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Stereoselective determination of venlafaxine and its three demethylated metabolites in human plasma and whole blood by liquid chromatography with electrospray tandem mass spectrometric detection and solid phase extraction

Maria Kingbäck[a], Martin Josefsson[b], Louise Karlsson[a], Johan Ahlner[a,b], Finn Bengtsson[a], Fredrik C. Kugelberg[a,b], Björn Carlsson[a,*]

[a] Department of Medical and Health Sciences, Division of Drug Research, Clinical Pharmacology, Faculty of Health Sciences, Linköping University, SE-581 85, Linköping, Sweden
[b] Department of Forensic Genetics and Forensic Toxicology, National Board of Forensic Medicine, Artillerigatan 12, SE-587 58, Linköping, Sweden

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ABSTRACT

A stereoselective method is described for simultaneous determination of the S- and R-enantiomers of venlafaxine and its three demethylated metabolites in human plasma and whole blood samples. This validated method involved LC/MS/MS with positive electrospray ionization and solid phase extraction. Chromatographic separation was performed on a 250 mm × 2.1 mm Chirobiotic V column with a total run time of 35 min. In plasma, calibration curves were in the range of 1–1000 nM for the S- and R-enantiomers of venlafaxine and O-desmethylvenlafaxine, and 0.5–500 nM for N-desmethylvenlafaxine and N,O-didesmethylvenlafaxine. In whole blood the corresponding concentrations were 10–4000 and 5–2000 nM, respectively. The intra-day precision was <6.3% and the inter-day precision was <9.9% for plasma and <15% and <19% for whole blood. LLOQ ranged between 0.25 and 0.5 nM. No ion suppression/enhancement or other matrix effects were observed. The method was successfully applied for determination of venlafaxine and its metabolites in plasma from patients and whole blood samples from forensic autopsy cases.

1. Introduction

Many of today's antidepressants are racemic drugs that consist of two or more enantiomers. Because of different pharmacological and toxicological activities it is of interest to study the individual enantiomers of chiral drugs in more detail [1–3]. This creates a need to develop stereoselective analytical methods for investigating the pharmacokinetics of the separate enantiomers of such drugs.

Venlafaxine (VEN) is used in the treatment of psychiatric disorders and it belongs to the pharmacodynamic class of dual serotonin and noradrenaline reuptake inhibitors (SNRIs). VEN is administered as a racemate, composed of the S-(+)- and R-(−) enantiomeric forms in equal amount [4]. Both of the enantiomers exhibit pharmacological activity, but interact with different signal molecules in the central nervous system. The R-enantiomer is a potent inhibitor of both serotonin and noradrenaline reuptake, while the S-enantiomer is more selective in inhibiting serotonin reuptake [4,5]. VEN is metabolised mainly by the cytochrome P-450 (CYP) system in the liver to its main metabolite O-desmethylvenlafaxine (ODV) and also to N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV) [4,6]. ODV exhibits a pharmacologic activity similar to that of VEN and its enantiomers also inhibit noradrenaline and serotonin reuptake. Compared to VEN and ODV, NDV and DDV display less potent effects on serotonin and noradrenaline reuptake [4,6,7].

Stereoselective metabolism has been observed both *in vitro* and *in vivo*, where CYP2D6 displays an appreciable stereoselectivity towards the R-enantiomer [6,8]. Since VEN is one of the most frequently prescribed antidepressant drugs worldwide, stereoselective determination of the concentrations of VEN and its metabolites is of importance for a further understanding of the mechanism of action of each enantiomer and their pharmacokinetic and pharmacodynamic properties.

The most frequently used methods for achiral quantification of VEN in biological samples are based on high performance liquid chromatography (HPLC) with ultraviolet (UV) [9–12] or fluorescence detection [11,13–16] and liquid chromatography/mass spectrometry (LC/MS) [8,17,18], although other methods, e.g. capillary electrophoresis, have also been reported [19,20]. Chiral methods using capillary electrophoresis for separation of the enantiomers of VEN an ODV have been described by Fanali et al. [20] and Rudaz et al. [19]. Wei et al. [17] have published a LC/MS method for stereoselective determination of VEN and ODV. However, only stereoselective methods involving single MS that provide analysis

* Corresponding author. Tel.: +46 13 221091; fax: +46 13 104195.
E-mail address: Bjorn.Carlsson@lio.se (B. Carlsson).

