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Direct stereoselective assay of fluoxetine and norfluoxetine enantiomers in human plasma or serum by two-dimensional gas–liquid chromatography with nitrogen–phosphorus selective detection

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Abstract

A method was developed and validated for the direct enantioselective assay of fluoxetine and norfluoxetine in human plasma or serum by two-dimensional capillary gas–liquid chromatography (GC). A Rtx-1 fused-silica capillary (15 m×0.25 mm I.D., 1.0 μm film thickness) and a hydrodex-β-6-TBDM fused-silica capillary (25 m×0.25 mm I.D., 0.25 μm film thickness) were used. A three-step liquid–liquid extraction was used for sample preparation with fluvoxamine and nisoxetine as internal standards. The method provided linear calibration between about 5 and 250 ng/ml for (*R*)- and (*S*)-fluoxetine as well as 15 and 250 ng/ml for (*R*)- and (*S*)-norfluoxetine. The limits of detection were about 1.5 and 6 ng/ml, respectively. Intra-day precision (coefficient of variation) was estimated as being between 5.4 and 12.7% at plasma levels of 25, 100 and 200 ng/ml for the four enantiomers. Inter-day precision was between 5.3 and 9.1% at 100 ng/ml. The enantioselective separation of some racemic psychopharmaceuticals was tested with various cyclodextrin GC-capillaries. Advantages and disadvantages of direct enantioselective GC are discussed for the assay of racemic psychopharmaceuticals. Samples from a patient who was treated with racemic fluoxetine were measured. In agreement with literature, plasma levels of the (*R*)-enantiomers of fluoxetine and norfluoxetine were considerably decreased in comparison to the (*S*)-enantiomers.

Keywords: Enantiomer separation; Fluoxetine; Norfluoxetine

1. Introduction

Fluoxetine is an antidepressant drug marketed under the trade name Prozac™ in the USA since 1988. It is now the most widely prescribed antidepressant in the USA and it rapidly reached a considerable portion of the psychotropic drug prescriptions in several other countries. Fluoxetine was the first substance of a new class of psychotropic drugs, the selective serotonin reuptake inhibitors. A more favorable spectrum of adverse drug effects and less acute toxicity is regarded as advantage of fluoxetine in contrast to the so-called tricyclic and tetracyclic antidepressants [1,2]. The assay of plasma levels of the classic antidepressant drugs was introduced into clinical practice (therapeutic drug monitoring). Relationships between plasma levels and clinical variables were investigated since about 1970. Thus, the relationship between plasma levels of fluoxetine and therapeutic effect was also investi-

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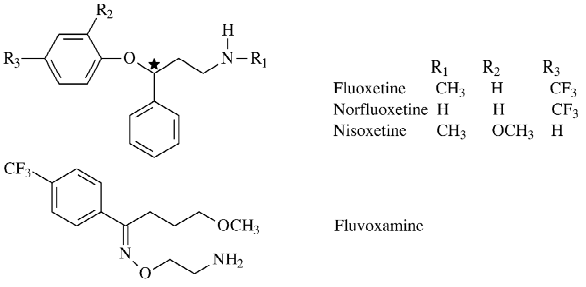
gated as an extension of basic pharmacokinetic investigations. However, no significant plasma level–therapeutic effect (or -adverse effect) relationships were found [1–4].

The primary metabolite of fluoxetine is norfluoxetine (Fig. 1) and steady-state plasma levels of this active metabolite are even higher than of the parent drug. Moreover, fluoxetine and norfluoxetine are chiral molecules with higher pharmacological activities of the (*S*)-enantiomers, i.e. the factor of relative activity is about 1.5 for fluoxetine and 20 for norfluoxetine [2]. Because fluoxetine is prescribed as a racemate, four active compounds occur in patients and, therefore, these four compounds must be measured for the investigation of the plasma level–therapeutic effect relationship. However, as a fundamental criticism, enantioselective assays were not used in the relevant studies [3].

In extension of achiral methods [3], several enantioselective methods were described for the assay of (*S*)- and (*R*)-fluoxetine as well as (*S*)- and (*R*)-norfluoxetine in serum or plasma. Thus, derivatization with (*S*)-trifluoroacetylprolyl chloride and separation of the resulting diastereoisomers with achiral capillary gas–liquid chromatography (GC)–electron-capture detection [5] (or -mass spectrometry [6]) were applied. This indirect approach was also used in HPLC methods, i.e. derivatization of the extracts with (*R*)-1-(1-naphthyl)ethyl isocyanate and separation by achiral normal-phase HPLC with fluorescence detection [7,8]. A direct separation of the enantiomers of fluoxetine and norfluoxetine, i.e. the application of chiral stationary phases, was described recently using a chiral β-cyclodextrin column in HPLC [9].



	R_1	R_2	R_3
Fluoxetine	CH_3	H	CF_3
Norfluoxetine	H	H	CF_3
Nisoxetine	CH_3	OCH_3	H

Fluvoxamine

Fig. 1. Chemical structures of the analytes and internal standards. The asterisk indicates the chiral center.

