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Improved enantioselective assay for the determination of fluoxetine and norfluoxetine enantiomers in human plasma by liquid chromatography

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Abstract

A simple and innovative assay is described which allows the chiral separation of the four enantiomers of fluoxetine and norfluoxetine, with performance characteristics adequate for therapeutic drug monitoring. The assay requires liquid–liquid extraction into acetonitrile/*n*-hexane/isopropylic alcohol and re-extraction into phosphoric acid for clean-up. The acidic layer is injected onto the HPLC system after filtering. Separation of the analytes is achieved with a Chiralcel ODR column and a mobile phase consisting of potassium hexafluorophosphate/acetonitrile. Detection is made by ultraviolet absorbance at 227 nm. Standard curves are linear for each enantiomer ($r^2 \geq 0.992$) over the range of 10–1000 ng/ml with a limit of quantification of 10 ng/ml for each enantiomer. Within-day and between-day CV% are ≤10% for each enantiomer.

Keywords: Enantiomer separation; Fluoxetine; Norfluoxetine

1. Introduction

Although the selective serotonin reuptake inhibitor fluoxetine has been available for over 15 years and continues to be increasingly used for the treatment of depression and numerous other neuropsychiatric disorders, factors affecting its efficacy and safety have not been fully characterized. Among these, the occurrence of a marked pharmacokinetic variability is likely to play a major role [1–4]. Assessment of potential correlations between clinical response and plasma levels of fluoxetine and/or its active demethylated metabolite norfluoxetine, however, has been hampered for many years by lack of an adequate assay for the discrimination of the respective enantiomers. Such a discrimination is essential

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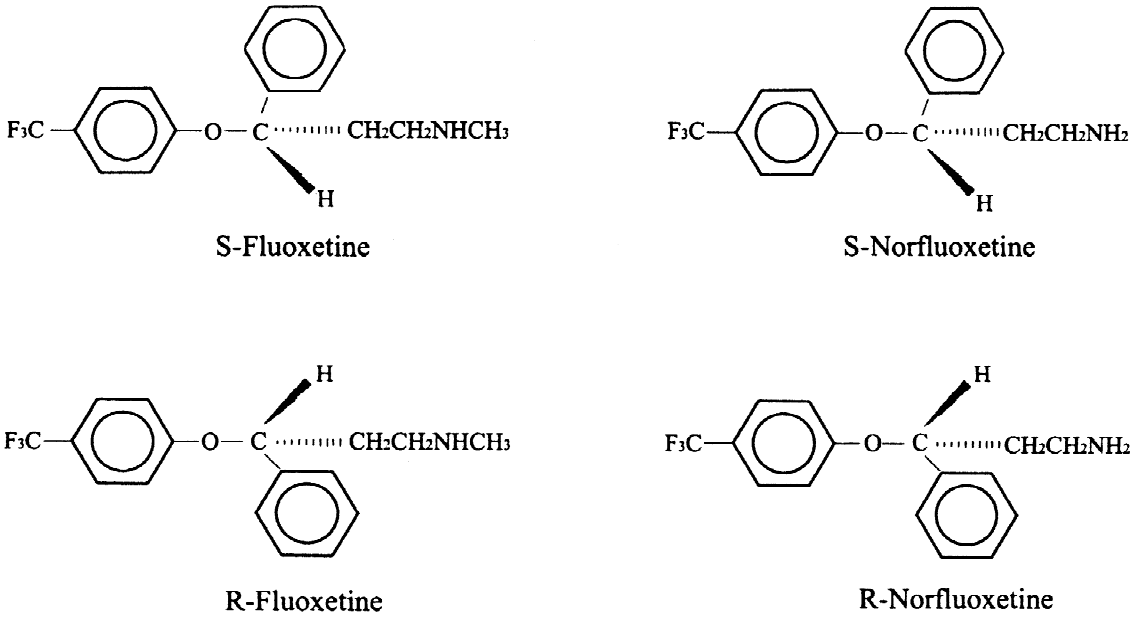

Fig. 1. Chemical structures of the enantiomers of fluoxetine and norfluoxetine.

because the chiral forms of fluoxetine and norfluoxetine (Fig. 1) differ considerably in pharmacological activity: in particular, the *S*-enantiomer of norfluoxetine is 20 times more potent than the *R*-enantiomer in inhibiting serotonin reuptake both in vitro and in vivo [5–8].