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of the enantiomeric concentrations of VEN and metabolites have been described previously [8,17,18]. For quantification of the enantiomers of VEN and its metabolites, chiral reagents have been used to make derivatives suitable for chromatography. In 1992, Wang et al. [13] reported a method involving derivatization of VEN using S-naproxen chloride as a chiral reagent for the quantification of the enantiomers. Macrocyclic antibiotics, such as vancomycin, was introduced by Armstrong et al. in 1994 [21] as a chiral stationary phase for HPLC. These have become valuable tools for the separation of a wide variety of chiral molecules. Vancomycin possesses several stereogenic centers and functional groups responsible for stereoselective interactions and a variety of racemic compounds have been resolved on it, including VEN [8,18,20]. For sample preparation of VEN and its metabolites, the most widely used technique for elimination of sample matrix effects is solid phase extraction [22–24] or liquid–liquid extraction for plasma or serum samples [9,12,15].

The relative lack of specificity and sensitivity of the HPLC methods has promoted the development of methods using MS and MS/MS for detection. The high sensitivity and specificity of LC/MS/MS has made it the method of choice for quantitative analysis of small molecules in biological specimens [23]. Non-enantioselective chromatographic methods for determination of VEN and its metabolites using LC/MS/MS instrumentation have been reported [23,25], but until now enantioselective methods using LC/MS/MS have not been described.

The aim of this study was to develop a method for stereoselective determination of VEN and its three demethylated metabolites using LC/MS/MS in human plasma and whole blood samples. The overall aim was to apply the present method to clinical and toxicological studies.

2. Experimental

2.1. Drugs and chemicals

Venlafaxine and metabolites were supplied from Wyeth-Ayerst Research (Rouses Point, VT, USA). Mexiletine was supplied from Boehinger Ingelheim (Germany). Acetonitrile, methanol and tetrahydrofurane (HPLC grade) were supplied from LabScan (Ireland). Ethanol (95%) was supplied from Apoteksbolaget AB (Sweden). Acetic acid, ammonia and formic acid (PA grade) were supplied from Merck (Germany). Triflouroacetic acid (PA grade) was obtained from Sigma Aldrich (Stockholm, Sweden). Ultrapure water was supplied from a Milli-Q station (Millipore AB, USA).

2.2. Instrumentation

The chromatographic system consisted of an Acquity LC-system (Waters, Milford, MA, USA) and a Sciex API 4000 tandem mass detector equipped with an electrospray ionisation (ESI) ion source (PE Sciex; Ontario, Canada). Chromatographic separation was performed on a Chirobiotic V column (5 μm particle size, 250 mm × 2.1 mm; Sigma–Aldrich) protected with a 5 μm in-line filter (VICI AB International, Switzerland). The column temperature was kept at 10 °C using a Jones Chromatography Model 7955 column chiller/heater (Hengoed, UK). The mobile phase used consisted of tetrahydrofuran:ammonium acetate (10 mM) pH 6.0 (10:90; v/v) with a flow rate of 0.2 ml/min. A solution composed of 0.05% formic acid in acetonitrile, delivered by a Gynkotek 480 pump (Dionex; Sunnyvale, CA, USA), was added post-column at a flow rate of 0.2 ml/min. Data acquisition and peak integration, recording the area of the peaks, were performed using Analyst 1.4 software (PE Sciex; Ontario, Canada).

2.3. Standards and quality control samples

Appropriate amounts of VEN and its metabolites were dissolved in ethanol to prepare stock solutions (1 mg/ml pure base). Stock solutions were further diluted in ethanol to obtain working solutions (200 μg/ml). Standard solutions were prepared by diluting the working solutions in human drug-free plasma. Seven different plasma standards were prepared with the following concentrations 1, 2.5, 10, 25, 100, 250 and 1000 nM (for each enantiomer of VEN and ODV) and 0.5, 1.25, 5, 12.5, 50, 125 and 500 nM (for each enantiomer of NDV and DDV). Plasma quality controls (QCs) were prepared with concentrations of 2 and 500 nM (for the enantiomers of VEN and ODV) and 1 and 250 nM (for the enantiomers of NDV and DDV). The internal standard used was mexiletine. For whole blood, standards were prepared with the following concentrations 10, 25, 100, 250, 1000, 2000 and 4000 nM (for each enantiomer of VEN and ODV) and 5, 12.5, 50, 125, 500, 1000 and 2000 nM (for each enantiomer of NDV and DDV). Whole blood QCs were prepared with concentrations of 15, 300 and 3000 nM (for the enantiomers of VEN and ODV) and 7.5, 150 and 1500 nM (for the enantiomers of NDV and DDV).