It was the aim of this study to investigate the potential of direct chiral GC methods for the enantioselective assay of some chiral psychotropic drugs in serum or plasma. Fluoxetine was chosen as a model compound because of its lower retention in comparison to other psychotropic drugs. Thus, moderate temperatures are sufficient for the separation which is important because chiral phases in GC have a limited maximal temperature. GC with nitrogen–phosphorus selective detection and without derivatization should be used as described previously in an achiral method [10]. Moreover, the GC separation of enantiomers of other chiral psychotropic drugs was investigated using various chiral phases.

2. Experimental

2.1. Standard solutions and solvents

Fluoxetine hydrochloride racemate (>98%), norfluoxetine hydrochloride racemate (>97%) and nisoxetine hydrochloride racemate (>98%) were purchased from Sigma (Deisenhofen, Germany). Fluvoxamine maleate (>99.5%) was kindly donated by Solvay-Duphar (Weesp, Netherlands). (*S*)- and (*R*)-fluoxetine as well as (*S*)- and (*R*)-norfluoxetine (enantiomeric ratio >98:2) were kindly donated by Chin B. Eap and Pierre Baumann (Unite de Biochimie et Psychopharmacologie Clinique, Hopital de Cery, Prilly-Lausanne, Switzerland). The organic solvents *n*-hexane, 2-propanol, ethyl acetate and toluene as well as NaCl, NaOH, HCl, acetic acid anhydride and dichlorodimethylsilane were from Merck (Darmstadt, Germany). The chemicals were of analytical or HPLC grade, except for dichlorodimethylsilane which was for synthesis; 1 *M* aqueous NaOH with 6% NaCl was prepared by dissolution of 20 g NaOH and 30 g NaCl in 500 ml of water; 0.1 *M* aqueous HCl was prepared by diluting 4.2 ml of concentrated HCl in 500 ml water. The extraction solvent was prepared by mixing 9 vol. of *n*-hexane with 1 vol. of 2-propanol. For the silanization of glassware, a solution of 5% dichlorodimethylsilane in toluene was used. Stock solutions of 100 μg/ml (base) were prepared for fluoxetine racemate, norfluoxetine racemate, nisoxetine racemate and fluvoxamine in water and stored at 4 °C. Volumes of 200 μl

each of nisoxetine and fluvoxamine stock solutions were added to 4.6 ml of water for the preparation of the internal standard solution daily (4 μg/ml each). Portions of fluoxetine racemate and norfluoxetine racemate stock solutions were diluted with water daily for the preparation of solutions which were used for spiking of plasma, for example, 500 μl each added to 4.0 ml of water (5 μg/ml of each of the four enantiomers).

2.2. Glassware

Glassware was silanized after every 5th extraction. Glassware was soaked in 5% dichlorodimethylsilane in toluene for 3 h, rinsed with methanol, soaked in methanol for another 3 h and dried in an oven at 80 °C. A special cleaning procedure, including 30 min sonification in 0.001 *M* HCl, was applied after the extraction to remove basic drugs, i.e. for the prevention of carry-over.

2.3. Extraction procedure

A volume of 100 μl of standard solution was added to plasma (2 ml) in a 10-ml glass tube. A volume of 0.5 ml aqueous NaOH (with 6% NaCl) and a volume of 4 ml of *n*-hexane–2-propanol (9:1, v/v) were added. The first extraction step was carried out by 30 min shaking with an overhead-rotary shaker. After 5 min centrifugation at 3000 min^{-1}, 3.0 ml of the organic layer was transferred to 1.25 ml of 0.1 *M* HCl in another 10-ml glass tube and shaken for 30 min. After 2 min of centrifugation at 3000 min^{-1} the organic layer was discarded. A volume of 1 ml of *n*-hexane–2-propanol (9:1, v/v) was added, the two phases were vortex-mixed for 30 s and separated again by 2 min centrifugation. A volume of 1.0 ml was taken away carefully from the lower phase (0.1 *M* HCl) and placed in a 4-ml glass tube. A volume of 150 μl of aqueous NaOH (with 6% NaCl) and a volume of 100 μl of *n*-hexane–2-propanol (9:1, v/v) were added and vortex-mixed for a time of 30 s. After 5 min centrifugation at 3000 min^{-1}, as much as possible of the organic layer (a volume of about 80 μl) was separated to a tapered 4-ml glass tube. The solution was evaporated to dryness for 5 min in a vacuum-evaporator and reconstituted in 20 μl of *n*-hexane–2-propanol (9:1, v/v). This final solution is evaporated at a temperature of about 35–40 °C to a volume of about 5–10 μl and 3 μl are injected into the GC. Tests for derivatization were carried out with ethyl acetate–acetic acid anhydride (9:1, v/v) at a temperature of 65 °C and for a time of 30 min.

