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Simultaneous stereoselective analysis of venlafaxine and *O*-desmethylvenlafaxine enantiomers in clinical samples by capillary electrophoresis using charged cyclodextrins

Serge Rudaz [a], Cinzia Stella [a], Androniki E. Balant-Gorgia [b], Salvatore Fanali [c], Jean-Luc Veuthey [a,*]

[a] *Laboratory of Pharmaceutical Analytical Chemistry, University of Geneva, 20 Bd d'Yvoy, 1211 Geneva 4, Switzerland*
[b] *Department of Psychiatry, Geneva University Hospital, Geneva, Switzerland*
[c] *Istituto di Cromatografia del C.N.R., Area della Ricerca di Roma, P.O. Box 10, 00016 Monterotondo Scalo, Roma, Italy*

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Abstract

Capillary electrophoresis (CE) was used for the simultaneous chiral determination of venlafaxine (Vx), a new antidepressant drug and its main active metabolite, *O*-desmethyl venlafaxine (ODV). Among the charged cyclodextrins (CD) tested, phosphated γ-CD was the most appropriate. Resolution of Vx and ODV was obtained with 50 mM phosphate buffer (pH 2.5) containing 20 mg/ml of phosphated γ-CD. After optimisation of the method (including robustness), validation was carried out. Vx and ODV concentrations, as well as the enantiomeric ratio, were investigated in clinical samples. Chiral determination of Vx and ODV was performed after a simple liquid–liquid extraction (LLE). In the tested concentration range (25–500 ng/ml), coefficients of correlation were superior to 0.996. Within-day and between-day accuracy and precision were determined at three different concentrations for each enantiomer. Analyses of clinical samples ($n = 16$) exhibited non-racemic ratios for Vx and ODV, which suggests a stereoselective metabolism in humans.

Keywords: Venlafaxine; Metabolites; Enantiomers; Cyclodextrins; Capillary zone electrophoresis

1. Introduction

1-2-(dimethylamino)-1-(4-methoxyphenyl)ethyl cyclohexanol hydrochloride (venlafaxine) is a second generation antidepressant which proves to be particularly effective [1]. Venlafaxine (Vx) exerts a dual mechanism of action on the monoaminergic system. It principally blocks neuronal re-uptake of noradrenaline, serotonine and, to a lesser extent, dopamine [2,3]. In humans Vx is metabolised into two minor metabolites, *N*-desmethyl and *N*,*O*-didesmethyl venlafaxine, and into a major active metabolite, *O*-desmethyl venlafaxine

* Corresponding author. Tel.: +41-22-702-6336; fax: +41-22-781-5193.
E-mail address: jean-luc.veuthey@pharm.unige.ch (J.-L. Veuthey)

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(ODV) which presents an activity profile similar to that of Vx [4–6].

As shown in Fig. 1, Vx has an asymmetric centre and both enantiomers exhibit different activities. In fact, the R (−) enantiomer inhibits both noradrenaline and serotonine re-uptake while the S (+) isomer inhibits primarily the serotonine re-uptake [7]. Furthermore, experimental data suggest that there is a difference potential in the *O*-desmethylation of the two Vx enantiomers [5]. It is important to determine the enantiomeric plasma concentrations for both Vx and its active *O*-desmethylated metabolite ODV in order to establish pharmacodynamic as well as pharmacokinetic relations and to understand the pharmacological contribution of each active enantiomer [4,5].

The methods used for venlafaxine analysis include gas chromatography with a nitrogen-phosphorus detector (GC-NDP) [8–10], high-performance liquid chromatography (HPLC) with UV [4,5,10], fluorimetric [3] and coulometric detections [11]. Enantiomeric separations of venlafaxine and/or its impurities have been carried out by HPLC [12].

