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Journal of Chromatography B, 682 (1996) 265–272

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simultaneous determination of plasma levels of fluvoxamine and of the enantiomers of fluoxetine and norfluoxetine by gas chromatography–mass spectrometry

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Received 15 November 1995; revised 6 February 1996; accepted 6 February 1996

Abstract

A gas chromatographic–mass spectrometric method is presented which allows the simultaneous determination of the plasma concentrations of fluvoxamine and of the enantiomers of fluoxetine and norfluoxetine after derivatization with the chiral reagent, (*S*)-(−)-N-trifluoroacetylprolyl chloride. No interference was observed from endogenous compounds following the extraction of plasma samples from six different human subjects. The standard curves were linear over a working range of 10 to 750 ng/ml for racemic fluoxetine and norfluoxetine and of 50 to 500 ng/ml for fluvoxamine. Recoveries ranged from 50 to 66% for the three compounds. Intra- and inter-day coefficients of variation ranged from 4 to 10% for fluvoxamine and from 4 to 13% for fluoxetine and norfluoxetine. The limits of quantitation of the method were found to be 2 ng/ml for fluvoxamine and 1 ng/ml for the (*R*)- and (*S*)-enantiomers of fluoxetine and norfluoxetine, hence allowing its use for single dose pharmacokinetics. Finally, by using a steeper gradient of temperature, much shorter analysis times are obtained if one is interested in the concentrations of fluvoxamine alone.

Keywords: Enantiomer separation; Fluvoxamine; Fluoxetine; Norfluoxetine

1. Introduction

Fluvoxamine (FLV) and fluoxetine (FLX) (see Fig. 1) are antidepressants belonging to the class of selective serotonin reuptake inhibitors (SSRIs). They show clinical efficacy comparable to classical tricyclic antidepressants but are devoid of some of the anticholinergic and cardiovascular adverse effects commonly associated with the latter drugs [1,2]. In the organism, FLV is biotransformed to several inactive metabolites [3] while the N-demethylation of FLX results in the formation of norfluoxetine (NFLX) which is pharmacologically active [4]. FLX is administered as a racemate, and in vitro studies have shown that (*S*)-FLX, (*R*)-FLX and (*S*)-NFLX, but not (*R*)-NFLX are potent serotonin reuptake inhibitors [5].

Several HPLC or GC methods have been published for the determination of either FLV [6–8] or FLX [9–13] in plasma or serum samples, taking into account [11–13] or not [9,10] the enantiomers of the latter drug. Recently, a method has been developed for the determination of the enantiomers of FLX and NFLX by gas chromatography with electron-capture

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0378-4347/96/$15.00
PII S0378-4347(96)00080-1

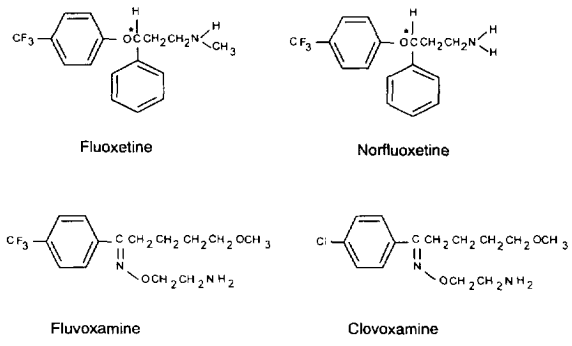


Fig. 1. Chemical structures of fluoxetine, norfluoxetine, fluvoxamine and clovoxamine (internal standard). The chiral centre is indicated by an asterisk.

detection after derivatization with (*S*)-(−)-trifluoroacetylprolyl chloride [12]. After having introduced this method, with some modifications, into our laboratory for therapeutic drug monitoring of FLX and NFLX by gas chromatography–mass spectrometry (GC–MS), we found that, using the same chiral reagent but with another internal standard, it was possible to simultaneously measure the concentrations of FLV and of the enantiomers of FLX and NFLX. Furthermore, by using a steeper gradient of temperature, much shorter analysis times are obtained if one is interested in the concentrations of FLV alone.

