with diode-array detection [6], gas chromatography (GC) with nitrogen–phosphorus detection (NPD) [7], GC with electron-capture detection (ECD) [8] and GC with mass spectrometric detection (MS) [9]. Fontaville et al. [7] achieved the best sensitivity with LLOQs of 0.3 and 2 ng/ml for fluoxetine and norfluoxetine, respectively. For a comprehensive overview of previous methods refer to Eap and Baumann [10]. In our laboratory we have already developed and validated a HPLC method with UV detection for the determination of fluoxetine (LLOQ of 3.77 ng/ml) and a GC method using ECD with LLOQs of 0.374 and 0.388 ng/ml for fluoxetine and norfluoxetine, respectively. These methods however, are still very time consuming. The GC methods usually involve a derivatization step (we used pentafluorobenzoylchloride), both methods need extensive sample clean-up and concentration to achieve the desired selectivity and sensitivity and both methods have rather long run times (~10 min). All these factors cause a decreased productivity and it was therefore decided to develop a new method involving the use of a mass-selective detector with tandem mass spectrometry (MS–MS) capabilities coupled to liquid chromatography (LC) to increase productivity and sensitivity.

This report describes a LC–MS–MS method for the simultaneous determination of fluoxetine and norfluoxetine in plasma with a simple liquid–liquid extraction procedure and a total chromatography time of 2.6 min, thereby enabling the operator to do more samples per day. The method is also more sensitive than previously described methods with a LLOQ of 0.15 ng/ml for both the analyte and metabolite.

2. Experimental

2.1. Materials and chemicals

A Phenomenex Luna C_{18} (2) 5 μm, 150×2.1 mm column (Phenomenex, Torrance, CA, USA) was used for separation at a flow-rate of 0.35 ml/min and injecting 20 μl onto the column. The mobile phase was delivered by a Hewlett-Packard Series 1050 pump (Hewlett-Packard, Palo Alto, CA, USA) and the samples injected by a Perkin-Elmer Series 200 autoampler. Detection was performed by a Perkin-Elmer Sciex API-2000 detector (Perkin-Elmer Sciex, Ontario, Canada) using TurboIonSpray ionisation (ESI) for ion production.

Isoamyl alcohol (analytical-reagent grade) was obtained from Merck (Darmstadt, Germany); formic acid (high purity grade) from BDH (Poole, UK); hexane and acetonitrile (Burdick and Jackson, high purity grade) were obtained from Baxter (USA), sodium hydroxide and sodium carbonate (analytical-reagent grade) were obtained from Fluka (Buchs, Switzerland). All chemicals were used as received. Water was purified by RO 20SA reverse osmosis and a Milli-Q polishing system (Millipore, Bedford, MA, USA). Sodium carbonate buffer (0.1 *M*) was prepared and adjusted to pH 12 with sodium hydroxide (5 *M*).

Fluoxetine–HCl, $C_{17}H_{18}F_3NO$–HCl, and norfluoxetine–HCl, $C_{16}H_{16}F_3NO$–HCl, were supplied by Mediat Spa (Italy). Doxepin–HCl internal standard was obtained from the FARMOVS Research Centre internal pure substance reference material library.

2.2. Extraction procedure

Fluoxetine and norfluoxetine standard solutions were made up in methanol and used immediately to spike plasma and discarded thereafter. Calibration standards and quality control standards were prepared in normal human plasma by spiking a pool of normal plasma which was then serially diluted with normal blank plasma to attain the desired concentrations (0.15–79.1 ng/ml for fluoxetine and 0.15–78.8 ng/ml for norfluoxetine). The calibration standards and quality control standards were aliquoted into tubes and stored under the same conditions as the trial samples; at −20°C. Fluoxetine and norfluoxetine have been shown to be stable at −20°C for at least 1 year [8].