2.4. Solid phase extraction

Prior to extraction the plasma samples were centrifuged. The extraction was performed with Isolute C8 columns 100 mg (International Sorbent Technology, Hengoed, UK). The columns were activated with 1 ml methanol and 1 ml ultrapure water. Thereafter, a mixture of 0.2 ml of the sample and 20 μl of the internal standard mexiletine (0.05 mM) was added to the columns. A gentle underpressure was used to drive out the solution through the column. The columns were then washed with 1 ml ultrapure water, followed by 2 ml methanol:ultrapure water (50:50; v/v) and 2 ml acetonitrile. After the wash, columns were dried for 1 min. VEN and its metabolites were then eluted with 1.5 ml acetonitrile with 10 mM trifluoric acid and evaporated with nitrogen at 50 °C in a block thermostat (Grant QBT2; Grant Instruments (Cambridge) Ltd., UK). The analytes were reconstituted in 50 μl mobile phase consisted of tetrahydrofuran:10 mM ammonium acetate pH 6 (10:90; v/v) and transferred to a vial. The samples were then placed in the autosampler and 5 μl of each sample was injected onto the chiral column for analysis. For whole blood, samples were extracted according to the method described for plasma with some modifications due to the higher viscosity and the expected higher concentrations. Briefly, 20 μl of the internal standard mexiletine (0.5 mM) was added to 0.2 ml blood and diluted with 3 ml ultrapure water. After vortex mixing and sonification for 5 min, the solution was centrifuged. After conditioning of the extraction columns with methanol and ultrapure water, the centrifuged samples were poured on to the columns and thereafter extracted according to the original procedure described for plasma. After evaporation, the samples were reconstituted in 100 μl mobile phase and 2 μl of each sample was injected onto the chiral column.

2.5. LC/MS/MS optimization and settings

The analytes were ionized in electrospray positive mode at 5000 V. Nitrogen was used as nebulizer, auxiliary, curtain and collision gas and was set at 50, 70 and 30 psi and a value of 5. The auxiliary gas temperature was set at 600 °C. Multiple reaction monitoring (MRM) mode was used for quantification. The two most abundant transitions originating from product ions of the protonated molecular ions for VEN, its metabolites and the internal standard were used. General parameters for all transitions were as follows; declustering potential (DP): 50 V, entrance potential (EP): 12 V and collision cell exit potential: 20 V. The

Table 1
LC/MS/MS parameters and retention times for the enantiomers of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV), N,O-didesmethylvenlafaxine (DDV) and for the internal standard mexiletine. Relative intensities calculated with respect to the S-enantiomer of VEN (N.C. = not calculated).

Compound	Transition (Q1/Q3)	Collision energy (V)	Relative area intensity (%)		Retention time (min)	
			S-enantiomer	R-enantiomer	S-enantiomer	R-enantiomer
VEN	278/58	45	100	102	19.6	23.4
	278/260	20				
ODV	264/58	40	98.1	104	14.4	16.2
	264/246	20				
NDV	264/121	35	49.2	54.2	19.1	21.6
	264/246	20				
DDV	250/107	40	52.7	57.8	13.9	15.2
	250/232	18				
Mexiletine	180/58	22	N.C.	N.C.	N.C.	N.C.
	180/105	27	N.C.	N.C.	N.C.	N.C.

remaining parameters and the retention times are shown in Table 1.

2.6. Method validation

To evaluate linearity, calibration curves were prepared from duplicate determinations at each concentration level. Stability of calibration was evaluated by comparison of two consecutive calibrations analysed as singlets with at least 1 week in between. The inter-day variation of the method was evaluated in plasma and whole blood by determining QC samples at different concentration levels in five replicates at 5 different days determined from the same calibration. Five replicates at each concentration were used to evaluate the intra-day variation. Matrix effects were evaluated by comparing the concentrations found of known amounts of working standards with those measured in control plasma and whole blood spiked with the same amount of analytes before or after extraction [26]. Furthermore, the influence of matrix components on ESI was studied using single-state MS (Q1) scanning for phospholipids and proteins in plasma and whole blood samples (m/z 400–750 and 1200–1800, respectively). The stability of VEN and its metabolites in reconstituted samples was evaluated by analyzing QC samples exposed to different time and temperature conditions. The extraction recoveries in plasma and whole blood were determined by comparing extracted spiked blank samples with unextracted reference samples prepared at the same concentrations. Samples at two different concentration levels in five replicates were analysed. The recoveries for the method were measured as the percentage difference.

2.7. Application of method

The present method was applied to human plasma and whole blood samples. The plasma samples were obtained from patients ($n = 5$) on chronic treatment with VEN for major depressive disorder. The whole blood samples were obtained from forensic autopsy cases ($n = 5$) with four different causes of death (intoxication, hanging, drowning and road traffic fatality).

3. Results

3.1. Linearity and lower limit of quantification

For calibration, quadratic regression with inverse x ($1/x$) weighting was used. Calibration curves prepared from drug-free plasma in the range of 1–1000 nM for the enantiomers of VEN and ODV, and 0.5–500 nM for NDV and DDV showed a good correlation with a correlation coefficient of $\geq$0.999 (Fig. 1). For whole blood, calibrations curves in the range of 10–4000 nM for the enantiomers of VEN and ODV, and 5–2000 nM for NDV and DDV showed a good correlation with a correlation coefficient of $\geq$0.999. Calibrations were found to be stable for at least 1 week. When two consecutive calibrations were compared 90–110% accuracy was found for each calculated standard concentration. The resolution for the enantiomers of each compound calculated from QC (Fig. 2) was as follows: VEN 1.79, ODV 1.48, NDV 1.25 and DDV 1.13. Due to differences in signal intensity the lower limit of quantification was 0.5 nM for the enantiomers of VEN and ODV, and 0.25 nM for NDV and DDV (Table 1). The LC/MS/MS chromatograms of blank plasma and whole blood did not show any interfering peaks in the matrix.