2. Experimental

2.1. Reagents

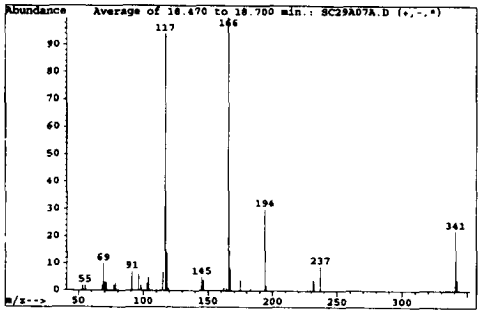
Fluvoxamine maleate and clovoxamine fumarate (internal standard) were obtained from Solvay Duphar (Weesp, Netherlands). Fluoxetine hydrochloride, norfluoxetine hydrochloride, (*R*)-fluoxetine hydrochloride, (*S*)-fluoxetine hydrochloride, (*R*)-norfluoxetine hydrochloride and (*S*)-norfluoxetine hydrochloride were provided by Eli Lilly (Indianapolis, IN, USA). Stock solutions of FLV and clovoxamine and of FLX and NFLX were made at 1 mg base/ml in methanol and in 0.1 *M* HCl, respectively. These solutions were divided into small aliquots and stored at −20°C. Working solutions of FLV, FLX and NFLX were made every day from freshly thawed aliquots at 10 ng/μl and at 1 ng/μl in 0.1 *M* HCl and of clovoxamine at 2 ng/μl in 0.1 *M* HCl. (*S*)-(−)-N-Trifluoroacetylprolyl chloride was purchased from Aldrich (Fluka Chemie, Buchs, Switzerland). All other reagents were of analytical or HPLC grade.

2.2. Instrumentation and chromatographic conditions

Analyses were performed on a Hewlett-Packard HP 5890 Series II gas chromatograph equipped with a splitless capillary and an electronic control pressure system and linked to a quadruple HP 5972 mass spectrometer operating in the electron impact (EI) mode. The MS conditions were: ionization potential 50 eV, emission 50 μA, ion source temperature around 180°C (heated by the interface), and GC–MS capillary direct interface 280°C. Splitless injections of 3 μl were made into a fused-silica Optima 5 capillary column (Macherey-Nagel, Oensingen, Switzerland), 15 m×0.25 mm I.D., 0.25 μm film thickness, with helium as the carrier gas. The column head-pressure was set to maintain a constant flow, with a pressure of 2 p.s.i. (14 kPa) at 145°C as starting conditions. The total flow is set to 50 ml/min and the septum purge to 3 ml/min. GC conditions, when measuring FLV and/or the enantiomers of FLX and NFLX, were: initial temperature 145°C, initial time 0.5 min, heating rate 30 C°/min until 207°C (207°C during 7 min), 1 C°/min until 221°C and 30 C°/min until 290°C, final temperature 290°C and injector temperature 250°C. GC conditions, when measuring fluvoxamine only, were: column head pressure set to maintain a constant flow with a pressure of 2 p.s.i. (14 kPa) at 160°C as starting conditions, initial temperature 160°C, heating rate 30 C°/min until 290°C. Analyses were performed in the selected-ion monitoring mode for the ions at *m/z* 117 (FLX and NFLX) and 166 (FLV and clovoxamine), with a dwell time of 200 ms.

2.3. Extraction and derivatization conditions

To a 1-ml volume of heparinized plasma was added 150 μl clovoxamine (internal standard, 2 ng/μl), 0.5 ml 1 *M* carbonate buffer pH 9.4, and 6 ml *n*-heptane–ethyl acetate (80:20, v/v). Extraction was performed on a rotatory shaker for 15 min. After



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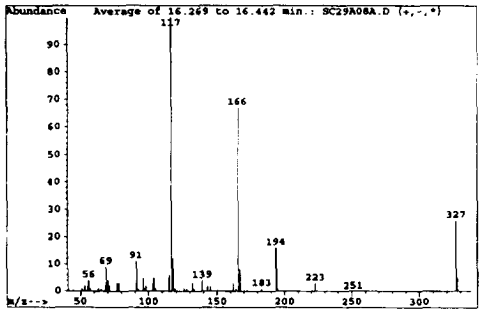