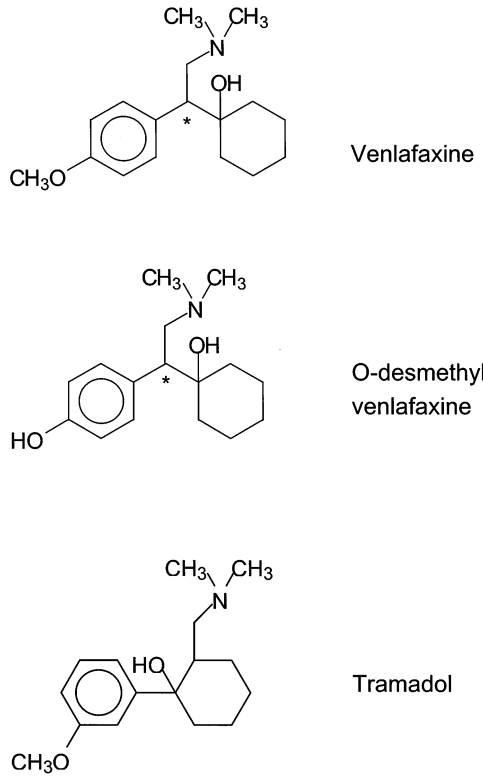

Fig. 1. Chemical structure of venlafaxine, ODV and tramadol as internal standard, showing the position of the chiral carbons.

Capillary electrophoresis (CE) is known to be a powerful separation technique in a large number of analytical fields, and particularly for the determination of drugs in biological fluids [13,14]. Compared to chromatographic techniques such as HPLC, its high-resolution power and efficiency present several advantages especially in the separation of complex drug metabolite mixtures [15,16]. Moreover, CE exhibits excellent results in the field of optical isomer separation [17–20]. Chiral discrimination by CE is generally achieved with the direct separation method where the chiral selector is simply added to the background electrolyte. Cyclodextrins and their derivatives have been predominantly used as chiral selector [21]. Due to the chemical reactivity of the hydroxy groups, native charged cyclodextrins (CD) have been modified to produce chiral selectors with properties different from those of the parent CD [22,23]. Charged and ionisable CD have been widely and successfully employed [24,25]. For positively charged compounds, negatively charged chiral selectors have already been used by several authors [26,27], often leading to higher resolution than with neutral cyclodextrins [28,29].

Recently, the enantiomeric separation of Vx by CE was achieved with neutral cyclodextrins [30,31] but to our knowledge, no separation of Vx and its active metabolite enantiomers has been carried out with charged cyclodextrins as chiral selectors in biological samples. In this paper, a phosphated γ-CD was used for the simultaneous determination of Vx and ODV enantiomers.

2. Materials and methods

2.1. Chemicals

Alpha-CD phosphate sodium salt (degree of substitution (DS) ~ 6–10), γ-CD phosphate sodium salt (DS ~ 3), γ-CD phosphate sodium

salt (DS ~ 6–10), carboxymethyl β-CD (DS = 3.5) (CMB) were purchased from Cyclolab (Budapest, Hungary). Sulfobuthylether (SBE; DS = 4.0) and sulfated β-CD was a gift from Dr P. Morin (Institute of Organic and Analytical Chemistry, Orléans University, France). Racemic Vx and racemic ODV were kindly supplied from Wyeth–Ayerst Research (Princeton, NJ, USA). (+)-Tramadol (T) was a gift from Professor P. Dayer (Department of Clinical Pharmacology, Geneva University Hospital, Geneva, Switzerland). Phosphoric acid (85% w/w) was purchased from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)-aminomethane (Tris) was purchased from Aldrich (Milwaukee, USA). Hexane, ethyl acetate and methanol (MeOH) were obtained from BDH (Poole, England) and all other reagents were of analytical or HPLC grade. Deionised water was obtained from a Milli-Q RG ultrapure water system (Millipore, Molsheim, France).

2.2. Instrumentation

A HP ^{3D}CE automatic electrophoresis apparatus (Hewlett-Packard, Waldbronn, Germany) equipped with a diode-array detector set at 195 nm was used for experiments. Electrophoretic runs were carried out in an untreated fused silica capillary of 64.5 cm (effective length 56 cm) × 75 μm I.D., 375 μm O.D., with an extended light path (bubble factor 2.7; Hewlett-Packard, Waldbronn, Germany). The applied voltage and the capillary temperature were set at 20 kV and 25°C, respectively. Sample injection was performed at the anodic end of the capillary by pressure, as following: (i) injection of the sample (50 mbar, 7.5 s); and (ii) injection of the running buffer (50 mbar, 1 s).