To 1.0 ml plasma containing both fluoxetine and norfluoxetine in a 10-ml amber glass ampoule was added 50 μl doxepin internal standard solution (546 ng/ml in water), 200 μl 0.1 *M* sodium carbonate

buffer (pH 12) and 4 ml of a 2% solution of isoamyl alcohol in hexane. The sample was vortex mixed for 1.5 min and centrifuged at 1300 *g* for 1 min at 8°C.

The aqueous phase was frozen at −30°C on a Fryka Polar cooling plate (Kältetechnik, Esslingen, Germany) and the organic phase decanted into a clean 5-ml amber glass ampoule containing 200 μl of a 2% formic acid solution. After vortex mixing for 1.5 min and centrifuging at 1300 *g* for 1 min at 8°C, the aqueous phase was again frozen at −30°C and the organic phase discarded. The aqueous phase was thawed at room temperature, transferred to an autosampler vial insert and 20 μl injected onto the HPLC column.

2.3. Liquid chromatography

All chromatographic solvents were degassed with helium before use. Chromatography was carried out at ambient temperature with a mobile phase consisting of acetonitrile–0.02% formic acid (340:660, v/v) at a flow-rate of 0.35 ml/min.

2.4. Mass spectrometry

Electrospray ionisation was performed in the positive ion mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 80, 50 and 52 (respective arbitrary values). The TurboIonSpray temperature was set at 400°C. The instrument response was optimised for fluoxetine, norfluoxetine and doxepin by infusing a constant flow of a solution of the drugs dissolved in mobile phase into the stream of mobile phase eluting from the column. The pause time was set at 5 ms and the dwell time at 200 ms.

The Perkin-Elmer Sciex API 2000 LC–MS–MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions *m/z* 309.9, 295.9 and 280.2 to the product ions *m/z* 43.6, 133.8 and 107.1 (for fluoxetine, norfluoxetine and doxepin, respectively). Fig. 1 shows the single parent (*m/z* 309.9) to product ions MS–MS of fluoxetine and Fig. 2 shows the single parent (*m/z* 295.9) to product ions MS–MS of norfluoxetine. The molecular structures and proposed fragmentation patterns for the analyte and metabolite are also indicated in these figures. TurboIonSpray ionisation (ESI) was used for ion production and the collision gas (N_2) set at 3 (arbitrary value). The instrument was interfaced to an Apple Macintosh computer running Perkin-Elmer MASSCHROM version 1.1 with MACQUAN version 1.6 softwares.

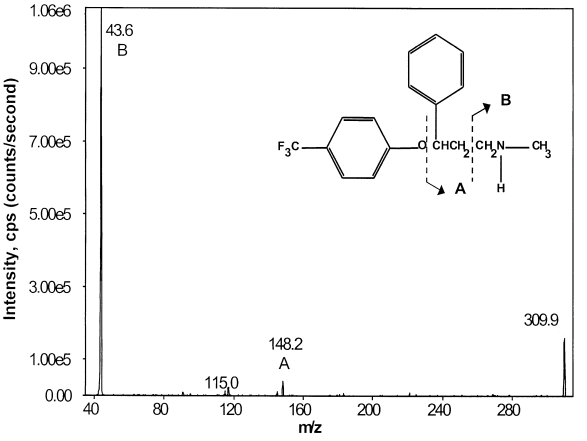

Fig. 1. Full mass spectrum of the protonated fluoxetine molecular ion (*m/z* 309.9, molecular structure and proposed fragmentation given) and the principal product ions formed at *m/z* 43.6 and 148.2 after collision (MS–MS).