3.2. Precision

The intra- and inter-day precision was evaluated in plasma and whole blood by analyzing five replicates of different concentrations on the same day and over 5 different days (Table 2). The intra-day precision was <6.3% and the inter-day precision was <9.9% for plasma and <15% and <19% for whole blood.

3.3. Extraction recovery, matrix effect and application of the method

The mean extraction recoveries for the enantiomers of VEN and its metabolites are shown in Table 3. The extraction recoveries in plasma and whole blood ranged between 75–110% and 68–107%, respectively.

The matrix effect was studied using the procedure described by Matuszewski et al. [26]. The S- and R-concentrations of the plasma and whole blood samples analysed were 100 nM for the enantiomers of VEN and ODV, and 50 nM for the enantiomers of NDV and DDV. Five replicates on each concentration were analysed (Table 4). The matrix effect data for VEN and its metabolites were between 100% and 108% in plasma and between 90.1% and 97.0% in whole blood, indicating no severe matrix effects (Table 4). No presence of phospholipids or proteins in the sample matrix was found by scanning (see Section 2.6).

In general higher concentrations were found in postmortem blood compared to patient plasma (Table 5). For S-VEN concentrations between 409 and 2415 nM were observed compared to plasma ranging between 42.5 and 376 nM. The S-enantiomer of the major active metabolite ODV was found in higher concentrations than S-VEN in plasma ranging from 327 to 546 nM. With the exception of blood samples 1 and 3, higher levels of S-ODV than S-VEN were also seen in the postmortem samples. In general, lower concentrations of the minor metabolites NDV and DDV were observed in both plasma and postmortem blood. Examples of chromatograms from analysis in the different matrices are displayed in Fig. 3.