2.4. Apparatus

A Hewlett-Packard 5890 series II gas chromatograph from Agilent (Waldbronn, Germany) equipped with a nitrogen–phosphorus selective detector and a split-splitless injector was used for the analysis. According to the main procedure, separation was obtained with a Rtx-1 fused-silica capillary (first capillary) 15 m×0.25 mm I.D., 1.0 μm film thickness (100% dimethyl polysiloxane) from Restek (Sulzbach, Germany) which was connected to a hydrodex-β-6-TBDM fused-silica capillary 25 m× 0.25 mm I.D., 0.25 μm film thickness (50% heptakis-(2,3-di-*O*-methyl-6-*O*-*tert*.-butyldimethylsilyl)-β-cyclodextrin in 14% cyanopropylphenyl-86% dimethylpolysiloxane (OV 1701)) from Macherey-Nagel (Düren, Germany). Hydrogen (1.45 ml/min at 170 °C and 1.20 ml/min at 201 °C, 140 kPa) was used as carrier gas. Septum vent flow was 0.2 ml/min. Flow-rates of the detector gases were for air 120 ml/min, for hydrogen 1.3 ml/min and for the auxiliary gas helium 13 ml/min. The injector was operated at 230 °C in the split-splitless mode. The split (30 ml/min) was opened 0.1 min after injection. A temperature program was used for the oven (T_1= 170 °C for 7 min, T_2=201 °C, ramp=1 °C/min (sum 38 min)). The detector was maintained at a temperature of 300 °C. The nitrogen–phosphorus selective detector was operated at a baseline of about 50 pA. HP GC ChemStation software from Agilent (Waldbronn, Germany) was used for the construction and analysis of chromatograms.

2.5. Validation of the method

Calibration curves were constructed by plotting the peak-area ratios of fluoxetine versus nisoxetine and norfluoxetine versus fluvoxamine obtained from blank plasma which was spiked with the above-mentioned reference solutions of fluoxetine and norfluoxetine racemates. Concentrations of the four

enantiomers of 25, 50, 100, 125, 150, 200, 225 and 250 ng/ml were used. Intra-day precision at 25, 100 and 200 ng/ml as well as inter-day precision at 100 ng/ml were estimated by the coefficient of variation of repeated measurements ($n=8$). Accuracy was estimated by the mean plasma levels of each of the four enantiomers measured in samples ($n=8$) relative to the known concentration of 100 ng/ml added. Recovery was calculated from the ratio of peak areas of the four analytes in extracts and in solutions without extraction. Several psychotropic drugs were tested for interferences with the analytes in the chromatogram. The chiral resolution R_S and the chiral separation factor α were calculated according to Eqs. (1) and (2).

$$R_S = 1.177\frac{t_{r2} - t_{r1}}{w_{h2} - w_{h1}} \quad (1)$$

$$\alpha = \frac{t_{r2} - t_0}{t_{r1} - t_0} \quad (2)$$

where $t_{r2,1}$ are the retention times of peaks 1 and 2; t_0, the time for mobile phase to pass the capillary; and $w_{h1,2}$, the peak widths at half height of peaks 1 and 2.

2.6. Test of other enantioselective capillaries

The following capillaries were tested: lipodex C (heptakis-(2,3,6-tri-*O*-*n*-pentyl)-β-cyclodextrin); lipodex D (heptakis-(2,6-di-*O*-*n*-pentyl-3-*O*-acetyl)-β-cyclodextrin); lipodex E (octakis-(2,6-di-*O*-*n*-pentyl-3-*O*-butyryl)-γ-cyclodextrin); lipodex G (octakis-(2,3-di-*O*-*n*-pentyl-6-*O*-methyl)-γ-cyclodextrin); hydrodex-β-PM (10% heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin in 14% cyanopropylphenyl–86% dimethylpolysiloxane (OV 1701)) and hydrodex-β-3P (50% heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin in 14% cyanopropylphenyl–86% dimethylpolysiloxane (OV 1701)) (in each case 25 m×0.25 mm I.D., 0.25 μm film thickness from Macherey-Nagel (Düren, Germany)) as well as betadex 325 (25% heptakis-(2,3-di-*O*-methyl-6-*O*-*tert*.-butyldimethylsilyl)-β-cyclodextrin in 20% diphenyl–80% dimethylpolysiloxane (SPB-20)) and gammadex 325 (25% octakis-(2,3-di-*O*-methyl-6-*O*-*tert*.-butyldimethylsilyl)-γ-cyclodextrin in 20% diphenyl–80% dimethylpolysiloxane (SPB-20)) (in each case 30 m×0.25 mm I.D., 0.25 μm film thickness from Supelco (Taufkirchen, Germany)). The parameters of the GC-apparatus were the same as for the hydrodex-β-6-TBDM-capillary except for the two 30-m capillaries with slightly increased pressure of carrier gas. In addition to fluoxetine and norfluoxetine, the chiral psychopharmaceuticals and metabolites citalopram, desmethylcitalopram, nisoxetine, trimipramine, desmethyltrimipramine, mianserin and E-10-hydroxyamitriptyline were tested (40 ng each in 2 μl of solvent) with a slightly modified temperature program ($T_1=200$ °C, $T_2=230$ °C, ramp = 1 °C/min) and inlet pressure (100 kPa). Fluoxetine and citalopram were also tested at lower temperatures and with 10 m of the hydrodex-β-6-TBDM-capillary.