2.5. Validation

The method was validated by analysing plasma

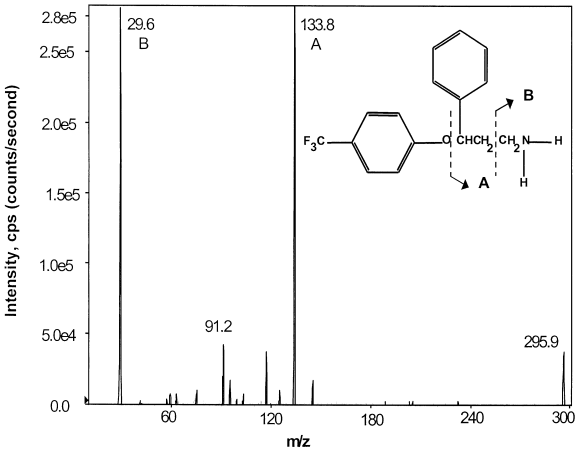

Fig. 2. Full mass spectrum of the protonated norfluoxetine molecular ion (*m/z* 295.9, molecular structure and proposed fragmentation given) and the principal product ions formed at *m/z* 133.8 and 29.6 after collision (MS–MS).

quality control samples five times at seven different concentrations i.e. 72.2, 36.1, 18.1, 1.48, 0.74, 0.37 and 0.19 ng/ml for fluoxetine and 72.2, 36.3, 18.2, 1.48, 0.75, 0.37 and 0.19 ng/ml for norfluoxetine to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing ten different concentrations spanning the concentration range (79.1–0.15 ng/ml for fluoxetine and 78.8–0.15 ng/ml for norfluoxetine). Calibration graphs were constructed using a weighted linear regression (1/concentration) of the drug/internal standard peak-area ratios of the product ions for fluoxetine, norfluoxetine and the internal standard, versus nominal drug concentrations. Several regression types were tested and the weighted linear regression (1/concentration) was found to be simplest regression, giving the best results.

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionisation) was investigated by extracting 'blank' biological fluids from six different sources, reconstituting the final extract in injecting solvent containing a known amount of the analyte, analysing the reconstituted extracts and then comparing the peak areas of the analytes.

Absolute recoveries of the analyte and metabolite were determined in triplicate in normal plasma by extracting drug free plasma samples spiked with fluoxetine and norfluoxetine. Recoveries were calculated by comparison of the analyte peak areas of the extracted samples with those of the unextracted system performance verification standard mixtures (prepared in the injection vehicle) representing 100% recovery. The recoveries were calculated using the system performance verification standard since no difference in ionisation between extracted samples and pure solutions was observed.

3. Results and discussion

The mean absolute recovery of fluoxetine determined in triplicate at 72.2, 18.1 and 0.74 ng/ml was 96 (RSD 2.3%), 98 (RSD 2.8%) and 100% (RSD 3.1%), respectively, while for norfluoxetine, the mean recovery at concentrations of 72.2, 18.2 and 0.75 ng/ml was 95 (RSD 2.6%), 97 (RSD 3.4%) and 99% (4.4%), respectively. No matrix effect for fluoxetine and norfluoxetine was observed for the six different plasma pools. The peak areas of the six reconstituted samples had RSDs of 5% for fluoxetine and 3.8% for norfluoxetine indicating that the extracts were 'clean' with no undetected co-eluting compounds that could influence the ionisation of the analytes.

The LLOQ is defined as that concentration of fluoxetine and norfluoxetine which can still be determined with acceptable precision (RSD$<$20%) and accuracy (bias$<$20%) and was found to be 0.15 ng/ml for the analyte and its metabolite. Results from the intra-day validation assays indicate a valid calibration range of 0.15–79.1 ng/ml for fluoxetine and 0.15–78.8 ng/ml for norfluoxetine. The intra- and inter-day assay method performance statistics are presented in Tables 1–4.

On-instrument stability was inferred from special stability samples which were prepared and included in the validation batch. No significant degradation could be detected in the cooled samples (4°C) left on the autosampler for at least 7 h.