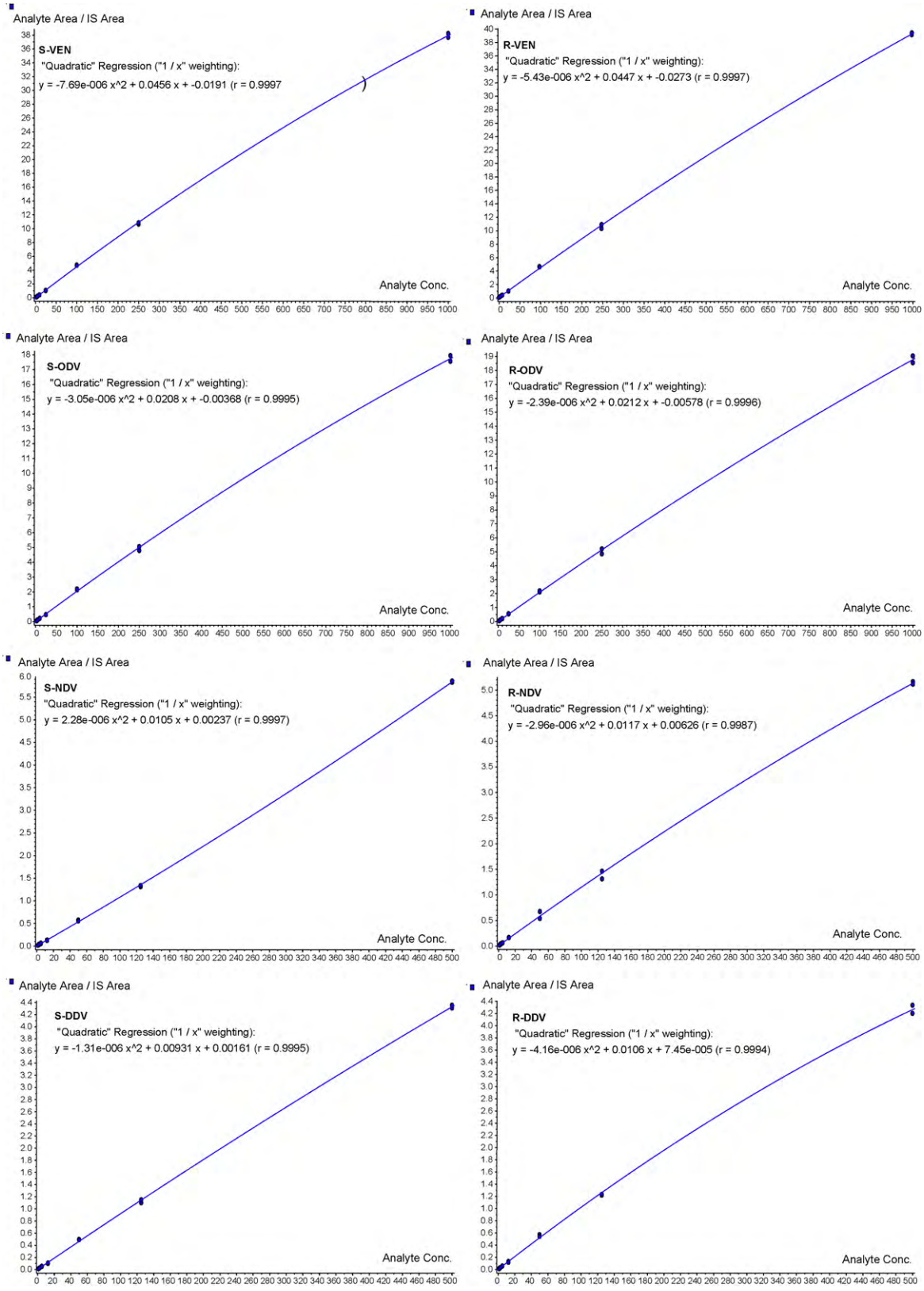

Fig. 1. Calibration curves for the enantiomers of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV) in plasma.

Table 2
The intra- and inter-day precision for the enantiomers of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV).

Compound	Plasma ($n=5$)					Whole blood ($n=5$)				
	Concentration (nM)	Intra-day precision		Inter-day precision		Concentration (nM)	Intra-day precision		Inter-day precision	
		Mean (%) ± SD	RSD	Mean (%) ± SD	RSD		Mean (%) ± SD	RSD	Mean (%) ± SD	RSD
S-VEN	2	102 ± 2.8	2.7	92.3 ± 7.2	6.6	15	104 ± 3.0	2.8	103 ± 9.4	9.2
	500	95.1 ± 4.0	4.3	91.3 ± 11	9.9	300	95.4 ± 6.9	7.2	93.0 ± 15	16
						3000	87.2 ± 10	12	79.9 ± 12	14
R-VEN	2	97.5 ± 4.8	4.9	92.0 ± 5.9	5.4	15	101 ± 3.9	3.8	99.9 ± 11	12
	500	96.3 ± 3.8	3.9	92.7 ± 10	9.5	300	95.7 ± 8.5	8.9	94.4 ± 15	16
						3000	85.2 ± 8.3	9.7	81.5 ± 13	16
S-ODV	2	101 ± 4.4	4.4	91.7 ± 5.9	5.4	15	101 ± 4.3	4.2	103 ± 1.6	1.6
	500	92.7 ± 2.3	2.5	94.4 ± 10	9.9	300	94.7 ± 11	12	93.5 ± 14	16
						3000	82.9 ± 9.4	11	81.2 ± 11	3.1
R-ODV	2	98.5 ± 6.2	6.3	94.0 ± 4.0	3.8	15	101 ± 6.8	6.7	103 ± 5.0	4.8
	500	94.5 ± 2.2	2.3	96.1 ± 8.4	8.1	300	93.8 ± 9.4	10	93.5 ± 12	13
						3000	84.5 ± 8.8	10	90.6 ± 16	14
S-NDV	1	99.8 ± 4.7	4.7	102 ± 4.9	5.0	7.5	101 ± 10	10	103 ± 12	12
	250	97.6 ± 6.1	6.2	97.1 ± 9.9	9.6	150	97.3 ± 9.0	9.3	91.6 ± 12	14
						1500	87.9 ± 8.9	10	86.4 ± 16	19
R-NDV	1	103 ± 5.1	4.9	93.4 ± 7.6	7.1	7.5	98.5 ± 9.3	9.5	104 ± 5.5	5.2
	250	96.4 ± 5.0	5.2	98.5 ± 4.4	4.3	150	99.5 ± 9.1	9.1	91.9 ± 12	14
						1500	94.9 ± 9.2	0.4	88.0 ± 11	13
S-DDV	1	102 ± 2.6	2.5	90.6 ± 7.0	6.4	7.5	96.7 ± 6.6	6.9	100 ± 7.0	7.0
	250	96.1 ± 5.4	5.6	99.4 ± 4.4	4.4	150	93.9 ± 14	15	95.4 ± 9.8	10
						1500	92.3 ± 4.2	4.6	85.2 ± 10	12
R-DDV	1	103 ± 1.8	1.7	96.0 ± 8.6	8.3	7.5	101 ± 10	10	111 ± 5.4	4.9
	250	91.5 ± 2.1	2.3	99.3 ± 3.9	3.9	150	91.0 ± 8.7	9.6	94.0 ± 3.1	3.3
						1500	85.2 ± 8.8	10	86.0 ± 9.9	12

Table 3
The extraction recovery of the enantiomers of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine in plasma and whole blood, respectively.

