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Short communication

A simple method to monitor serum concentrations of fluoxetine and its major metabolite for pharmacokinetic studies

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ABSTRACT

A rapid, selective and sensitive isocratic reversed-phase HPLC assay coupled with MS/MS detection for simultaneous quantification of fluoxetine and its major active metabolite in serum samples has been developed. Analytes were extracted with a simple three step liquid–liquid procedure and chromatographic separation was achieved on a C18 column.

Because of its sensitivity, this HPLC/MS/MS method is suitable both for routine therapeutic drug monitoring and for pharmacokinetic studies, due to its low limits of quantification.

1. Introduction

Patients with chronic hepatitis C achieve a sustained virological response after ribavirine and peg-interferon alpha 2b (peg-IFN-α2b) therapy (INF-α). Adverse psychiatric events occur in more than 20% of patients treated with INF-α. Severe depression and suicidal ideation are generally manifested in patients with a pre-existing history of depression, but they may also occur in individuals with no history of depression [1]. Patients should be closely monitored for depressive symptoms, and cessation of therapy with INF-α should be considered in patients experiencing depression.

Fluoxetine (FLU), due to its tolerability profile, may be administered in association with INF-α to prevent adverse psychiatric events. INF-α can, however, inhibit microsomal enzymes involved in the hepatic cytochrome P450 system (CYP1A2, CYP2D6 and CYP2C19) [2,3]. The mechanism by which INF-α inhibits hepatic CYPs is not definitively known. It has been suggested that the loss of cytochrome P450 can result from the generation of free radical species by the enzyme xantine oxidase [4]. A pharmacokinetic interaction with INF-α is predicted for any drug that is a CYP2D6 substrate; therefore, metabolism of FLU, when co-administered with INF-α, may be modified.

The aim of this study was to establish a simple and rapid high performance liquid chromatography (HPLC) method for simultaneous identification and quantification of FLU and norfluoxetine (N-FLU) to elucidate a possible effect of IFN-α on FLU pharmacokinetics.

Though several chromatographic methods have been developed for the determination of FLU and its major metabolite by means of HPLC/UV, HPLC/mass spectrometry or gas chromatography/mass spectrometry; these methods have a limit of quantification (LOQ) that is too high for a pharmacokinetic study [5–7]. In addition, some techniques require toxic reagents for derivatization such as (S)-trifluoroacetylpropyl chloride [8] or ethyl chloroformate [9].

To purify FLU and N-FLU from biological samples, we modified the extraction procedure proposed by Wong et al. [10]. To ameliorate the LOQ and limit of detection (LOD) we quantified FLU and N-FLU by means of LC/MS/MS detection.

This report describes the optimisation and validation of an HPLC assay coupled with MS/MS detection for quantification of FLU and its major and active metabolite. Sample handling and chromatographic run times were minimized to provide quantitative results while maintaining high sensitivity, specificity, accuracy and precision for the pharmacokinetic evaluation of FLU and N-FLU. In view of these applications the method, was tested on samples obtained from patients cotreated with IFN-α and FLU.

2. Experimental

2.1. Chemicals and reagents

FLU–HCl, N-FLU–HCl, and clomipramine–HCl (CLO) (internal standard) were purchased from Sigma–Aldrich (Milan, Italy). Acetonitrile, *n*-hexane, isoamyl alcohol, sodium carbonate, ammonium

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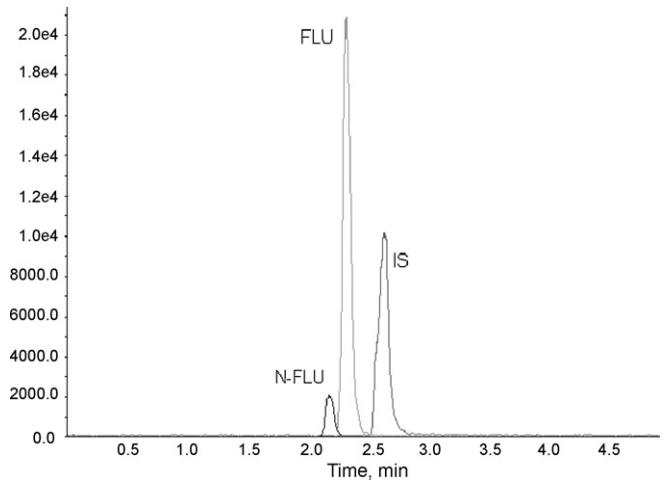