Due to the high specificity of MS–MS detection,

Table 1
Intra-day quality control results of fluoxetine ($n=5$)

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	% Nominal
72.2	71.6	2.7	99
36.1	38.2	1.5	106
18.1	18.9	2.5	104
1.48	1.60	1.0	108
0.74	0.81	1.2	109
0.37	0.40	2.5	109
0.19	0.22	1.8	117

Table 2
Intra-day quality control results of norfluoxetine ($n=5$)

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	% Nominal
72.2	71.3	2.4	99
36.3	37.1	2.6	102
18.2	18.1	3.6	99
1.48	1.48	4.0	100
0.75	0.74	5.9	99
0.37	0.39	3.1	103
0.19	0.17	2.8	93

Table 3
Inter-day quality control results of fluoxetine

Nominal (ng/ml)	0.19	0.37	0.74	18.1	36.1	72.2
Mean	0.20	0.42	0.82	18.2	35.8	70.3
RSD	8.1	9.7	3.9	10.5	9.1	5.5
% Nominal	106	114	112	101	99	97
n	9	10	9	10	10	10

no interfering or late eluting peaks were found when chromatographing blank plasma extracts from six different sources.

Several extraction procedures were tested to allow for a single extraction procedure for both fluoxetine and norfluoxetine which include solid-phase and liquid–liquid extraction methods using different organic solvents and buffers. A liquid–liquid back-extraction procedure of the analyte and metabolite from hexane into a 2% formic acid solution proved to be successful. This procedure not only saved solvent evaporation time, but also resulted in the introduction of much cleaner extracts with high recovery rates into the ionization source. The extraction yield was further improved by the addition of 2% isoamyl alcohol to the hexane. Doxepin was found to be a suitable internal standard (radio labelled isotopes for fluoxetine or norfluoxetine were not available at the time) as it had a good recovery (75%) and eluted close to the analyte and metabolite thereby minimising any potential matrix effects. As this was a pharmacokinetic study where only healthy volunteers are used, we decided to use doxepin as internal standard even though doxepin can be co-administered in treatment with fluoxetine. It is however, important to choose another internal standard when doing therapeutic drug monitoring in patients. The method was developed using 1 ml of plasma and can easily be adjusted to use less plasma due to the good sensitivity and small injection volume (20 μl).

Different concentrations of acetic acid, formic acid and ammonium acetate were tested for optimum ionisation of the analytes and it was found that 0.02% formic acid gave the best result. The principal product ions of fluoxetine (m/z 148.2, 43.6) and norfluoxetine (m/z 133.8, 29.6) differ only with the added methyl group to the fluoxetine product ions

Table 4
Inter-day quality control results of norfluoxetine

Nominal (ng/ml)	0.19	0.37	0.75	18.2	36.3	72.2
Mean	0.18	0.38	0.72	17.5	35.2	70.6
RSD	11.2	12.5	6.0	10.6	8.5	5.3
% Nominal	92	103	96	96	97	98
n	8	10	10	10	10	10

(fluoxetine is demethylated in the liver to norfluoxetine) (Figs. 1 and 2). Unlike the m/z 133.8 product ion of norfluoxetine, the m/z 148.2 product ion of fluoxetine does not seem stable, fragmenting further to the m/z 43.6 product ion. This may be due to the added methyl group on the m/z 148.2 product ion of fluoxetine destabilising the ion relative to the primary amine m/z 133.8 product ion of norfluoxetine. It was therefore decided to use the m/z 43.6 product ion of fluoxetine to quantitate the drug (m/z 133.8 was used for norfluoxetine).

Retention times were 2.35 and 1.97 min for fluoxetine and norfluoxetine, respectively, and 1.85 min for the internal standard doxepin. A total chromatography run time of 2.60 min was allowed, which made it possible to analyse large batches of samples per day (we analysed 300 samples per/day). Fig. 3 shows representative chromatograms obtained of fluoxetine (A) and norfluoxetine (B) at concentrations of 0.15 ng/ml (the LLOQs) and of study samples close to the limit of quantification at the elimination phase of the pharmacokinetic profile for each analyte.