3. Results

3.1. Separation of fluoxetine and norfluoxetine

The hydrodex-β-6-TBDM capillary provided almost baseline separation for the enantiomers of fluoxetine and norfluoxetine (temperature program $T_1=170$ °C, $T_2=200$ °C, ramp = 1 °C/min, carrier gas hydrogen 1.1 ml/min at 170 °C (90 kPa), other parameters as described above, Fig. 2). The chiral separation factors were $\alpha=1.024$ for fluoxetine and $\alpha=1.034$ for norfluoxetine with chiral resolutions $R_S=1.806$ and $R_S=2.197$, respectively. The chiral

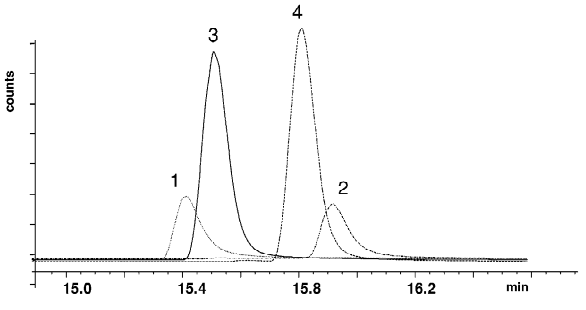


Fig. 2. Chiral separation of fluoxetine and norfluoxetine enantiomers (30 ng each) with hydrodex-β-6-TBDM capillary (25 m× 0.25 mm I.D., 0.25 mm film thickness), hydrogen as carrier gas; flow: 1.1 ml/min; oven: $T_1=170$ °C, $T_2=200$ °C, ramp = 1 °C/min); 1, (*S*)-norfluoxetine; 2, (*R*)-norfluoxetine; 3, (*S*)-fluoxetine; 4, (*R*)-fluoxetine.

resolution of fluoxetine was only slightly increased (12%) and decreased (7%) with decreased (0.4 ml/min) and increased (2.0 ml/min) carrier gas flow, respectively. A temperature program $T_1 = 160$ °C, $T_2 = 200$ °C, ramp = 1 °C/min increased the chiral resolution of fluoxetine (38%). The influence of carrier gas flow and temperature program was lower for the separation of norfluoxetine enantiomers. In general, peak areas of fluoxetine were considerably increased in comparison with norfluoxetine. No enantiomer separation was found at higher temperatures. For example, the fluoxetine peak appeared with only a shoulder at a retention time of $t_r = 6.53$ min ($T_1 = 200$ °C, $T_2 = 230$ °C, ramp = 1 °C/min). However, no separation of parent drug and metabolite was achieved with the hydrodex-β-6-TBDM capillary. The Rtx-1 capillary presented sufficient separation of parent drug and metabolite within 9 min at a temperature of 170 °C ($R_S = 3.47$). Alternative capillaries were tested, i.e. DB-17 30 m × 0.25 mm I.D., 0.25 μm film thickness (phenylmethyl polysiloxane, 50% phenyl) from J&W Scientific (Folsom, USA) with $R_S = 2.04$ and Rtx-200 30 m × 0.25 mm I.D., 0.5 μm film thickness (trifluoropropylmethyl polysiloxane) from Restek (Sulzbach, Germany) with $R_S = 3.20$. The retention times were increased and retention of the two analytes was inversed in comparison with the Rtx-1 capillary. Therefore, a combination of the Rtx-1 and hydrodex-β-6-TBDM capillaries was used for the simultaneous analysis of the four analytes in one chromatographic run and without derivatization. A heart-cut connection of the two capillaries was regarded as unnecessary because a very pure extract was obtained in the three-step sample preparation (see above). No major impurity will reach the enantioselective capillary in great excess and impair the separation of target analytes. However, the separation of enantiomers was worsened in this two-dimensional GC in comparison to the one-dimensional approach with $\alpha = 1.008$ for fluoxetine and $\alpha = 1.015$ for norfluoxetine and with chiral resolutions $R_S = 0.976$ and $R_S = 1.342$, respectively (Fig. 3). Moreover, the peaks of norfluoxetine enantiomers eluted with a tailing ($t_r = 24.68$ min for (*S*)-norfluoxetine and $t_r = 25.02$ min for (*R*)-norfluoxetine) in comparison to only a very slight tailing for the hydrodex-β-6-TBDM capillary alone. Fluoxetine enantiomers eluted without tailing neither with the hydrodex-β-6-TBDM capillary alone nor in the combination with the Rtx-1 capillary ($t_r = 25.39$ min for (*S*)-fluoxetine and $t_r = 25.60$ min for (*R*)-fluoxetine). Nevertheless, no interferences