2.3. Procedures

On every working day, the capillary was purged with water for 10 min with 0.1 M sodium hydroxide (10 min), water (5 min) and the background electrolyte (BGE) without chiral selector for 5 min. Between runs, the capillary was treated successively with water (1 min), 0.1 M sodium hydroxide (1 min), water (1 min), BGE without chiral selector (1 min) and BGE containing the chiral selector (1 min). The BGE consisted of a 50 mM phosphoric acid solution adjusted to pH 2.5 with a concentrated Tris solution (6 M).

2.3.1. Internal standard (IS)

Stock solution of (+)-tramadol hydrochloride in water containing 10 mg/ml was weekly prepared and stored at 4°C. Further dilution of the stock solution in water was done daily to obtain a final internal standard concentration of 10 μg/ml.

2.3.2. Standard solution of Vx and ODV

Stock solutions of (+) Vx, (−) Vx, racemic Vx and racemic ODV containing 1 mg/ml of each compound were prepared in methanol and stored at 4°C. Further dilution of the stock solutions with water were daily done to obtain standard solutions.

Before use, all solutions were filtered through a 0.45-μm pore size filter (Millipore Corp., Milford, MA, USA) and degassed in an ultrasonic bath for 10 min.

Migration times, resolution (Rs) and number of theoretical plates (N) were calculated by the HP ChemStation program. Due to the differential residence times of peaks within the detector, normalised peak areas (area divided by migration time) were used for calculations.

2.4. Liquid–liquid extraction (LLE)

A total of 0.5 ml of K_2CO_3 1 M and 100 μl of a 10-μg/ml aqueous solution of T(+) were added to 1 ml of serum. The mixture was shaken for 15 min after addition of 5 ml of an hexane-ethyl acetate solution (80:20, v/v). After centrifugation (1000 rpm, 5 min), the tubes were stored in a freezer for 1 h. The organic layer was saved and 100 μl HCl 5 M in methanol was added. The organic solution was evaporated to dryness under a gentle stream of nitrogen and the dry residue was redissolved in 100 μl HCl 0.01 M. This extraction procedure was applied to both the spiked serum samples and clinical samples.

2.5. Clinical samples

Clinical samples, from 16 patients who received venlafaxine orally, were taken after at least 4 days of treatment on a constant dosage regime (Geneva University Hospital, Geneva, Switzerland). After LLE, the enantioselective determination of Vx and ODV was carried out by CE. Total Vx and ODV concentrations were obtained by adding of the respective enantiomeric concentrations.

3. Results and discussion

3.1. Method optimisation

Preliminary experiments were carried out for the enantiomeric separation of Vx and its active metabolite, ODV using a Tris-phosphate buffer (50 mM) titrated to pH 2.5. In accordance with its chemical properties (pK_a 9.4), Vx migrated towards the detector as a cation. Preliminary data showed that the use of a 75 μm i.d. capillary with an extended light path was necessary in order to detect the therapeutic concentration. In absence of chiral selector, Vx and ODV were not separated from each other.

Charged cyclodextrins were selected to find the most appropriate chiral selector for the simultaneous stereoselective separation of Vx and ODV enantiomers. Among the negatively charged selectors tested (sulfated β-CD, phosphated α-CD, SBE, CMB), phosphated γ-CDs gave the best results for ODV and Vx enantiomeric separations. The substitution degree (DS) of γ-CD was found to be an important parameter for the stereoselective discrimination of the investigated analytes. Indeed, a complete resolution ($Rs > 1.5$) of Vx and ODV was achieved by using a phosphated γ-CD with a high DS ($DS \sim 6–10$), but with relatively long migration time (> 36 min). The phosphated γ-CD with a low DS ($DS \sim 3$) permitted a baseline enantioseparation of both analytes in a shorter analysis time. Thus, the latter CD was selected for subsequent studies. After investigation of several electrophoretic parameters, including the chiral selector concentration (2.5–30 mg/ml), buffer pH (2.5–4.0) and concentration (10–100 mM), temperature (15–40°C) and voltage (15–25 kV), the optimal conditions were: 20 mg/ml of phosphated γ-CD ($DS \sim 3$) in a 50 mM Tris-phosphate buffer, set at pH 2.5, 25°C and 20 kV. As shown in Fig. 2, these conditions allowed the complete resolution of both compounds ($Rs > 1.5$) in less than 25 min, with Vx migrating first.