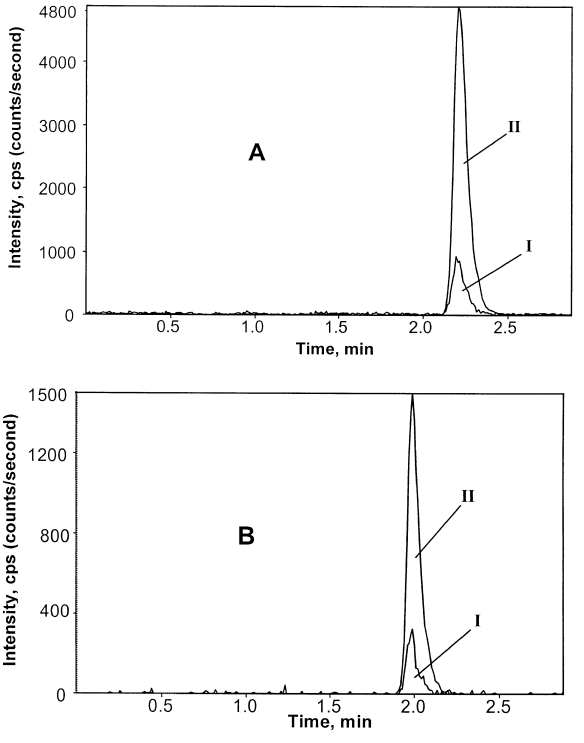

Fig. 3. (A and B) High-performance liquid chromatograms of the calibration standard at the limit of quantification (I) containing 0.15 ng/ml fluoxetine (A) and 0.15 ng/ml norfluoxetine (B) and of study samples (II) at the late elimination phase of the pharmacokinetic profile of each analyte containing 0.73 ng/ml fluoxetine (A) and 0.62 ng/ml norfluoxetine (B).

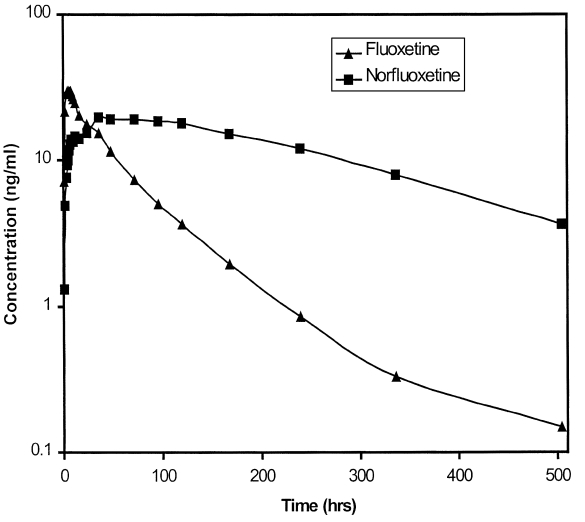

Fig. 4. Representative fluoxetine and norfluoxetine plasma concentrations vs. time profiles as obtained after a single 40-mg oral dose of fluoxetine (24 subjects, 2 phases).

The method was employed to analyse plasma samples containing fluoxetine and norfluoxetine obtained after a single oral dose of 40 mg fluoxetine hydrochloride per treatment phase in 24 healthy volunteers. Concentration vs. time profiles were constructed for up to 504 h for the analyte and metabolite (Fig. 4). The maximum fluoxetine plasma concentrations obtained varied between 26.0 and 51.8 ng/ml and between 12.8 and 33.1 ng/ml for the norfluoxetine metabolite. The elimination half-life of fluoxetine was 1.6 days and that of norfluoxetine 12 days which is consistent with values reported in the literature [2].

In this method we made use of the increased sensitivity and selectivity of MS–MS detection to decrease the sample preparation and chromatography time to enable us to do more samples per day thereby increasing productivity considerably.

4. Conclusion

A highly sensitive and selective method for the simultaneous determination of fluoxetine and its major active metabolite norfluoxetine in plasma was developed, using HPLC separation with tandem mass spectrometric detection. With a LLOQ of 0.15 ng/ml for both fluoxetine and norfluoxetine pharmacokinetic profiles of the drug and its metabolite could be constructed for up to 504 h after a single oral dose of 40 mg fluoxetine hydrochloride. The method is more sensitive than previously described methods and allows for a much higher sample throughput due to the short chromatography time (2.6 min) and relatively simple sample preparation.

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