Many analytical methods based on gas-chromatography (GC, Refs. [9–13]) and high-performance liquid chromatography (HPLC, Refs. [14–34]) have been validated for the determination of fluoxetine and its metabolite, but only a few of these allow chiral separation of the enantiomers through derivatization with a chiral agent [10,11,13,28,29] or use of a chiral stationary phase without derivatization [30–34]. Moreover, some of the methods using chiral columns only allow separation of the enantiomers of the parent drug (or its metabolite) and cannot be used to determine all four enantiomers in a single run [30,31].

The goal of the work described in this article was to develop a simpler and faster enantioselective assay for fluoxetine and norfluoxetine, which could be applied for the simultaneous monitoring of the enantiomers of these compounds in the plasma of patients receiving fluoxetine treatment.

2. Experimental

2.1. Standard, reagents and solvents

Racemic fluoxetine hydrochloride and norfluoxetine hydrochloride were obtained from Eli Lilly (Indianapolis, IN, USA). Pure *R*- and *S*-enantiomers of fluoxetine and norfluoxetine were kindly donated by Professor P. Baumann (Prilly-Lausanne, Switzerland). Fluvoxamine maleate (internal standard, I.S.) was obtained from Tocris Cookson Ltd (Avonmouth, Bristol, UK). HPLC grade solvents (acetonitrile, methanol, *n*-hexane) and phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany); sodium hydroxide (32%) and isopropyl alcohol were obtained from Baker (Milan, Italy), and potassium hexafluorophosphate from Aldrich (Milan, Italy). Water used for the mobile phase and for preparing sample solutions was deionized and degassed by filtering through a Millipore filter (Fluoropore 0.20 μm) before use.

2.2. Instruments and chromatographic conditions

Analyses were carried out on a Merck-Hitachi

apparatus (Merck, Darmstadt, Germany) consisting of a model L-6200 pump, a model AS-2000A autosampler and a LaChrom L-7400 variable wavelength detector set at 227 nm. The detector signal, processed by a D-2500 chromato integrator (Merck), was plotted at an attenuation of 3 (full scale=1 V). The analytical column was a Chiralcel ODR (250× 4.6 mm I.D., 10 μm, Daicel Inc., Schilling, Milan, Italy) heated to 37 °C by a model T-6300 thermostat (Merck). The mobile phase (pH 3.0) consisted of potassium hexafluorophosphate 100 m*M* and acetonitrile (65:35, v/v). Flow-rate was 0.5 ml/min. The pH of the mobile phase, adjusted with phosphoric acid 8.5%, was measured in the aqueous component alone.

2.3. Stock solutions and extraction procedures

Stock solutions were prepared in methanol to contain 1 mg/ml of each enantiomer (free base) or internal standard (fluvoxamine maleate). Working solutions were prepared by diluting stock solutions in water for fluoxetine and norfluoxetine enantiomers, and in a mixture of methanol and water (50:50) for the internal standard.

Biological samples consisted of human plasma collected in ethylenediaminetetraacetic acid (EDTA). Samples for the preparation of calibrators and controls were obtained from healthy volunteers not receiving any medication. The assay was also tested in samples collected from patients receiving fluoxetine as monotherapy or in combination with other psychotropic medications [35].

For extraction, 1 ml of plasma was mixed with 100 μl of internal standard (I.S., fluvoxamine maleate 4 μg/ml in methanol/water, 50:50, v/v) and 0.5 ml of acetonitrile. The samples were vortexed, 0.5 ml sodium hydroxide 0.5 *M* was added, and the vortexing was then repeated prior to adding 2.5 ml of a mixture of *n*-hexane/isopropyl alcohol (97:3, v/v). After 15 min on a reciprocating shaker, the samples were centrifuged at 1400 *g* for 5 min and the organic layer was transferred to a separate tube containing 400 μl of phosphoric acid 20 m*M*. After shaking (10 min), the samples were centrifuged again (1400 *g*, 5 min), the organic layer was discarded, and 80 μl of the acidic layer was injected onto the HPLC system after filtering through 0.45-μm nylon filters (Sigma, Deisenhofen, Germany).