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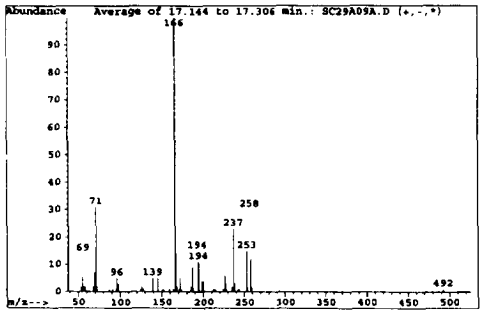

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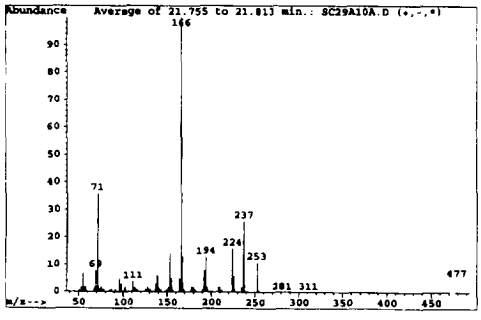

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Fig. 2. Electron-impact mass spectra of fluoxetine (A, M^+=502), norfluoxetine (B, M^+=488), fluvoxamine (C, M^+=511) and clovoxamine (D, M^+=477) after derivatization with (*S*)-(−)-trifluoroacetylprolyl chloride. Examples of the probable fragmentation modes are given.

Table 1
Statistical data concerning the analysis of fluoxetine, norfluoxetine and fluvoxamine

Parameter	FLV	(*R*)-FLX	(*S*)-FLX	(*R*)-NFLX	(*S*)-NFLX
Calibration ($n=5$)					
Range (ng/ml)	50–500	5–350	5–350	5–350	5–350
Slope: mean±S.D. (C.V.)	1.08±0.10 (10)	1.57±0.13 (8)	1.59±0.17 (11)	2.17±0.13 (6)	1.88±0.17 (9)
Intercept: mean (range)	−0.0811 (−0.126, −0.0188)	−0.0086 (−0.064, 0.0317)	−0.0142 (−0.0568, 0.0235)	−0.0264 (−0.0687, −0.0002)	−0.0328 (−0.0739, −0.0048)
Coefficient of correlation: mean (range)	0.996 (0.993, 1.0)	0.996 (0.990, 0.999)	0.997 (0.991, 0.999)	0.998 (0.994, 1.0)	0.997 (0.990, 1.0)
Recovery ($n=8$)					
Concentration used (ng/ml)	100	50	50	50	50
Recovery (in %): mean±S.D. (C.V.)	59±9 (15)	66±4 (6)	66±3 (4)	56±3 (6)	50±3 (5)
Concentration used (ng/ml)	400	250	250	250	250
Recovery (in %): mean±S.D. (C.V.)	58±7 (12)	54±8 (15)	54±7 (14)	55±6 (10)	50±7 (13)
Within-day variation ($n=7$)					
Theoretical values (ng/ml)	100	50	50	50	50
Measured values (ng/ml): mean±S.D. (C.V.)	97.0±3.4 (4)	48.2±4.2 (9)	46.8±4.4 (10)	51.4±6.7 (13)	53.1±6.2 (12)
Percentage of theory	97	96.4	93.6	102.8	106.2
Theoretical values (ng/ml)	400	250	250	250	250
Measured values (ng/ml): mean±S.D. (C.V.)	414.3±26.5 (6)	267.0±11.8 (4)	259.1±12.2 (5)	255.6±22.1 (9)	263.9±23.7 (9)
Percentage of theory	103.6	106.8	103.6	102.2	105.6
Day-to-day variation ($n=10$)					
Theoretical values (ng/ml)	100	50	50	50	50
Measured values (ng/ml): mean±S.D. (C.V.)	97.8±8.7 (9)	50.4±2.9 (6)	48.7±4.1 (9)	48.4±4.1 (8)	48.8±3.8 (8)
Percentage of theory	97.8	100.8	97.4	96.8	97.6
Theoretical values (ng/ml)	400	250	250	250	250
Measured values (ng/ml): mean±S.D. (C.V.)	389.0±40.5 (10)	248.2±15.2 (6)	247.9±14.0 (6)	243.3±25.9 (11)	248.7±25.9 (10)
Percentage of theory	97.3	99.3	99.2	97.3	99.5
Limit of quantitation ($n=9$)					
Theoretical values (ng/ml)	2	1	1	1	1
Measured values (ng/ml): mean±S.D. (C.V.)	2.10±0.22 (10)	1.07±0.13 (12)	0.99±0.14 (14)	0.95±0.10 (10)	1.08±0.12 (11)

