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Short communication

Comparison of liquid chromatography with fluorescence detection to liquid chromatography–mass spectrometry for the determination of fluoxetine and norfluoxetine in human plasma

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Abstract

A comparison study on fluoxetine (FL) and norfluoxetine (NORFL) quantitation in human plasma was carried out between the recently developed liquid chromatographic method with fluorescence detection (LC–FLD) and an earlier established liquid chromatography–mass spectrometry (LC–MS) laboratory procedure. Comparative method evaluation was based on the analysis of plasma samples obtained from Parkinsonian patients receiving 20 mg of FL per day. The LC–FLD method involves a two-step liquid extraction procedure without any derivatization, followed by direct chromatography on a Zorbax C8 reversed-phase column. The analytical results are discussed in terms of the method validation and the corresponding experimental protocol ($r \geq 0.998$; CV < 9%; LOQ 20 μg/l). There was good correlation between FL, as well as NORFL, plasma levels as determined by the LC–MS and LC–FLD techniques ($r = 0.9597$, $N = 16$ and $r = 0.9852$, $N = 14$ for FL and NORFL, respectively). The results confirm that direct FL/NORFL fluorimetric determination is acceptable for routine use in pharmacokinetic and clinical studies.
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1. Introduction

Fluoxetine (FL), a bicyclic derivative of phenyl-propylamine [D,L-*N*-methyl-3-phenyl-3-(α,α,α-trifluoro-*p*-tolyloxy)-propylamine hydrochloride], is the drug which belongs to the group of selective serotonin inhibitors (SSRIs). These drugs enhance serotonergic neurotransmission, but do not inhibit norepinephrine uptake [1–3]. FL metabolism involves *N*-demethylation to norfluoxetine (NORFL), although there is still uncertainty about the metabolism of 50% of an administered dose [5–10]. NORFL, the most important FL metabolite, is a slightly more potent inhibitor of serotonin neuronal reuptake than its parent compound [1].

Extraction of FL and NORFL from biological specimens is easily achieved with either liquid–liquid or solid-phase extraction (SPE). The recovery values are 75–85% for FL and 65–70% for NORFL in methods using liquid–liquid extraction, and 75–90% for FL and 75–85% for NORFL in SPE methods [11–13].

Several methods have been described for the determination of FL and NORFL in human plasma. The most widely used methods involve high-performance liquid chromatography (HPLC) with UV detection [4,11,12,14–18]. FL/NORFL levels can also be measured in biological samples using gas chromatography (GC) coupled with MS [21,22], flame ionization [23], electron capture [24,25] or nitrogen phosphorus detection [26]. Enantioselective methods are growing in number and importance, since NORFL enantiomers have different potency and a formulation containing a single FL enantiomer is currently under development [27,28].

Among the HPLC methods with fluorescence detection (FLD) described, only one is a direct measurement (without derivatization) [11], while the others involve a derivatization

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step to provide better sensitivity or allow enantioselective analyte determination [19,20].

Our research group has recently developed a sensitive and selective HPLC–MS method [29] used for the analysis of plasma samples from Parkinsonian patients. We were able to calculate pharmacokinetic profiles of the drug after single and multiple dosing and consequently to monitor and assess established pharmacotherapy in this specific population of patients. The purpose of this study was to evaluate a simple, sensitive, rapid and highly selective HPLC–FLD method for the determination of FL and NORFL in human plasma and to compare it to a previously established HPLC–MS method [29].

2. Experimental

Experimental conditions for the iquid chromatography–mass spectrometry (LC–MS) method have been described previously [29], so only details of the liquid chromatographic method with fluorescence detection (LC–FLD) are given here.