2.4. Calibration curves and determination of unknowns

Calibrators were prepared from drug-free human plasma by adding appropriate aliquots of working solution of each enantiomer to obtain concentrations of 0, 10, 20, 50, 100, 250, 500 and 1000 ng/ml for each analyte. A complete set of calibrators was prepared weekly.

Calibration curves were constructed by plotting the fluoxetine and norfluoxetine enantiomers to internal standard peak height ratio as a function of the enantiomers' concentrations in the calibrators. The concentration of each enantiomer in the unknowns was calculated from the least-squares linear regression equation of the calibration curve.

Three quality control samples containing 20, 250 and 1000 ng/ml, respectively of each enantiomer were assayed with each run of unknowns.

2.5. Assay performance characteristics

Recovery was determined by comparing peak heights from extracted calibrators with those obtained after injection of known volumes of stock solution ($n=6$).

Reproducibility (precision) and accuracy were evaluated at three concentration levels. Replicates of samples spiked with 20, 250 or 1000 ng/ml of each enantiomer were processed according to the procedure used for unknowns and the measured concentrations were used to calculate mean values and coefficients of variation (CV%) for within-day and between-day variability. The deviation of the mean measured value from the theoretical value (Measured value/Theoretical value)×100, was used as a measure of accuracy.

The lowest limit of detection (LOD) was defined as the concentration yielding a signal-to-noise ratio of 3, whereas the limit of quantitation (LOQ) was considered as the lowest tested concentration (value of the lowest calibrator) at which CV% was <15%.

Specificity was evaluated by injecting into the chromatograph solutions containing several drugs

potentially prescribed in combination with fluoxetine (see Section 3.4).

2.6. Stability

The stability of the enantiomers in plasma was evaluated by comparing assay results in freshly spiked samples ($n=6$) at three different concentrations (20, 250 and 1000 ng/ml) and aliquots of the same samples analyzed after storage under different conditions (see Section 3). Stability was also evaluated for samples stored in extraction solvent (phosphoric acid 20 m*M*) for at least 24 h in autosampler vials.

2.7. Calculations and statistical analysis

Capacity factors (K') were calculated as $(T_r - T_0)/T_0$ where T_r and T_0 are the retention times for the analyte and for a non-retained substance (injection solvent), respectively. Comparisons among stability parameters were assessed by one-way ANOVA. A two-tailed *P*-value ≤ 0.05 was considered significant. Statistical analysis was carried out using Graphpad software (GraphPad, San Diego, CA, USA).

All concentration results are reported as ng/ml of free base.

3. Results

3.1. Chromatographic separation

Under the chromatographic conditions described above, optimal separation of the enantiomers was obtained with peak retention times (RT) of 12.5 min for fluvoxamine (I.S.), 17.5 min for *S*-norfluoxetine, 18.7 min for *R*-norfluoxetine, 20.1 min for *S*-fluoxetine and 21.3 min for *R*-fluoxetine (Fig. 2). The enantioselectivity factors (α) were 1.10 for fluoxetine and 1.09 for norfluoxetine.

3.2. Linearity and sensitivity

The calibration curves for each enantiomer were linear over the concentration range investigated, with mean slopes of 0.0034 for *R*-fluoxetine, 0.0037 for *S*-fluoxetine, 0.0035 for *R*-norfluoxetine and 0.0037 for *S*-norfluoxetine. Correlation coefficients were ≥ 0.992 for all calibration curves (Table 1).

For all enantiomers, the lowest limit of detection was estimated to be 5 ng/ml, whereas the lowest limit of quantitation was 10 ng/ml.