Fig. 1. Chromatogram [10.6 ng mL^{-1} fluoxetine (FLU); 2.84 ng mL^{-1} norfluoxetine (N-FLU) and 100 ng clomipramine (IS)] of a serum sample obtained 4 h after an oral administration of 40 mg of fluoxetine.

acetate (Merck KgaA, Darmstadt, Germany), methanol (Carlo Erba, Milan, Italy), and formic acid (Sigma–Aldrich, Milan, Italy) were analytical-grade reagents. MilliQ water was filtered and deionised with an Ultra Pure Water System, MilliQ-plus (Millipore, USA).

2.2. Chromatographic analysis

FLU and its main metabolite, N-FLU, were measured by means of an HPLC technique coupled with MS detection. FLU, N-FLU and CLO were separated on a Beckman C18 column (ODS-150 mm × 2.1 mm, 5 μm) at a flow-rate of 0.2 mL min^{-1}. Separation was carried out in isocratic conditions with a solution of acetonitrile/water/formic acid + 2 mM ammonium acetate (68/32/0.1, v/v/v). Under these conditions, N-FLU, FLU and CLO retention times were 2.2, 2.4 and 2.6 min, respectively (Fig. 1). Total run time was less than 5 min for each injection.

Chromatographic equipment consisted of HPLC LC-200 pump (PerkinElmer, USA), and column effluent was introduced into the mass spectrometer using a fused silica capillary. A Q-trap LC/MS/MS Systems (MDS Sciex, Ontario, Canada) was equipped with an electrospray source, operating in the positive ion mode (ESI). Data were acquired and processed with *Analyst 1.4.* (Applied Biosystems Package, MDS Sciex, Ontario, Canada). Samples were detected in multiple-reaction monitoring (MRM) (FLU 310.1/44.1; N-FLU 296.1/134.0; CLO 315.1/86.1). In order to optimise the MS parameters, a standard solution of analytes and IS was infused into the mass spectrometer using an infusion pump. The optimised parameters were: curtain gas, ion source gas 1 and 2 25, 35 and 45 units, respectively; dwell time 300 ms; source temperature 400 °C; ionspray voltage 5.5 kV.

2.3. Preparation of stock and work solutions

Stock solutions of FLU, N-FLU and CLO were prepared separately in methanol at the concentration of 1 mg mL^{-1}. Working solutions, for the preparation of calibration curves and quality control samples, were made by diluting, in methanol, stock solutions. Stock and work solutions were stored at −80 °C.

2.4. Calibrators and quality control samples

Calibrators and quality control samples containing FLU and N-FLU were prepared adding known amounts of analytes to blank serum. They were included in each batch of patient samples.

Calibration curves and quality control samples ranged from 0.2 to 50 ng mL^{-1} and 0.3 to 45 ng mL^{-1} for FLU and N-FLU, respectively.

2.5. Sample preparation

Extraction was a simple three-step procedure as follows: alkalinization, organic extraction and back-extraction. One milliliter of serum was transferred to a polypropylene tube, followed by the addition of 10 μL of internal standard (CLO 10 ng μL^{-1}) and 200 μL of sodium carbonate (100 mM, pH 12). The tubes were vortexed and samples were extracted with 4 mL of *n*-hexane/isoamyl alcohol (99/1, v/v) by rotation for 1.5 min and centrifuged at 1500 rpm for 10 min. The organic layer was transferred, for back-extraction, to another polypropylene tube containing 200 μL of formic acid (0.2%). The tubes were rotated for 1.5 min and centrifuged at 1500 rpm for 10 min. The organic phase was discharged, the lower aqueous acid layer was transferred to a small test tube and 20 μL were injected into HPLC system.