Compound	Concentration (nM)	Plasma ($n=5$)		Whole blood ($n=5$)	
		Mean (%) ± SD	RSD	Mean (%) ± SD	RSD
S-VEN	1	80.6 ± 3.8	4.7	86.3 ± 1.9	2.2
	1000	102 ± 4.7	4.6	104 ± 7.1	6.8
R-VEN	1	75.2 ± 8.5	11	101 ± 3.4	3.4
	1000	101 ± 7.3	7.3	107 ± 7.7	7.2
S-ODV	1	94.2 ± 2.7	2.9	91.9 ± 2.0	2.2
	1000	99.8 ± 3.6	3.6	97.3 ± 9.9	10
R-ODV	1	96.2 ± 3.3	3.4	83.8 ± 2.6	3.1
	1000	104 ± 8.9	8.5	100 ± 7.9	7.9
S-NDV	0.5	89.9 ± 5.8	6.4	87.5 ± 0.7	0.8
	500	110 ± 9.5	8.6	72.6 ± 5.0	6.9
R-NDV	0.5	87.0 ± 4.3	4.9	81.4 ± 3.4	4.2
	500	108 ± 9.1	8.4	73.2 ± 6.1	8.4
S-DDV	0.5	90.9 ± 2.5	2.8	78.1 ± 1.2	1.6
	500	105 ± 8.6	8.2	64.6 ± 7.2	11
R-DDV	0.5	96.3 ± 5.8	6.0	78.8 ± 2.9	3.7
	500	110 ± 8.2	7.5	67.9 ± 8.7	13

Table 4
Matrix effects for the enantiomers of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV).

Compound	Concentration (nM)	Plasma ($n=5$)	Whole blood ($n=5$)
		Mean (%)	Mean (%)
S-VEN	100	102	95.4
R-VEN	100	102	97.0
S-ODV	100	101	93.1
R-ODV	100	100	90.1
S-NDV	50	103	94.1
R-NDV	50	108	92.2
S-DDV	50	101	93.4
R-DDV	50	100	91.0

3.4. Post-column additive

To the mobile phase 0.05% formic acid in acetonitrile was added post-column at a flow rate of 0.2 ml/min to increase the sensitivity of the method, which increased markedly (2-, 6-, 4- and 7-fold for VEN, ODV, NDV and DDV, respectively) after this modification.

3.5. Stability

The stability of the enantiomers of VEN and its metabolites in reconstituted samples was evaluated by reanalyzing QC samples. No degradation of either compound was observed when placed in refrigerator (+4 °C) for 3 weeks or in the freezer (−20 °C) for 4 weeks. The reconstituted sample extracts placed at room temperature were stable for 1 week.

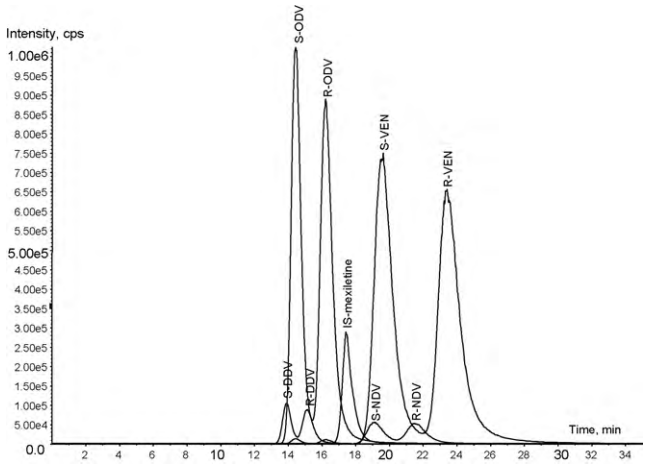

Fig. 2. Representative chromatogram of a plasma quality control sample prepared with concentrations of 500 nM for the enantiomers of venlafaxine (VEN) and O-desmethylvenlafaxine (ODV), and 250 nM for N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV). Internal standard mexiletine.