3.3. Recovery, precision and accuracy

Recoveries (means±SD) over the whole calibration range were 77±6% for *R*-fluoxetine, 76±6% for *S*-fluoxetine, 72±14% for *R*-norfluoxetine and 66±6% for *S*-norfluoxetine.

Within- and between-day accuracy and precision are reported in Table 1. For all analytes and for all concentrations tested, the coefficient of variation (CV%) was always ≤10% for both within-day ($n=6$) and between-day ($n=6$) variation. Precision at the limit of quantitation (10 ng/ml), evaluated on a reduced calibration curve (range 0–50 ng/ml) was 5.6% for *S*-norfluoxetine, 5.0% for *R*-norfluoxetine, 7.9% for *S*-fluoxetine and 5.8% for *R*-fluoxetine. Overall, the accuracy of the assay based on the deviation of the mean measured value from the theoretical (spiked) value ranged from 90 to 116%.

3.4. Specificity

No interfering peaks were found in chromatograms obtained after extraction of different sets of blank plasma samples ($n=6$) from drug-free healthy volunteers (Fig. 1). Potential interference from some of the most commonly used antidepressants, antipsychotics, anticonvulsants and other agents was also tested by direct injection of 80 μl of solutions containing appropriate (3 μg/ml) concentrations of each compound. The compounds tested included amitriptyline, carbamazepine, carbamazepine-10,11-epoxide, clomipramine, clozapine, desipramine, dextromethorphan, diazepam, doxepin, ethosuximide, gabapentin, imipramine, lamotrigine, lorazepam, nortriptyline, phenobarbital, phenytoin and valproic acid. As shown in Table 2, most of the compounds tested did not interfere with the determination of fluoxetine and norfluoxetine enantiomers. Moreover, acidic compounds are not expected to be extracted with the extraction procedure, a prediction which was confirmed by testing, in separate experiments, plasma samples spiked with phenobarbital, phenytoin