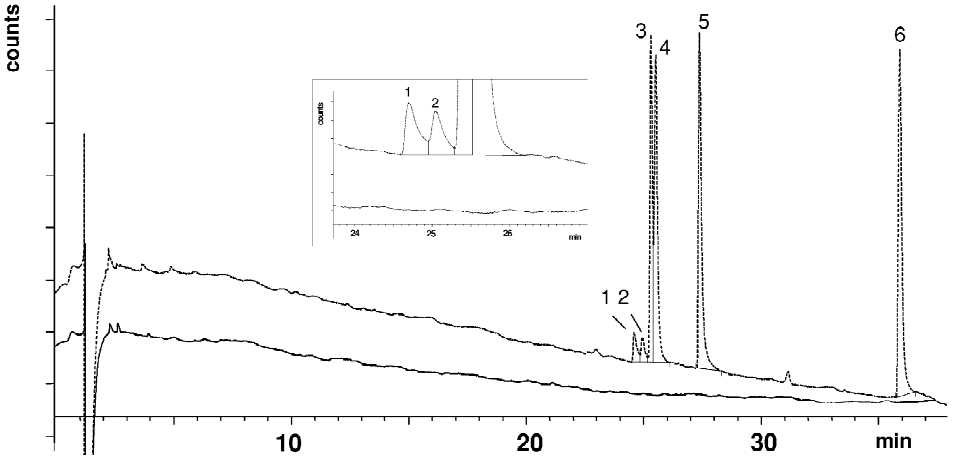


Fig. 3. Two-dimensional gas chromatograms of extracts of drug-free plasma and of plasma spiked with 50 ng/ml each of (*S*)- and (*R*)-fluoxetine as well as (*S*)- and (*R*)-norfluoxetine, Rtx-1 (first capillary) 15 m × 0.25 mm I.D., 1.0 μm film thickness connected to hydrodex-β-6-TBDM (second) capillary 25 m × 0.25 mm I.D., 0.25 μm film thickness; carrier gas: hydrogen (1.45 ml/min at 170 °C and 1.20 ml/min at 201 °C, 140 kPa); temperature program: $T_1 = 170$ °C for 7 min, $T_2 = 201$ °C, ramp = 1 °C/min, nitrogen–phosphorus selective detection; 1, (*S*)-norfluoxetine; 2, (*R*)-norfluoxetine; 3, (*S*)-fluoxetine; 4, (*R*)-fluoxetine; 5, fluvoxamine (internal standard); 6, nisoxetine (internal standard).

Table 1
Calibration parameters for the assay of fluoxetine and norfluoxetine enantiomers in plasma by direct chiral two-dimensional gas–liquid chromatography

Analyte	*A*	*B*	*r*	s_a	s_b
(*S*)-Fluoxetine	−0.1135	0.01	0.995	0.0627	0.0004
(*R*)-Fluoxetine	−0.0184	0.01	0.994	0.0687	0.0004
(*S*)-Norfluoxetine	−0.0489	0.0024	0.995	0.0151	0.0001
(*R*)-Norfluoxetine	−0.0269	0.0020	0.995	0.0122	0.0001

A, intercept; *B*, slope; *r*, regression coefficient; s_a, standard deviation of intercept; s_b, standard deviation of slope.

were found with endogenous substances of plasma or with impurities of the chemicals used. Extracts of spiked plasma with 50 ng/ml each of the four analytes provided sufficient peaks and chromatograms for a quantitative analysis (Fig. 3). Therefore, a method validation was carried out to investigate the potential of this new approach in more detail.

Derivatization with acetic acid anhydride provided a decreased separation for norfluoxetine enantiomers ($\alpha = 1.009$, $R_S = 0.973$) and no separation for fluoxetine enantiomers. The retention times considerably increased and higher temperatures were needed. In contrast to no derivatization, peak areas were similar for parent drug and metabolite.

3.2. Method validation

Results of least-squares linear regression according to the equation $y = A + Bx$ with A = intercept, B = slope, y = peak-area ratio of analyte and internal standard and x = plasma level of analyte in ng/ml are summarized in Table 1. Linear calibration curves were found, however, the calibration curve crossed the origin only for (*R*)-fluoxetine, i.e. the standard deviation of intercept s_a did not include the origin for the other three analytes. Results for precision and accuracy are shown in Table 2. The results of calibration and precision of norfluoxetine were poor if the peak area was calculated as the ratio of the peak area of nisoxetine (not shown).

Recoveries were calculated as 8.2, 8.7, 6.6 and 4.7% for (*S*)- and (*R*)-fluoxetine as well as (*S*)- and (*R*)-norfluoxetine, respectively (extracts of 25–125 ng/ml). No difference was found for different plasma levels. The maximum recovery that can be achieved is only 16.8% when taking into account the loss of volume during sample preparation. Thus, the extraction yield is calculated as being about 50% for (*S*)- and (*R*)-fluoxetine as well as being about 34% for (*S*)- and (*R*)-norfluoxetine. The limit of detection was estimated from chromatograms of spiked plasma (25 ng/ml) and with a ratio signal-to-noise of 3. The limits of detection were about 1.5 ng/ml for (*S*)- and (*R*)-fluoxetine as well as 6 ng/ml for (*S*)- and (*R*)-norfluoxetine.