2.6. Method validation

Method validation was performed to evaluate the specificity, linearity and sensitivity, accuracy and precision, recovery and matrix effect according to the currently approved US Food and Drug Administration (FDA) bioanalytical method validation guidelines [11].

The specificity of the method was investigated by analysing six different batches of drug-free human serum for the exclusion of any endogenus co-eluting interferences at the peak region of each analyte and IS.

The calibration standard were prepared and assayed in triplicate on 5 different days to demonstrate the linearity of the method.

The extraction recovery was determined comparing the analyte-to-IS ratios in QC prepared in drug-free human serum and reconstituted in the mobile phase and QC prepared in the mobile phase directly.

Intra- and inter-day precision and accuracy were assessed by extracting and analysing five replicates of each of the eight quality control concentration levels.

The presence of matrix effect was determined by applying the procedures recommended by Annesley [12].

The LOD was defined at a signal-to-noise (S/N) ratio of 3:1; the LOQ was defined the lowest quantifiable concentration of analyte with accuracy within 20% and a precision <20%.

2.7. Pharmacokinetic validation

After approval by the Institutional Review Board for the protection of human subjects according to the Helsinki declaration, five patients suffering from chronic hepatitis C who were candidates to peg-IFN-α2b plus ribavirin treatment, were prospectively admitted to the study; all patients received a single 40 mg oral dose of FLU. Blood samples were collected immediately before fluoxetine administration, and 1, 2, 4, 6, 8, 12 and 24 h and 2–7, 14, 21 and 28 days following its administration. Blood samples were centrifuged at 3000 rpm for 10 min and serum samples were transferred to polypropylene tubes and frozen in liquid nitrogen until processing.

3. Results

3.1. Specificity

The specificity of the method was evaluated for potential endogenous interferences by analysing blank serum samples from six different batches. Potential ribavirine, peg-IFN-α2b interferences were assessed. Carry-over effects were assessed by

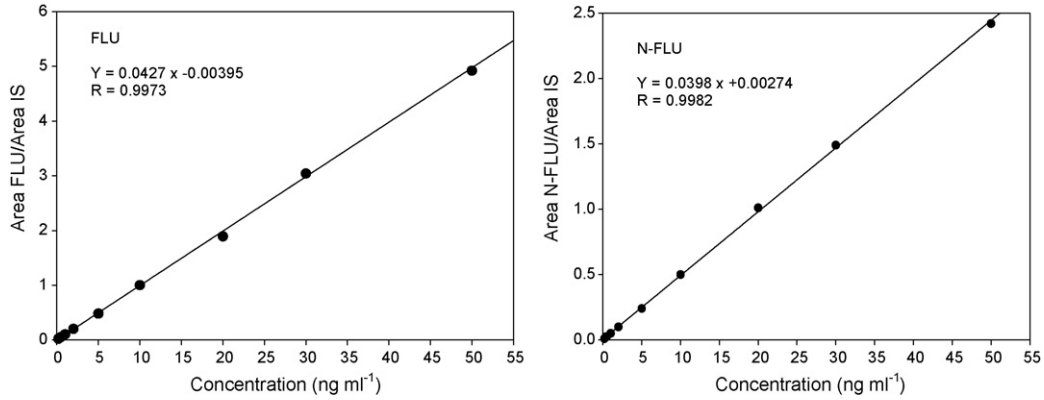

Fig. 2. Calibrations curves of fluoxetine (FLU) and norfluoxetine (N-FLU).

injecting an extract containing ribavirine (2 μg mL^{-1}), peg-IFN-α2b (2 ng mL^{-1}), followed by five blank extracts and observing any potential residual peaks at the retention time of each analyte. This process was repeated five times. No interferences were observed in any of these samples.