Fluvoxamine (FLV), (*R*)- and (*S*)-fluoxetine [(*R*)- and (*S*)-FLX], (*R*)- and (*S*)-norfluoxetine [(*R*]- and (*S*)-NFLX), standard deviation (S.D.), coefficient of variation (C.V., in %).

centrifugation (8 min, 3400 *g*), the organic layer was transferred to another tube containing 1.2 ml of 0.1 *M* HCl. After 15 min shaking and centrifugation, the aqueous phase was transferred to another tube containing 1 ml of 1 *M* carbonate buffer (pH 9.4) and 150 μl toluene–isoamyl alcohol (85:15, v/v). After 15 min shaking and 2 min centrifugation, the solvent was transferred to a conical tube and evaporated to dryness under a stream of nitrogen at 40°C.

The residue was dissolved with 100 μl of the chiral reagent [made every day with 100 μl (*S*)-(−)-N-trifluoroacetylprolyl chloride in 2.5 ml toluene] and left for 1 h at 60°C in a small closed vial. The reagent was then evaporated to dryness under a stream of nitrogen at 40°C, the derivatized drug was reconstituted in 100 μl toluene–isoamyl alcohol, and 3 μl was injected onto the GC–MS system.

3. Results and discussion

FLX is a secondary amine, and NFLX and FLV are primary amines (Fig. 1): a derivatization step is useful to improve their chromatographic properties (data not shown). As FLX and NFLX are chiral compounds, the choice of a chiral reagent also allows the separation of the enantiomers on a non-chiral column by the formation of diastereoisomeric pairs. (*S*)-(−)-N-Trifluoroacetylprolyl chloride was used for the derivatization of FLX and NFLX in our laboratory according to Ref. [12], with the method being adapted to allow the determination of these compounds by GC–MS. As a method was also needed for FLV, it was found that the same reagent was also suitable for this latter compound, hence allowing the simultaneous determination of these two