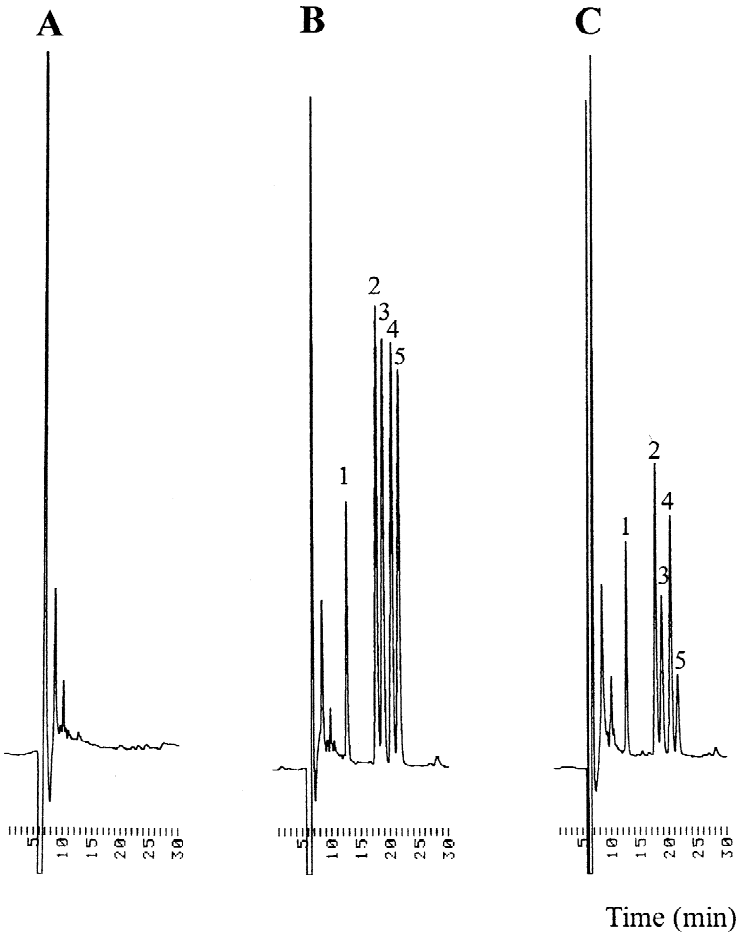

Fig. 2. Representative chromatograms of blank plasma (A), a calibration sample (B, 250 ng/ml of each enantiomer) and a sample of a patient (C) taking fluoxetine at a dosage of 20 mg/day. The signals in (C) correspond to fluvoxamine (1, internal standard), 194 ng/ml of *S*-norfluoxetine (2), 115 ng/ml of *R*-norfluoxetine (3), 183 ng/ml of *S*-fluoxetine (4) and 66 ng/ml of *R*-fluoxetine (5).

and valproic acid. Carbamazepine, doxepin, and desipramine showed, under the conditions used in this assay, K' values similar to *S*-norfluoxetine, *S*-fluoxetine and *R*-fluoxetine, respectively. When assaying samples from patients receiving concomitant treatment with these drugs, chromatographic conditions should be modified by reducing the proportion of organic solvent in the mobile phase or by modifying the pH of the mobile phase until a satisfactory separation is obtained. For example, when assaying samples from patients taking carbamazepine, a reduction of the proportion of acetonitrile from 35 to 33% produces a good separation of carbamazepine from *S*-norfluoxetine, with RT values (min) of 14.8–15.7 for fluvoxamine (I.S.), 18.2–19.4 for carbamazepine, 22.6–24.5 for *S*-norfluoxetine, 24.4–26.5 for *R*-norfluoxetine, 26.4–28.7 for *S*-fluoxetine and 28.4–31.0 for *R*-fluoxetine.

In assaying samples from patients taking clozapine, an interfering peak was detected whose RT was similar to that of the internal standard fluvoxamine. The peak does not correspond to the retention time of clozapine, and therefore it is probably related to a metabolite.

Table 1
Performance characteristics of the assay of the *R*- and *S*-enantiomers of fluoxetine (FLX) and norfluoxetine (NFLX)

Parameter	*R*-FLX	*S*-FLX	*R*-NFLX	*S*-NFLX
Calibration parameters (n=6)				
Slope: mean (CV%)	0.0034 (6.9)	0.0037 (5.4)	0.0035 (11.9)	0.0037 (6.8)
Intercept: mean±SD	−0.018±0.027	−0.014±0.023	−0.029±0.031	−0.020±0.020
Correlation coefficient: mean	0.998	0.997	0.997	0.998
(range)	(0.996–1.0)	(0.994–1.0)	(0.992–1.0)	(0.994–1.0)
Recovery (n=6)				
Recovery %: mean±SD (CV%)	77±6 (7.8)	76±6 (7.9)	72±14 (19)	66±6 (9.1)
Within-day variation (n=6)				
Theoretical value	20	20	20	20
Measured value: mean±SD	23.3±2.0	21.3±2.1	18.7±0.8	21.0±1.3
Precision (CV%)	(8.4)	(9.9)	(4.3)	(6.2)
Accuracy (%)	116	106	94	105
Theoretical value	250	250	250	250
Measured value: mean±SD	244.2±6.8	240.1±2.8	224.8±7.6	227.6±9.9
Precision (CV%)	(2.8)	(1.2)	(3.4)	(4.4)
Accuracy (%)	98	96	90	91
Theoretical value	1000	1000	1000	1000
Measured value: mean±SD	937.4±34.8	938.9±17.8	902.4±41.1	914.8±13.2
Precision (CV%)	(3.7)	(1.9)	(4.6)	(1.4)
Accuracy (%)	94	94	90	92
Between-day variation (n=6)				
Theoretical value	20	20	20	20
Measured value: mean±SD	19.4±1.7	19.1±0.8	18.6±1.7	20.8±2.1
Precision (CV%)	(8.9)	(4.3)	(9.1)	(10.0)
Accuracy (%)	97	96	93	104
Theoretical value	250	250	250	250
Measured value: mean±SD	250.4±3.7	249.1±3.6	245.9±8.4	245.4±8.5
Precision (CV%)	(1.5)	(1.4)	(3.4)	(3.5)
Accuracy (%)	100	100	98	98
Theoretical value	1000	1000	1000	1000
Measured value: mean±SD	1004.9±22.4	1007.6±21.6	1000.5±25.7	1000.8±24.4
Precision (CV%)	(2.2)	(2.1)	(2.6)	(2.4)
Accuracy (%)	100	101	100	100

The concentration range of calibration curves was 10–1000 ng/ml. All concentration values are expressed in ng/ml.