2.1. Chemicals

Analytical standards of FL ($C_{17}H_{18}F_3NO$-HCl) and NORFL ($C_{16}H_{16}F_3NO$-HCl) were obtained from Eli Lilly (Basingstoke, UK). Stock FL and NORFL solutions (1 mg/ml) were prepared by dissolving FL and NORFL analytical standards in methanol. All standards were prepared by further dilution of the stock solution with purified water so that application of adequate volume of the standard solution to "drug-free" plasma provided a series of FL and NORFL concentration covering the range from 40 to 800 μg/l. Acetonitrile, hexane, isoamyl alcohol, triethylamine and glacial acetic acid were of chromatography-grade purity. Sodium hydroxide solution (5 M) was prepared from the solid substance, which was of analytical grade and used as received without further purification. Chemicals were obtained from Merck (Darmstadt, Germany). Water purified by a Millipore Milli-Q system was used for the preparation of all solutions.

Prozac® capsules (one capsule contains 20 mg of fluoxetine hydrochloride) were manufactured by Eli Lilly.

2.2. Extraction procedure

A 60-μl aliquot of aqueous solution of internal standard (IS) (paroxetine; 10 mg/l) was added to 1 ml of plasma containing 40–800 μg/l of FL and NORFL, or to 1 ml of patient plasma. After the addition of 200 μl of sodium hydroxide solution (5 M), extraction was performed with 5 ml of hexane/isoamyl alcohol mixture (97:3, v/v) for 20 min. After centrifugation (10 min) the organic layer was carefully removed and back extraction was performed with 0.1 M hydrochloric acid (vortex-mixed for 2 min). A volume of 100 μl was injected.

2.3. Liquid chromatography

The extracts were analyzed using a Hewlett-Packard 1100 series chromatograph (Agilent), equipped with an automatic sample injector and a fluorescence detector (1100 series, Agilent). The column was an Eclipse XDB C8 (150 × 4.6 mm, 5 μm, Zorbax). The mobile phase contained acetonitrile/water/triethylamine (35:65:0.4, v/v/v). The pH was adjusted to 4.00 with glacial acetic acid (approximately 1 ml). Chromatography was performed at a flow rate of 1.0 ml/min and the column temperature was set at 30 °C. The column effluent was monitored at excitation and emission wavelengths of 230 and 310 nm, respectively, for a run time of 12 min.

2.4. Assay validation

Calibration samples were prepared by adding FL and NORFL solution to blank ("drug-free") human plasma. The linearity of the assay was demonstrated on five separate occasions at five separate concentrations over the range 40–800 μg/l for both FL and NORFL. The calibration curves were obtained by plotting FL and NORFL peak area ratios (FL/IS or NORFL/IS) against the FL and NORFL concentration and analyzed using weighted least-square linear regression. The detection limit was determined for FL and NORFL by extraction of plasma spiked with decreasing analyte concentrations until a response equivalent to three times the background was obtained. Recovery was determined for each concentration as the mean (±S.D.) of five samples by comparing the peak areas of extracted and non-extracted samples. Precision is expressed as percentage variation (coefficient of variation, CV) of the value determined for each concentration in the calibration curve. Intra-day CV values were obtained by analyzing five samples for each FL/NORFL plasma level on the same day. Inter-day CV values (CV^*) were obtained for each drug/metabolite concentration in the same manner, but on five different days.

2.5. Correlation procedure

Plasma samples were obtained from Parkinsonian patients receiving 20 mg of FL daily. Blood sampling for the determination of FL and NORFL plasma levels was carried out before drug administration (at time zero) and at 4, 6 and 8 h and 14 days thereafter, and at time zero and 6 h and 7, 45 and 75 days thereafter of chronic treatment with FL. Blood samples (heparin was used as anticoagulant) were centrifuged and plasma was stored at −20 °C until analyzed. Plasma samples previously analyzed using the LC–MS method were then stored again at −20 °C and were reanalyzed 9 months later using the LC–FLD method. Plasma FL and NORFL levels determined with the two methods were compared and the correlation pattern data was defined using a linear-correlation procedure.

3. Results and discussion

Representative HPLC–FLD chromatograms of an extracted blank plasma sample, plasma spiked with FL and NORFL, and a Parkinsonian patient's plasma sample are shown in Fig. 1.