Tricyclic and tetracyclic antidepressant drugs (for example amitriptyline, imipramine and maprotiline), the tricyclic phenothiazine antipsychotic drugs (for

Table 2
Precision and accuracy for the assay of fluoxetine and norfluoxetine enantiomers in plasma by direct chiral two-dimensional gas–liquid chromatography

Analyte	Within-day precision			Between-day precision		Accuracy (%)
	Plasma level (ng/ml)			Plasma level (ng/ml)		
	25 CV (%)	100 CV (%)	200 CV (%)	100		
				Found (ng/ml)	CV (%)	
(*S*)-Fluoxetine	5.4	8.1	6.8	105.0±6.4	6.1	105
(*R*)-Fluoxetine	5.8	8.4	7.8	105.2±5.6	5.3	105
(*S*)-Norfluoxetine	6.1	7.4	9.8	104.9±6.5	6.2	105
(*R*)-Norfluoxetine	11.2	6.8	12.7	100.8±9.2	9.1	101

CV, coefficient of variation.

example chlorpromazine and promethazine) and clozapine did not interfere with the analytes or internal standards. The retention times were considerably higher than 38 min. This also applied to benzodiazepines (for example alprazolam, diazepam and nordiazepam), zolpidem, venlafaxine, sertraline, paroxetine and biperidene. Other antipsychotic drugs such as haloperidol, fluphenazine and perphenazine are known to have higher retention times than the tricyclics. A test was regarded as unnecessary. Chlormethiazole had a retention time of 3.8 min. Melperone had a retention time of 27.5 min, i.e. close to fluvoxamine which was used as an internal standard. However, in fact, melperone did not impair the analysis of fluvoxamine although baseline separation was not found (Fig. 4).

3.3. Enantiomer stability

The pure enantiomers of fluoxetine and norfluoxetine did not give signals of their misnomers in the analysis of test solutions (3 μl of 20 ng/μl). Extracts which were obtained from spiked samples of the pure enantiomers did not yield peaks of the corresponding enantiomer in the chromatograms, too. Aqueous solutions of the pure enantiomers were stable for about 1 year at a temperature of 4 °C.

3.4. Plasma levels of fluoxetine and norfluoxetine enantiomers in a patient

The course of trough plasma levels of (*S*)- and (*R*)-fluoxetine as well as (*S*)- and (*R*)-norfluoxetine is shown for a patient who was treated with racemic fluoxetine (Fig. 5). The highest plasma levels were consistently found for (*S*)-fluoxetine, e.g. 218.4 ng/ml at a dose of 40 mg/day after about 5 weeks of treatment. Lower plasma levels occurred for the other three analytes, e.g. 61.3, 39.1 and 41.7 ng/ml for (*S*)-norfluoxetine, (*R*)-norfluoxetine and (*R*)-fluoxetine, respectively. The enantiomer ratio (*R*)-enantiomer versus (*S*)-enantiomer was considerably lower for fluoxetine than for norfluoxetine, i.e. 0.19 and 0.64, respectively.

3.5. Separation with other enantioselective capillaries

The lipodex capillaries did not separate the enantiomers of test racemates. Moreover, no peaks were found for the analytes containing N–H-bonds, i.e. fluoxetine, norfluoxetine, desmethylcitalopram, nisoxetine and desmethyltrimipramine. The hydrodex-β-3P provided a little chiral separation for mianserin with $R_S = 0.509$ and $\alpha = 1.006$. No chiral

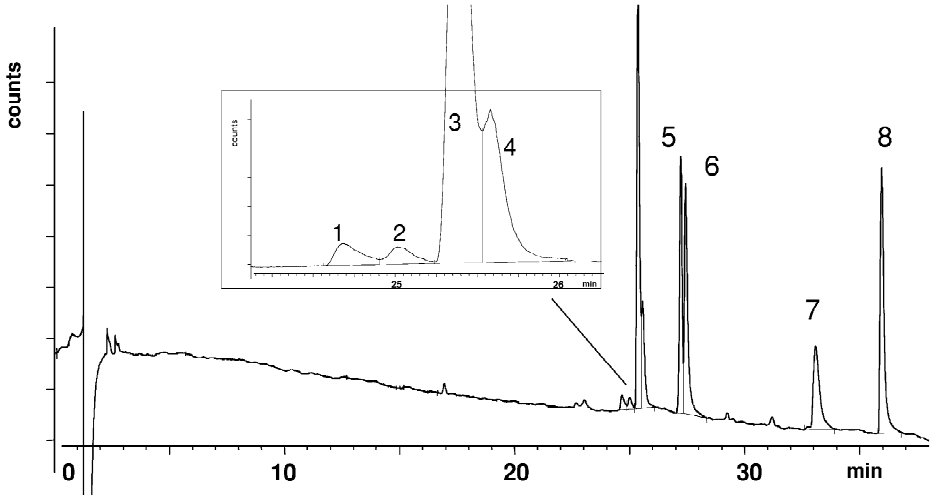


Fig. 4. Two-dimensional gas chromatogram for plasma of a patient who was treated with 20 mg/day of racemic fluoxetine, chromatographic parameters as described in Fig. 3; 1, (*S*)-norfluoxetine (44.4 ng/ml); 2, (*R*)-norfluoxetine (27.7 ng/ml); 3, (*S*)-fluoxetine (132.9 ng/ml); 4, (*R*)-fluoxetine (29.6 ng/ml); 5, melperone (concurrent medication); 6, fluvoxamine (internal standard); 7, unknown peak (probably concurrent medication); 8, nisoxetine (internal standard).