3.2. Linearity and recovery

The linearity of our method, checked by analysing control samples in quintuplicate, was in the range of 0.3–50 ng mL^{-1} for FLU and N-FLU (Fig. 2 and Table 1).

The extraction recoveries ranged from 95.2 to 97.1% for both analytes. These values were estimated at five different concentrations.

3.3. Intra- and inter-assay

A series of quality control samples were prepared at 8 different concentrations in the range of 0.3–50 ng mL^{-1} for both analytes. Intra-day accuracy was calculated after five replicate runs of the same extracted sample; inter-day accuracy was calculated after analysis on 5 consecutive days (Table 1).

3.4. Matrix effect

To evaluate the effect of the sample matrix on MS response, we compared the instrument response for quality control samples injected into the mobile phase with responses for the same amount of FLU and N-FLU added to unextracted and extracted samples. No significant decrease (<4%) of signal was observed.

3.5. Limit of quantification and limit of detection

The LOQ was 0.17 and 0.18 ng mL^{-1} for FLU and N-FLU, respectively, and the LOD was 0.06 ng mL^{-1} for both analytes.

3.6. Pharmacokinetics

Peak serum concentrations (C_{max}) of FLU ranged from 7.17 to 10.10 ng mL^{-1} [8.30 ± 1.11 ng mL^{-1} (mean ± S.D.)], and time to reach C_{max}, ranged from 2 to 8 h [4.8 ± 2.28 h (mean ± S.D.)]. The mean serum concentrations of FLU and N-FLU after a single oral dose (40 mg) of FLU are shown in Fig. 3.

Peak serum concentrations (C_{max}) of N-FLU, after a 40 mg single dose, ranged from 8.94 to 23.8 ng mL^{-1} [16.20 ± 5.30 ng mL^{-1} (mean ± S.D.)], and time to C_{max}, ranged from 48 to 96 h [67.20 ± 20.07 h (mean ± S.D.)]. The area under the curve (AUC) (calculated by means trapezoidal role) ranged from 0 to 672 h was, 311.52 ± 55.57 ng h mL^{-1} and 4684.35 ± 2257.24 ng h mL^{-1} for FLU and N-FLU, respectively. The elimination half-life ($t_{1/2}$) ranged from 20.35 to 59.47 h [32.40 ± 15.68 h (mean ± S.D.)] and 154.51 to 270.07 h [208.16 ± 54.81 h (mean ± S.D.)] for FLU and N-FLU, respectively. The $t_{1/2}$ values were calculated with WinNonlin Software (Pharsight Product, USA).

4. Discussion

A specific and sensitive LC/MS/MS method was developed and validated for the quantification of FLU and its major metabolite in serum samples. A number of HPLC methods have been previously described in the literature to measure FLU and N-FLU concen-

Table 1
Linearity and intra- and inter-day precision.