IS, NORFL and FL were separated and eluted at retention times of 3.2, 9.8 and 11.2 min, respectively. With a total run time of 12 min, there were no interfering peaks in patient plasma samples. With this run time, the method is consistent

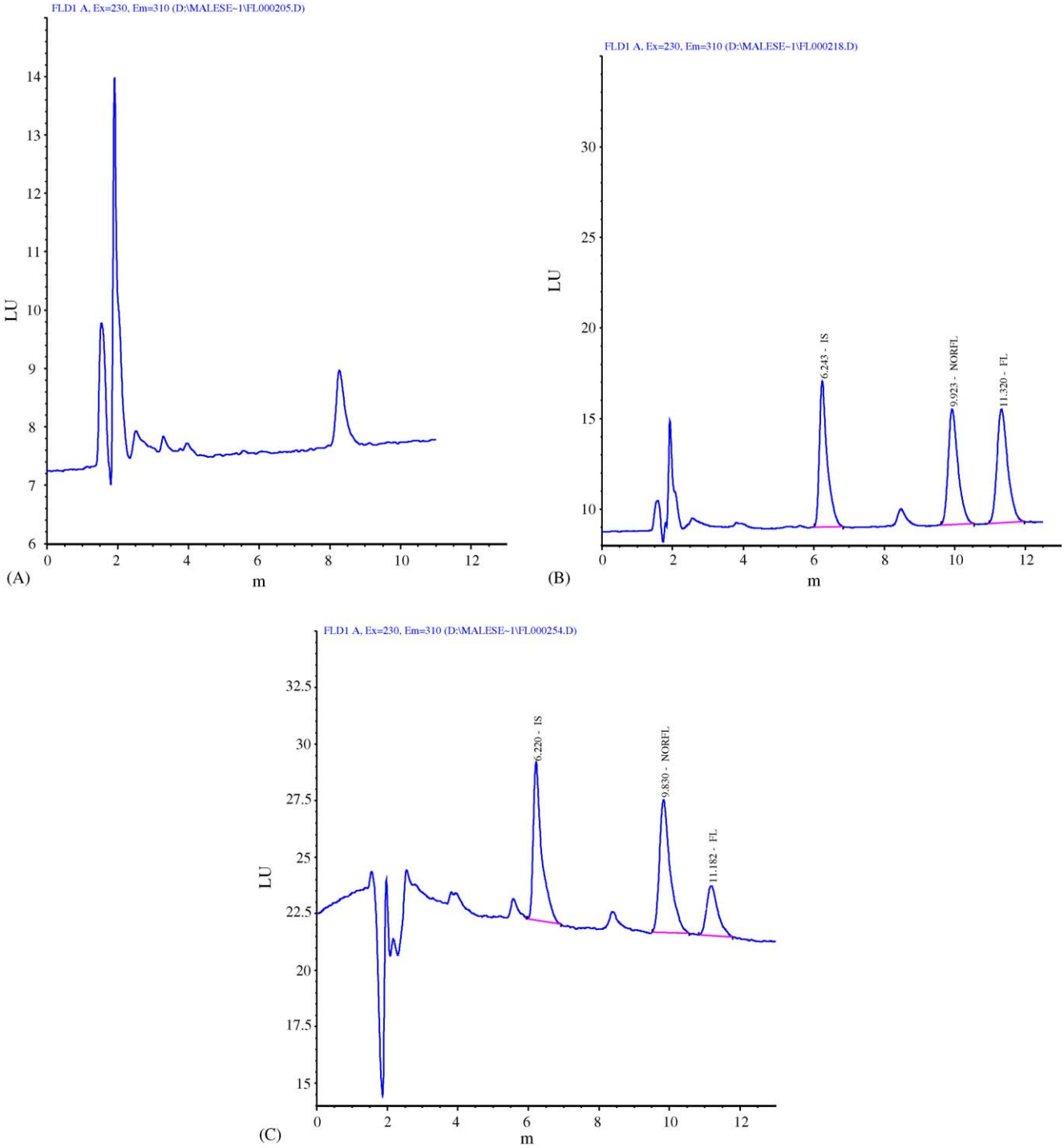

Fig. 1. Chromatograms of extracted: (A) blank (drug-free) plasma; (B) plasma spiked with standard mixture of FL, NORFL (200 μg/l) and paroxetine (IS); and (C) a Parkinsonian patient's plasma sample (FL = 181.96 μg/l, NORFL = 95.25 μg/l).