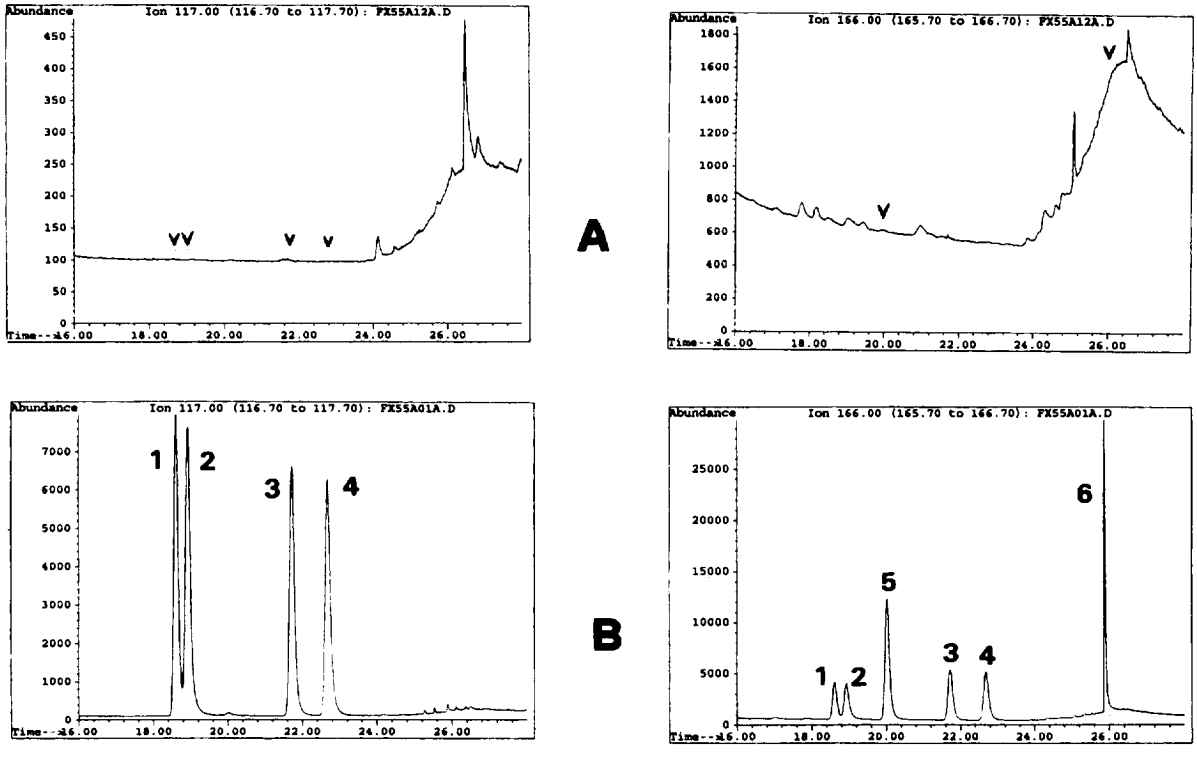

Fig. 3. Selected-ion monitoring (SIM) tracing of a 1-ml blank plasma (A) and a 1-ml blank plasma spiked with 250 ng/ml racemic fluoxetine and norfluoxetine and 300 ng/ml fluvoxamine (B). Peaks: 1=(*S*)-norfluoxetine (18.61 min); 2=(*R*)-norfluoxetine (18.93 min); 3=(*R*)-fluoxetine (21.72 min); 4=(*S*)-fluoxetine (22.68 min); 5=fluvoxamine (5, 20.03 min); 6=clovoxamine (internal standard, 25.89 min). Left, *m/z* 117; right, *m/z* 166.

SSRIs. Nevertheless, the internal standard L-alprenolol was replaced by clovoxamine, as the former compound was found to be poorly recovered at the last step of derivatization (drying of the derivatization solvent) and to elute between NFLX and FLX as does FLV.

Fig. 2 shows the EI mass spectra of FLX, NFLX, FLV and clovoxamine after derivatization with (*S*)-(−)-trifluoroacetylprolyl chloride, with the probable fragmentation mode. Table 1 shows a summary of the statistical data on the analysis of FLX, NFLX and FLV. To summarize, the mean coefficients of correlation of the calibration curves (seven concentrations from 10 to 750 ng/ml for racemic FLX and NFLX and seven concentrations from 50 to 500 ng/ml for FLV) obtained from five separate experiments ranged from 0.996 to 0.998. As pure standards of the derivatized compounds are not available, recovery was calculated by dividing mean areas ($n=8$) obtained after the complete extraction and derivatization procedure of plasmas containing 100 or 400 ng/ml FLV and 100 or 500 ng/ml racemic FLX and NFLX by mean areas obtained after direct derivatization of the same quantities of pure standards. Recovery was found to be satisfactory for all compounds, ranging from 50 to 66%. The variability of the assays, as assessed by the coefficients of variation (C.V.) measured at two concentrations for each substance, was always less than 13%, both for the intra-day ($n=7$) and inter-day ($n=9$) experiments. The deviations from the theoretical concentrations, which represent the accuracy of the method, were all within ±7%. The limits of quantitation, defined as the concentrations for which the mean value of replicate determinations ($n=9$) is within 20% of the actual value, the C.V. less than 20% and which gives a signal-to-noise ratio of at least 10, were found to be 2 ng/ml for FLV and 1 ng/ml for each enantiomer of FLX and NFLX. For the purpose of a kinetic experiment over a long period of time