3.5. Stability

Stability data are summarized in Table 3. No significant loss (one-way ANOVA, NS) was observed when samples were stored for 24 h at room temperature, for up 6 months at −20 °C, or after three freeze/thaw cycles during a 7-day period. All four enantiomers were also stable when stored in phosphoric acid 20 m*M* for at least 24 h in the autosampler vials at room temperature (Table 3).

4. Discussion

This method offers significant advantages in terms of simplicity and turnaround time compared with the

Table 2
K' values of compounds tested for potential interference

Compound	K'
Fluvoxamine (I.S.)	0.87
S-norfluoxetine	1.63
R-norfluoxetine	1.78
S-fluoxetine	1.99
R-fluoxetine	2.18
Amitriptyline	3.55
Carbamazepine	1.57
Carbamazepine-10,11-epoxide	0.64
Clomipramine	4.44
Clozapine	0.64
Desipramine	2.12
Dextromethorphan	1.34
Diazepam	5.81
Doxepin	1.96
Ethosuximide	3.38
Gabapentin	ND
Imipramine	3.02
Lamotrigine	0.40
Lorazepam	1.44
Nortriptyline	ND
Phenobarbital	0.92
Phenytoin	3.85
Valproic acid	1.37

K' values of the internal standard (fluvoxamine) and of the *R*- and *S*-enantiomers of fluoxetine and norfluoxetine are shown for comparison purposes.

majority of assays described to date [9–34]. Time consuming solvent evaporation and sample reconstitution are avoided because the required sensitivity is retained without need for sample concentration steps. Moreover, separation of the *R*- and *S*-enantiomers of fluoxetine and norfluoxetine is obtained using a single chiral column, therefore avoiding time consuming derivatization steps. The double step purification, involving an alkaline extraction followed by re-extraction into phosphoric acid, eliminates potential interference by acidic compounds such as phenobarbital, phenytoin and valproic acid. For each of the analytes, recoveries are similar to those reported for other published methods [19]. Optimal chromatographic separation of the four enantiomers is obtained in less than 25 min, a time comparable to that observed in methods using chiral derivatization [10,28,29] and much shorter than the 40–45 min typically required in previously published methods using chiral stationary phases [32,34]. Only one method, applied to pharmaceutical solutions and not to biological samples [33], resolved the four enantiomers in 25 min using a chiral column (cyclodextrin β).

The present method also represents an advance over an enantioselective assay described previously by our own group [32,36], which was associated with significant problems when testing samples exposed to prolonged storage. In fact, while in the previous assay no instability problems were encountered in EDTA plasma stored for up to 8 days at 4 °C and for up to 21 days at −20 °C, chromatograms from samples exposed to longer storage showed an interfering peak which was ascribed to release of a degradation product from the matrix during storage. In the improved assay described in the present report, this interference was eliminated by modifying the extraction conditions, and no instability problems were observed even in samples stored at −20 °C for up to 6 months. On the other hand, storage at room temperature for 24 h did result in a modest, statistically non-significant, decrease in assayed values. This is consistent with findings by Binsumait et al. [37], who also detected a 22.4% decrease in fluoxetine concentration in plasma stored at room temperature for 2 weeks. In the latter study, no significant loss was observed in plasma stored at 5 °C and −20 °C for 12 weeks.