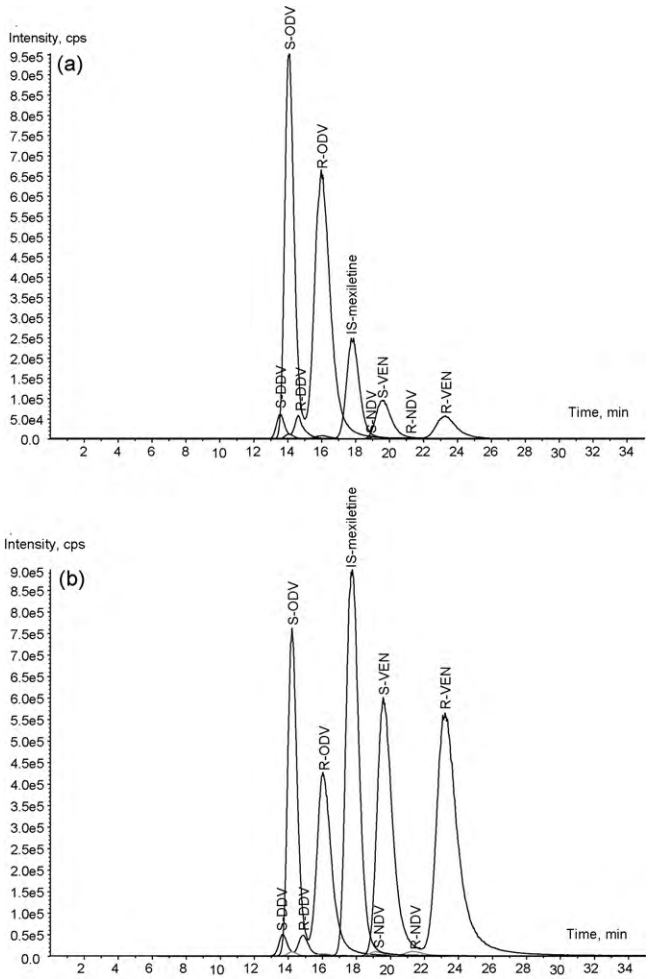

Fig. 3. Representative chromatograms from drug analyses of the enantiomers of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV) in (a) patient plasma sample nr 4 in Table 5, (b) postmortem blood sample nr 5 in Table 5.

4. Discussion

Development of stereoselective analytical methods for investigation of the metabolism and pharmacokinetics of the separate enantiomers of racemic drugs is of great importance in clinical pharmacology and toxicology [1–3]. In the present study, a sensitive stereoselective method for simultaneous analysis of the enantiomers of VEN and its three most relevant metabolites with LC/MS/MS has been developed and validated. The main metabolite ODV is known to be pharmacologically active. The method was successfully applied for the determination of the S- and R-enantiomers of VEN, ODV, NDV and DDV in human plasma and postmortem blood samples. In some cases, the chiral drug analysis revealed major differences in the enantiomeric disposition of the parent compound and its metabolites.

Initially, a method for stereoselective determination of the enantiomers of VEN and its metabolites was developed based on liquid chromatography with UV-detection (LC-UVD). The enantiomers of each compound were separated, but only a limited selectivity was achieved between the pairs. VEN and NDV, and ODV and DDV, respectively, showed overlapping peaks and could not be determined individually. To overcome this problem, the LC/MS/MS method was developed with unique transitions for determination of the individual compounds. This allowed the overlapping VEN and NDV as well as ODV and DDV to be detected separately. For each compound the same transition was used for the S- and R-enantiomer. Thanks to chromatographic separation, NDV and ODV could be determined individually even if the same transitions were used. The chiral column gave, due to limited column efficiency, limited resolution and broad peaks. The enantiomers were not completely baseline separated, but the separation was sufficient for quantification of the separate enantiomers. However, large differences in the concentrations between individual enantiomers within a pair may influence the accuracy of the quantification of each enantiomer. Inaccurate results are more likely at extreme enantiomeric concentration ratios if the resolution of the peaks is

Table 5
Determination of the concentrations of the enantiomers and the enantiomeric ratios (S/R) of venlafaxine (VEN), O-desmethylvenlafaxine (ODV), N-desmethylvenlafaxine (NDV) and N,O-didesmethylvenlafaxine (DDV) in patient plasma and post-mortem blood.

Matrix	Sample	Concentration (nM)											
		S-VEN	R-VEN	S/R-VEN	S-ODV	R-ODV	S/R-ODV	S-NDV	R-NDV	S/R NDV	S-DDV	R-DDV	S/R DDV
Patient plasma	1	96.9	22.4	4.33	546	625	0.87	5.69	0.00	-	11.5	24.6	0.47
	9	376	130	2.89	468	539	0.87	12.4	4.26	2.91	13.5	32.4	0.42
	3	42.5	42.1	1.01	352	403	0.87	29.2	20.8	1.40	136	144	0.94
	4	51.8	39.5	1.31	417	467	0.89	19.9	14.7	1.35	135	131	1.03
	5	151	113	1.34	327	332	0.98	32	25.9	1.24	111	94	1.18
Postmortem blood	1	2415	2236	1.08	1472	1896	0.78	289	273	1.06	272	265	1.03
	9	409	503	0.81	896	1071	0.84	171	136	1.26	280	281	1.00
	3	1654	2811	0.59	348	28.3	12.3	528	1148	0.46	88.8	44.6	1.99
	4	642	579	1.11	1023	1472	0.70	99.5	65.2	1.53	250	224	1.12
	5	836	1019	0.82	966	827	1.17	67.4	91.8	0.73	362	401	0.90