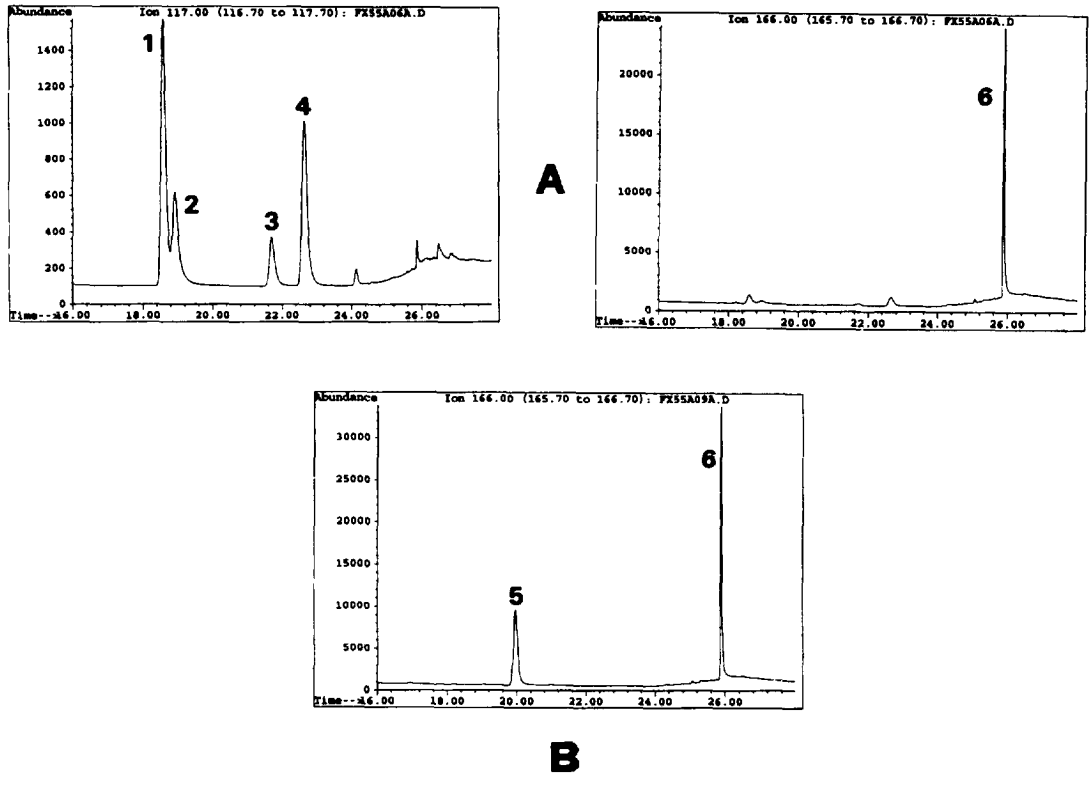

Fig. 4. SIM tracing of a 1-ml plasma analysis from a patient treated with 20 mg racemic fluoxetine per day (A) and a 1-ml plasma analysis from a patient treated with 300 mg fluvoxamine per day (B). For retention times, see Fig. 3.

after a single oral dose of racemic FLX, we checked whether the limits of quantitation of FLX and NFLX could be lowered by extracting 2 ml of plasma (instead of 1 ml) and dissolving the dry residue at the end of the derivatization steps in 20 μl toluene–isoamyl alcohol (instead of 100 μl). The limits of quantitation, using the same definitions as above, were found to be 100 pg/ml for the (*R*)- and (*S*)-enantiomers of FLX, while the presence of a trace contaminant prevented the accurate determination of the enantiomers of NFLX at the picogram level (data not shown). Finally, the stability of these three compounds was evaluated by analysing spiked plasmas stored at −20°C for different periods of time. No loss was noted after storage of up to three months.

Fig. 3 shows the SIM tracing of a blank plasma and of blank plasma spiked with 250 ng/ml FLX and NFLX and 300 ng/ml FLV. Fig. 4 shows an example of a chromatogram obtained from the plasma analysis of one patient receiving 20 mg racemic FLX per day for nine months and of another receiving 300 mg FLV per day for four months. The measured concentrations of (*S*)-NFLX, (*R*)-NFLX, (*R*)-FLX, (*S*)-FLX and FLV were 38, 22, 11, 31 and 244 ng/ml, respectively. This confirms a stereoselectivity in the metabolism of fluoxetine, which has been the subject of several reports [11–13]. Finally, when only the plasma concentration of FLV is needed, a much shorter time is necessary for analysis (see Fig. 5) owing to the use of a steeper gradient of temperature (see Experimental).