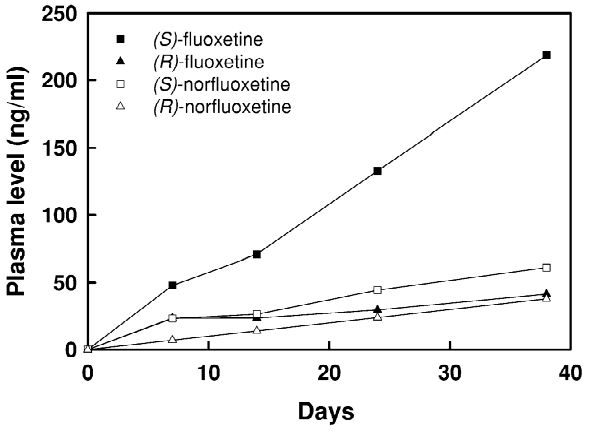


Fig. 5. Course of plasma levels of fluoxetine and norfluoxetine enantiomers in a female patient (62 years of age, body weight 85 kg, body height 168 cm, nonsmoker) with diagnosis of paranoid-hallucinatory psychosis and depression, dose of 20 mg/day of racemic fluoxetine (after 7, 14 and 24 days), dose of 40 mg/day (after 38 days), concurrent medication: clozapine, lorazepam, melperone, levothyroxine, amlodipine.

separation was found for the other racemates and even no peaks appeared for norfluoxetine, desmethylcitalopram and nisoxetine. The hydrodex-β-PM provided some chiral separation for norfluoxetine ($R_S = 0.634$, $\alpha = 1.010$), a very poor chiral separation for fluoxetine ($R_S = 0.300$, $\alpha = 1.004$) and no chiral separation for the other racemates (desmethylcitalopram no peak). No chiral separation was found for both the betadex 325 and the gammadex 325 with even no peaks for desmethylcitalopram and desmethyltrimipramine. Chiral separation was found with the hydrodex-β-6-TBDM-capillary for mianserin ($R_S = 0.957$, $\alpha = 1.013$) and E-10-hydroxy-amitriptyline ($R_S = 0.866$, $\alpha = 1.010$), however, there was no chiral separation for citalopram, desmethylcitalopram, nisoxetine, trimipramine and desmethyltrimipramine. With 10 m of the hydrodex-β-6-TBDM capillary and at a temperature of 130 °C (ramp 1 °C/min to 160 °C), the chiral resolution R_S improved (61%) for fluoxetine. An improvement of R_S (28%) was also found for citalopram at a temperature of 150 °C (ramp 1 °C/min to 180 °C). No peaks appeared at lower temperatures for both analytes.

4. Discussion

Capillary GC with an improved cyclodextrin phase (hydrodex-β-6-TBDM) is a new approach for the direct chiral separation of fluoxetine and norfluoxetine enantiomers. The parameters of the method were optimized or a compromise was found, for example, in the case of carrier gas flow and temperature program between resolution and time of separation. A diminution of chiral separation is inherent for the two-dimensional approach because the zone of analyte-dispersion is increased after passing the first capillary in contrast to the normal injection of analyte to the second capillary, i.e. the capillary with chiral stationary phase. An improvement may be possible, for example, by heart-cutting the fluoxetine and norfluoxetine peaks and separately trapping them in a zone of decreased temperature prior to analysis in the capillary with chiral stationary phase, i.e. complicated technical equipment is needed. Alternatively, a 50-m hydrodex-β-TBDM-capillary should be tried for the separation of the four analytes with one capillary. Nevertheless, with the simple connection of two capillaries, a sensitive and specific method, which includes a three-step liquid–liquid extraction as sample preparation, was developed and validated for the assay of fluoxetine and norfluoxetine enantiomers in human plasma or serum.

Although the recovery of internal standards was not investigated, nisoxetine appears to be an ideal internal standard for fluoxetine because of the very similar chemical structure (Fig. 1). The importance of the NH_2-group of norfluoxetine for the extraction and GC is expressed by the better calibration and precision if norfluoxetine peak areas are calculated as the ratio of the peak area of fluvoxamine which also contains a NH_2-group. Despite the fact that nisoxetine has a chemical structure more similar to norfluoxetine, it was not a good internal standard for norfluoxetine. Nevertheless, another lipophile and basic substance with a molecular mass of about 250 g/mol and a NH_2-moiety may be better suited as internal standard for norfluoxetine because fluvoxamine is an antidepressant drug on its own and it may be present in the plasma of patients.