with other published methods using either UV/FLD detection, where separation demands a run time of at least 8 or even 20 min, or with gas-chromatographic methods, where good resolution is achieved within at least 10 min or longer [11,15,18,19,26]. Consequently, the method described allows the possibility of analyzing up to 100 samples per day. The LC–FLD described in this paper is a direct method, not including derivatization step, which allows higher recovery values. In the literature, there has been found one direct method with fluorescence detection only. However, there are several advantages of our method. Firstly, the method described here is more selective than previously published one. This fact is due to the specific 3D detector that has been used. The detector provides fluorescence specter for each compound eluted from column, which is very important when analyzing biological samples obtained from patients on combined therapy. That way, it is possible to have great certainty

in analytical results that are the basis for further pharmacokinetic analysis. Secondly, our method was more sensitive (LOQ 20 and 30 μg/l; for FL and NORFL, respectively) comparing to the earlier published one (LOQ 30 μg/l), which is of great importance, since FL level in all samples taken from chronically treated patients, under steady state conditions, can be measured with this HPLC–FLD procedure. It is not a case with the published one (FL steady state plasma concentrations may be ranged between 20 and 30 μg/l).

Drugs that might also be present in patient plasma, such as L-dopa, biperiden, bromocriptine, phenobarbital, carbamazepine, diazepam (and/or its metabolites), caffeine, salicylates and paracetamol (acetaminophen), have been checked for analytical interference. No interference was found. While treating the patients with FL some other antidepressant drug (particularly from the group of selective serotonin inhibitors) must not be prescribed. Combined antidepressant therapy is never used as a pharmacotherapy approach in the treatment of depression implicating paroxetine to be a preferable substance to be chosen for IS.

The regression equations for FL and NORFL were: $Y = 0.0069X - 0.1425$ ($r = 0.9985$, covering the range 40–800 μg/l) and $Y = 0.0061X - 0.1233$ ($r = 0.9993$, covering the range 40–800 μg/l).

The method was found to be reproducible, with intra- and inter-day CV values of <7 and <9%, respectively. Due to the extraction step and the high specificity of FLD detection, no interfering or late-eluting peaks were found when chromatographing blank plasma samples from six different sources. Recovery was high and reproducible, with values of 90.75–94.48% and 69.09–83.43% for FL and NORFL, respectively. CV and recovery values are shown in Table 1.

Correlation between the LC–MS and LC–FLD methods for determination of FL/NORFL plasma levels is shown in Fig. 2.

LC–MS was the more sensitive method. Limit of quantification (LOQ) values, of the LC–MS, were 2.5 and 10 μg/l for FL and NORFL, respectively. However, the LC–FLD sensitivity is high enough (LOQ 20 μg/l) to allow its routine application in the determination of FL/NORFL plasma levels in steady-state conditions (achieved 2 weeks after therapy initiation), which provides good support for different pharmacokinetic requirements (therapy individualization, drug interaction assessment, patient compliance). Moreover, the LC–FLD method produced results as reliable as those by the LC–MS method, as judged by the CV values, which were <9% for both analytical procedures, confirming the similar robustness of these methods. The certainty in identification and determination of FL/NORFL plasma levels was the same for both methods due to the very high selectivity of the detectors used. The lack of analytical interference is very important for valid measurement, especially in conditions when human subjects are on combined therapy (Parkinsonian patients). Finally, it has to be pointed out that although LC–MS has recently become a more commonly applied technique, it still cannot be considered standard equipment, especially in clinical laboratories, which is a very important practical issue.