Because fluoxetine is often prescribed in combination with different psychotropic agents and other drugs, it was important to assess potential interferences from potentially co-administered compounds. Among those tested, only carbamazepine, doxepin and desipramine were found to be co-eluted with one of the fluoxetine or norfluoxetine enantiomers, and additional interference was detected from a substance present in the plasma of patients treated with clozapine. When assaying samples from patients taking the latter agents (or imipramine, which is biotransformed to desipramine), careful readjustment of chromatographic conditions is indicated. Of course, the assay is not suitable for the analysis of samples from patients taking fluvoxamine, which is used as internal standard. However, there is no rationale for the simultaneous administration of two different serotonin reuptake inhibitors, and this type of combination therapy is extremely unlikely to be encountered in clinical practice.

In conclusion, the present assay combines a vari-

Table 3
Stability of fluoxetine (FLX) and norfluoxetine (NFLX) enantiomers in plasma under different storage conditions

Storage conditions	Spiked value	*R*-FLX	*S*-FLX	*R*-NFLX	*S*-NFLX
Controls	20	23.3±2.0	21.3±2.1	18.7±0.8	21.0±1.3
	250	244.2±6.8	240.1±2.8	224.8±7.6	227.6±9.9
	1000	937.4±34.8	938.9±17.8	902.4±41.1	914.8±13.1
24 h at room	20	18.5±2.9	18.1±3.5	20.3±4.1	18.7±2.2
temperature	250	234.7±3.7	238.2±4.5	231.8±4.6	231.3±4.7
	1000	898.0±22.2	911.7±21.1	875.5±25.4	878.8±26.1
3 days at −20 °C	20	26.5±4.0	28.7±4.4	26.7±5.6	27.0±4.8
	250	241.6±5.6	244.5±3.4	240.4±6.9	241.3±6.8
	1000	917.5±10.4	923.9±11.1	916.8±12.7	920.5±14.4
8 days at −20 °C	20	23.9±3.9	27.0±5.2[a]	26.3±4.5[a]	24.5±5.1[a]
	250	241.5±3.0	244.0±1.8	231.6±5.9	233.1±6.1
	1000	906.6±34.7	910.0±36.5	909.0±44.1	912.4±43.7
50 days at −20 °C	20	19.0±3.1	26.1±1.9	21.0±3.7	27.3±4.7
	250	233.6±11.5	235.6±14.0	245.5±13.1	248.5±13.6
	1000	922.2±42.9	927.6±44.1	977.3±56.6	987.7±54.2
3 months at −20 °C	20	20.8±2.6	18.1±1.9	18.0±2.6	20.9±3.6
	250	236.4±7.7	238.1±7.4	236.9±10.9	234.8±10.4
	1000	933.8±54.2	941.8±52.8	976.0±53.4	977.8±42.6
6 months at −20 °C	20	26.2±7.2	26.1±6.1	22.9±5.5	26.3±5.4
	250	228.4±3.9	227.6±3.5	195.0±3.8	216.8±6.0
	1000	874.3±12.6	876.6±12.9	783.0±80.3	876.6±13.0
Three freeze/thaw cycles	20	23.4±0.8	24.6±1.2	22.5±1.2	24.0±0.8
	250	235.2±4.8	237.9±4.0	226.6±4.2	227.3±3.7
	1000	899.3±14.7	905.2±16.1	863.7±18.7	867.6±19.9
24 h in autosampler vial[c]	20	27.4±1.4	26.6±1.4	23.5±1.3	24.6±1.1
	250	245.8±3.9	249.9±4.5	236.3±5.3	238.0±4.3
	1000	927.9±10.2[b]	933.9±8.5[b]	890.6±8.4[b]	894.2±8.3[b]

Values are mean±SD. All concentrations are expressed in ng/ml; $n=6$, unless indicated otherwise.
[a] $n=5$.
[b] $n=3$.
[c] Sample stored in extraction solvent (phosphoric acid).

ety of convenient features, being relatively simple, accurate, precise and endowed with acceptable specificity. The sensitivity is adequate for the monitoring of patients receiving therapeutic doses of fluoxetine. The feasibility of this application could be confirmed in our laboratory by conducting a prospective study where samples from 131 patients were analysed to determine the influence of genetic and environmental factors on the enantioselective pharmacokinetics of fluoxetine and norfluoxetine. The results of this study are reported in a separate publication [35].

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