not complete (e.g. 9:1 or 1:9) [28]. To further improve and verify precision and accuracy it would be of value to have controls with different amount of each enantiomer [29,30]. Unfortunately as all enantiomers are not available commercially, the controls had to be made from the racemic mixtures. To achieve an acceptable separation of the analytes, the run time was about 35 min for each sample. This long analysis time meant that a limited number of samples could be processed each day and therefore the extent of validation of the method was hampered. Historical calibration was used, which was controlled by consecutively including freshly prepared QC samples within each run. The inter-day precision was satisfactory (RSD <19%) for all analytes.

Compared to the most frequently used enantioselective LC-UVD approach, LC/MS/MS exhibits a higher specificity and sensitivity [27]. For determination of VEN and metabolites with LC-UVD, Matoga et al. [9] reported a LLOQ of 181 and 361 nM for VEN and ODV, respectively, and Hicks et al. [12] reported a LLOQ for VEN and ODV of 36 nM. None of the studies included analysis of NDV or DDV. This can be compared to the present study where the LLOQs for VEN and metabolites are shown to be markedly lower, 0.5 nM for VEN and ODV, and 0.25 nM for NDV and DDV for each enantiomer. This higher sensitivity is desired for analysis in pharmacokinetic studies and therapeutic drug monitoring. The mobile phase used consisted of tetrahydrofuran and ammonium acetate buffer and the best chromatographic separation was achieved at pH 6.0. The selected pH for the mobile phase was unfavourable for electrospray ionisation, demonstrating that what is optimal for chromatography is not necessarily optimal for electrospray ionisation and mass detection. However, by adding a post-column additive consisting of formic acid in acetonitrile, the pH could be adjusted giving a more effective ionisation and ion evaporation resulting in improved detector response and increased peak area for all target analytes.

The electrospray ionisation is an important factor determining the dynamic range for quantification since saturation in the ion source is observed at higher concentrations due to competition of charges [31]. The calibration curves were linear within limited concentration range. At the high end of the curves, some saturation was observed thus a quadratic curve fit was used. Ion suppression or enhancement is a well-known phenomenon in LC/MS/MS analysis especially observed with ESI, and depends mainly on the sample matrix constitution, sample preparation procedure, quality of chromatographic separation, mobile phase additives and ionisation mode. Ion suppression or enhancement may affect LOD, LLOQ, reproducibility and accuracy of the method [31]. For the analysis of biological samples, the most widely used techniques for elimination of sample matrix components are liquid/liquid extraction and/or solid phase extraction (SPE) [31]. Reversed phase SPE was used for the pretreatment of the samples. SPE was chosen as the extraction method for the ability to concentrate the samples as well as environmental health reasons. The samples were concentrated twofold for whole blood and fourfold for plasma. The high sensitivity of the method meant small sample volumes could be used which is favourable for the SPE procedure since the columns do not clog up as easily when whole blood is analyzed. The extraction procedure resulted in clean extracts and neither extracted plasma nor whole blood samples showed matrix effects in the LC/MS/MS analysis, proven by qualitative and quantitative results. The lack of matrix effects was also evidenced by the fact that the precision of the method was high even at lower concentrations; however VEN and NDV showed a difference of around 20% in extraction recovery between low and high concentrations.

In toxicology it is desirable to have access of analytical methods that cover both therapeutic and toxic concentrations of drugs. This is also evident from the results showed in Table 5 which compares therapeutic plasma samples with postmortem blood [32]. Compared to the calibration curve used for the plasma samples a different calibration curve with a wider concentration range was used for the postmortem blood samples. In some cases it was even necessary to re-run samples if the concentrations exceeded the high end of the calibration curve. This was carried out by diluting the samples or by using a smaller sample volume for the extraction.

The concentrations of the S- and R-enantiomers of VEN and its metabolites varied in both the five plasma samples and the five whole blood samples (see Table 5). Since the enantiomers of VEN have different pharmacodynamic properties [4,5] a different enantiomeric disposition could have significant effects on the therapeutic response after treatment with racemic VEN. Hence, the results from Table 5 indicate that enantioselective analysis can give extra information when interpreting the concentrations of VEN and its metabolites. However, the observed variation in S/R ratios needs to be evaluated in more detail in a larger material of individuals and we are currently in progress with such studies.

In conclusion, the present LC/MS/MS method for enantioselective determination of the enantiomers of venlafaxine can be applied for several biomatrices over a wide concentration range. The three major metabolites of venlafaxine can be analysed within a single run, and furthermore, with the addition of post-column additive, the method has a high sensitivity. The intention is to apply the present method in the future for clinical and toxicological studies.

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