Precision and accuracy of the method were shown to meet the requirements of therapeutic drug moni-

toring and of clinical studies which investigate the plasma level–therapeutic effect relationship of fluoxetine. The analysis of the enantiomers of parent drug and metabolite would be a principal advantage in comparison to previous clinical studies of this type. The method is well suited for the investigation of fluoxetine enantiomers in single-dose pharmacokinetics because of the detection limit of 1.5 ng/ml and because a maximum racemic plasma level of 15–55 ng/ml was described after a time of 6–8 h (dose 30 mg) in healthy volunteers with a half-life of 1–4 days [11]. Single-dose pharmacokinetics was investigated in experimental animals with a method having a detection limit of only 8 ng/ml [9].

However, some advantages and disadvantages are evident for the present method. Moderate temperatures have to be used because of the instability of cyclodextrin phases at high temperatures (maximum temperature 230 or 200 °C for the present capillaries) and, therefore, the time of separation is increased. For comparison, the achiral separation of psychopharmaceuticals with capillary GC is completed in our laboratory within 4–12 min at temperatures of 240–290 °C [12,13]. A baseline separation of enantiomers was not completely achieved due to a slight tailing of peaks. Accordingly, negative intercepts were found in the calibrations of the analytes except for (*R*)-fluoxetine. This will provide an increased error of quantification if one enantiomer occurs at considerably lower concentrations than its misnomer. This was not taken into account in the validation. A principal disadvantage of the chiral phases investigated is the poor separation of drug and metabolite. This was found not only for fluoxetine and norfluoxetine but also for citalopram and desmethylcitalopram as well as for trimipramine and desmethyltrimipramine. Thus, in the present work, a two-dimensional approach was used for fluoxetine at a cost of a deterioration of chiral separation. Both chiral and achiral separation of fluoxetine and norfluoxetine enantiomers were slightly better with the indirect GC and HPLC methods [6–8]. As an additional advantage of the indirect GC method [6], the sensitivity of norfluoxetine is similar to fluoxetine. In contrast, due to the NH_2 group of norfluoxetine, a restriction occurs in the direct chiral GC (without derivatization) and peak areas are considerably lower than for fluoxetine. This was also described for an achiral method without derivatization [10]. On the other hand, perhaps as the most important disadvantage, a cancerogenic reagent was used for derivatization in the indirect GC method [6]. It appears as an advantage of the direct capillary GC method in comparison with the HPLC methods that chromatograms of blank plasma were completely devoid of interfering peaks. This important advantage of a good baseline of extracts of blank sample should not be underestimated. This is obvious, for instance, if Fig. 3 is compared with chromatograms shown for the direct chiral HPLC method [9]. In agreement, the indirect HPLC method was not linear over the entire range of calibration and an interfering peak appeared at least with (*S*)-fluoxetine [7]. Finally, the sensitivity for fluoxetine enantiomers of the direct HPLC method [9] was considerably lower (limit of detection 8 ng/ml) than in the present direct GC method.

The results of the assay of the four analytes in a patient are in agreement with a recent report [14]. The plasma levels of the (*R*)-enantiomers of fluoxetine and norfluoxetine are considerably decreased in comparison with the (*S*)-enantiomers. The low ratio of norfluoxetine versus fluoxetine for instance of the (*S*)-enantiomers may indicate a poor metabolizer status of cytochrome CYP2D6 of the present patient [14].

The investigation of separation of chiral psychopharmaceuticals and metabolites with various chiral capillaries shows that the hydrodex-β-TBDM-capillary provides the best performance. The pure cyclodextrin phases (lipodex) have only a poor performance for the low volatile psychopharmaceuticals investigated in this study. Some examples of chiral separation were detected only for the more complex phases. This conclusion applies only for the moderate temperatures of 170–230 °C which were used in this study. The chiral resolution was improved in some preliminary experiments at lower temperatures of 130–150 °C and with a shorter capillary of 10 m. Thus, this approach should be investigated further in the future.

For a more general discussion, and taking into account more chiral drugs than only fluoxetine [15], such as citalopram [16], thalidomide [17],

thioridazine [18], non-steroidal anti-inflammatory drugs [19] as well as mexiletine [20] and vigabatrine [21], for example, it may be tentatively concluded that direct enantioselective GC methods are less suitable for the separation of chiral psychopharmaceuticals than direct enantioselective HPLC-methods, at least with the recently available chiral capillaries. Fluoxetine may be one exception because of its higher volatility compared with other psychopharmaceuticals. However, taking into account the success of enantioselective capillary GC in the analysis of flavors and fragrances [22–24], i.e. in the analysis of more volatile analytes, the development of improved chiral phases is expected to enable considerable progress for the enantioselective analysis of psychopharmaceuticals and metabolites by capillary GC.

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