Drug	Conc. (ng mL^{-1})	Linearity (n = 5) (mean ± S.D.)	CV%	Intra-day (n = 5) (mean ± S.D.)	CV%	Inter-day (n = 5) (mean ± S.D.)	CV%
FLU	0.3	0.29 ± 0.02	6.90	0.29 ± 0.02	6.90	0.31 ± 0.02	6.45
	0.6	0.61 ± 0.03	4.92	0.59 ± 0.04	6.78	0.62 ± 0.04	6.45
	0.8	0.76 ± 0.03	3.95	0.81 ± 0.04	4.94	0.77 ± 0.03	3.90
	1.5	1.39 ± 0.05	3.60	1.49 ± 0.06	4.03	1.51 ± 0.08	5.30
	8.0	8.29 ± 0.14	1.69	8.18 ± 0.11	1.34	8.24 ± 0.13	1.58
	15.0	15.12 ± 0.25	1.65	15.22 ± 0.20	1.31	15.22 ± 0.18	1.18
	30.0	29.84 ± 0.54	1.81	30.22 ± 0.49	1.62	29.79 ± 0.58	1.95
	50.0	50.65 ± 0.47	0.93	49.87 ± 0.23	0.46	50.15 ± 0.56	1.12
N-FLU	0.3	0.31 ± 0.02	6.45	0.30 ± 0.02	6.67	0.32 ± 0.02	6.25
	0.6	0.58 ± 0.04	6.90	0.51 ± 0.03	5.88	0.53 ± 0.03	5.66
	0.8	0.78 ± 0.04	5.13	0.79 ± 0.04	5.06	0.76 ± 0.05	6.58
	1.5	1.52 ± 0.07	4.61	1.55 ± 0.08	5.16	1.55 ± 0.08	5.16
	8.0	8.09 ± 0.11	1.36	8.07 ± 0.13	1.61	8.11 ± 0.09	1.11
	15.0	15.24 ± 0.15	0.98	15.14 ± 0.11	0.73	15.14 ± 0.18	1.19
	30.0	29.54 ± 0.32	1.08	29.27 ± 0.36	1.23	29.75 ± 0.50	1.68
	50.0	50.05 ± 0.56	1.12	49.78 ± 0.44	0.88	49.86 ± 0.35	0.70

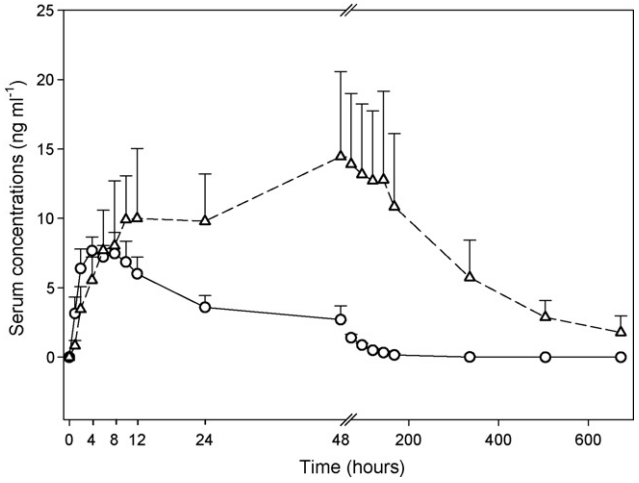

Fig. 3. Mean (±S.D.) serum total fluoxetine (FLU; circles) and norfluoxetine (N-FLU; triangles) concentrations in five patients with chronic hepatitis C.

trations at steady-state conditions. However, these methods were inadequate for pharmacokinetic purpose. The aim of this study was the implementation of an HPLC/MS method for the simultaneous quantification of FLU and its main metabolite in human serum.

The method combines a variety of convenient features in terms of simplicity, precision, accuracy, sensitivity, rapidity and very satisfactory reproducibility. Extraction is a simple three-step procedure consisting of alkalinization, organic and back-extraction with a high recovery rate. Because of the specificity of the MS/MS analysis, a rapid liquid–liquid extraction avoids the need for more extensive, time-consuming purification steps. By using this procedure, the extraction requires less than 30 min and chromatographic separation takes only 3 min.

Modifications we introduced to the method of Wong et al. [10] improved sensitivity (from 6 ng mL^{-1} for both analytes to 0.17 and 0.18 ng mL^{-1} for FLU and N-FLU, respectively) and recovery (from 55 to 60% and 79 to 86% for N-FLU and FLU, respectively, to 95.2 and 97.1% for both analytes). Finally high recovery and lower values of LOQ (FLU 0.17 ng mL^{-1} and N-FLU 0.18 ng mL^{-1}) allows the identification and quantification of low concentrations for pharmacokinetic studies. The method, thanks to its high sensitivity, was successfully applied to PK study of FLU and N-FLU and, thanks to its rapidity, this method has been demonstrated to be of great usefulness in our laboratory for therapeutic drug monitoring (TDM) too. The method is suitable also for testing concentrations at steady-state, when the higher drug levels achieved allows assessment even on smaller amounts of sample.

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