It should be mentioned that the same reagent was also tried for the derivatization of paroxetine and sertraline, two other SSRIs which are secondary amines (citalopram as the fifth and last SSRI on the market is a tertiary amine). Unfortunately, it was found that the derivatization has a poor yield re-

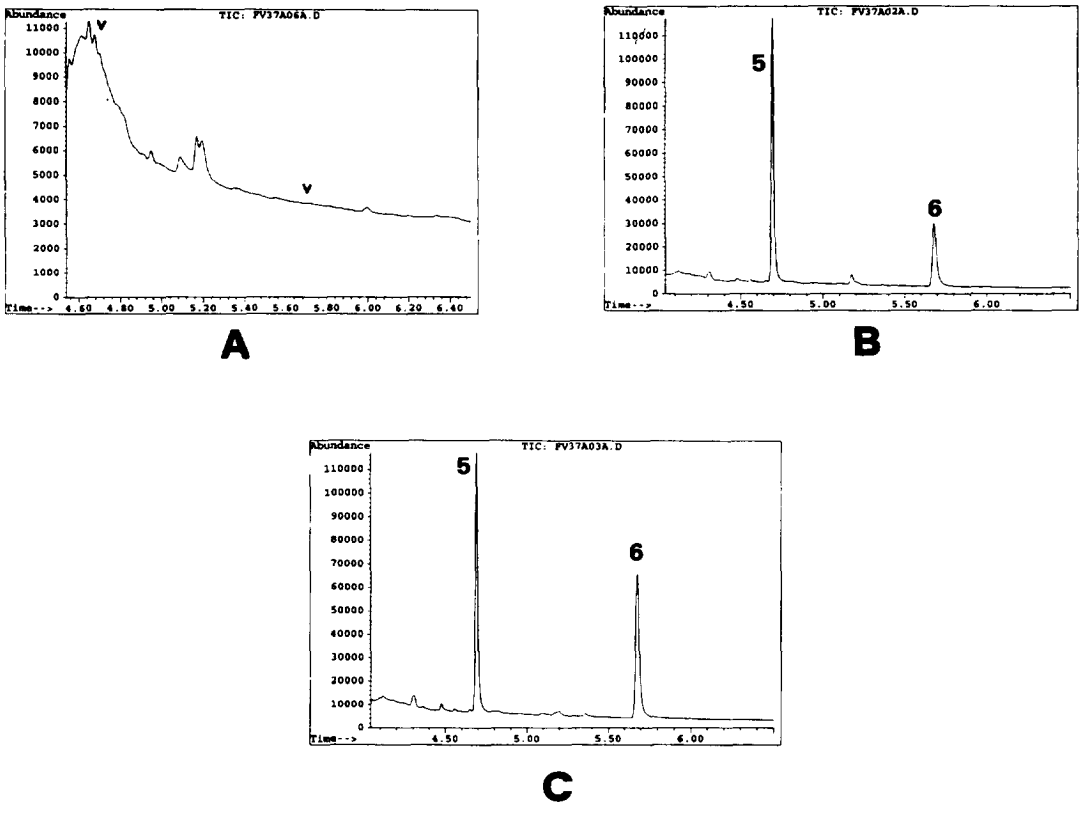

Fig. 5. SIM tracing with an increased gradient of temperature for the analysis of fluvoxamine alone. (A) Blank plasma; (B) blank plasma spiked with 400 ng/ml fluvoxamine; (C) plasma from a patient treated with 300 mg fluvoxamine per day. Peaks: 5=fluvoxamine (4.70 min); 6=clovoxamine (internal standard, 5.69 min).

sulting in a high limit of quantification (approximately 50 ng/ml, data not shown), thus unsuitable for clinical purposes.

To summarize, this method both sensitive and selective, allows the simultaneous quantification of FLV and of the enantiomers of FLX and NFLX in plasma. It can be used for single-dose pharmacokinetic studies and offers a good possibility for the analysis of blood samples drawn from psychiatric patients who are often co-medicated with many different drugs, often resulting in interfering or overlapping peaks when such samples are analysed by HPLC.

Acknowledgments

We gratefully acknowledge the editorial assistance of Mrs. C. Berstchi, and the bibliographic help of Mrs. L. Delassue, Mrs. M. Gobin and Mrs. T. Bocquet. We thank Solvay Duphar for providing us with fluvoxamine and clovoxamine and Eli Lilly for (*R*)-, (*S*)- and (*R*,*S*)-fluoxetine and norfluoxetine. This work was supported in part by the Swiss National Research Foundation (Project No. 32-27579.89).

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