Statistical analysis was performed on the basis of the concentrations in the range of 41.19–110.36 μg/l for FL and 48.17–201.09 μg/l for NORFOL, determined by LC–MS and LC–FLD methods. Linear regression showed that results obtained with the newly established LC–FLD method correlated well with those obtained with the LC–MS method (Fig. 2). Correlation coefficients for concentration values determined by the LC–MS and LC–FLD methods were 0.9597 and 0.9852 for FL and NORFL, respectively. This shows good correlation between the methods, since $r > 0.9$. Further analysis showed that the LC–FLD method gave a lower value of 15.56% for FL plasma concentrations compared to the LC–MS method. Since this difference is not statistically significant ($P \geq 0.1$), it can be concluded that LC–FLD is accurate in comparison with the reference LC–MS method. Regarding NORFL levels, the slope was 0.9824, which means that results obtained with LC–FLD are higher by 1.75% than those measured with LC–MS. In this case, the difference was also not statistically significant ($P \geq 0.2$). Compared to the reference LC–MS method, the accuracy of the LC–FLD method was also confirmed for metabolite determination. Due to the comparison results obtained, the LC–FLD method can be used instead of LC–MS and further applied in clinical pharmacokinetic investigations of FL and NORFL.

The correlation results can also be used to confirm the stability of the samples: no significant loss was observed for analysis of samples stored for 1.5 years at −20 °C or after a freeze/thaw cycle 9 months after they were analyzed for the first time (using the LC–MS method).

Table 1
Precision (intra-day, CV and inter-day, CV*) and recovery values ($n = 5$)

Compound	Concentration (μg/l)	Recovery (%) ($X_{averager} \pm$ S.D.)	CV (%)	CV* (%)
FL	40	93.24 ± 4.07	4.37	6.70
	100	94.48 ± 5.21	5.51	8.23
	200	92.14 ± 4.23	4.59	5.12
	400	90.75 ± 1.74	1.94	4.22
	800	92.17 ± 4.73	5.13	2.91
NORFL	40	74.47 ± 5.17	6.94	6.31
	100	83.43 ± 5.46	6.54	6.41
	200	76.54 ± 3.37	4.40	4.85
	400	76.31 ± 1.19	1.55	3.66
	800	69.09 ± 2.48	3.59	4.69

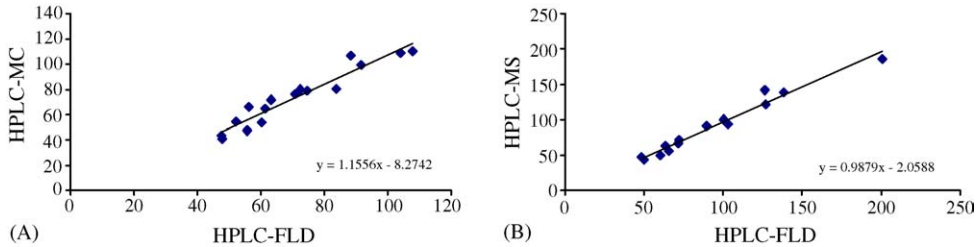

Fig. 2. Linear regression between FL (A) and NORFL (B) plasma levels, determined by LC–FLD (X-ose) and LC–MS method (Y-ose), ($r = 0.9597$, slope = 1.1556, for FL, and $r = 0.9852$, slope = 0.9879, for NORFL).

4. Conclusion

This paper describes a rapid, selective and direct LC method with fluorescence detection for quantification of FL and NORFL in human plasma, and its applicability to pharmacokinetic studies (determination of drug/metabolite levels under steady-state conditions). The method allows a high sample throughput due to the chromatographic run-time of 12 min. Fluorescence detection provides high reliability in the identification and determination of compounds of interest in plasma samples when patients are on combined therapy. Based on the comparison results obtained, this method can be used instead of the earlier described LC–MS method and can therefore be applied in a clinical laboratory for therapeutic drug monitoring, allowing individual dose optimization, detection of drug interactions and assessment of patient compliance.

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