Because of the variability generally observed in CE, robustness is an important point for method validation. Robustness is defined as the capability of an analytical procedure to remain unaffected by small but deliberate variations in the method parameters [32]. Therefore, an evaluation of the robustness was undertaken in function of the following selected factors: (1) buffer concentration (48–52 mM); (2) chiral selector concentration (19.5–20.5 mg/ml); and (3) buffer pH (2.4–2.6). A 2^3 full factorial design was chosen (i.e. eight experiments) [33–36]. Five points at the optimised experimental conditions (central values) were added to evaluate the standard deviation of the method, and the experiments were performed randomly. The responses measured were the Vx and ODV enantiomeric resolutions. As shown in Fig. 3, the zero was included in the confidence interval of all coefficients and no factors significantly influenced Vx and ODV resolution ($P = 0.05$) Furthermore, all the interactions between factors were negligible, and thus the small variations in the BGE preparation did not affect the stereoselective separation of both analytes. The method can therefore be considered as robust.

In separate experiments, injections of non-racemic mixtures were carried out to determine the enantiomer migration order. Only Vx was available as a pure enantiomer and (+) Vx was migrating first. For ODV, an index (1) was assigned to the first detected enantiomer and an index (2) was assigned to the second one.

3.2. Validation

In order to obtain both a clean electrophoretic sample profile and good extraction yields of Vx and its main metabolite, ODV, a number of organic solvent mixtures as well as different extrac-

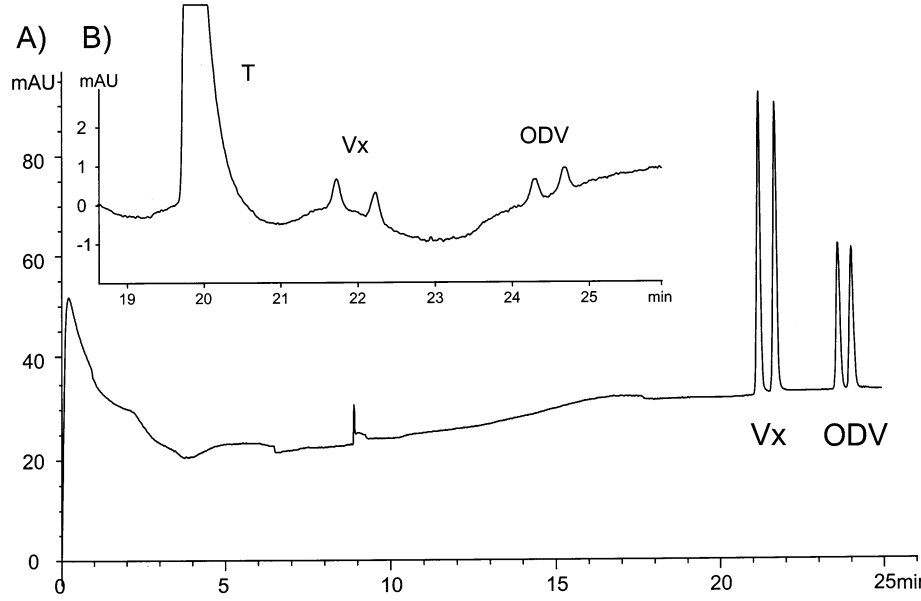

Fig. 2. Electropherograms of: (A) a standard mixture of 20 μg/ml racemic Vx and 10 μg/ml racemic ODV; and (B) a spiked serum standard of 50.0 ng/ml of Vx and ODV after LLE, T as internal standard, using a concentration of 20 mg/ml of γ-phosphated CD as chiral selector. For experimental conditions, see text.

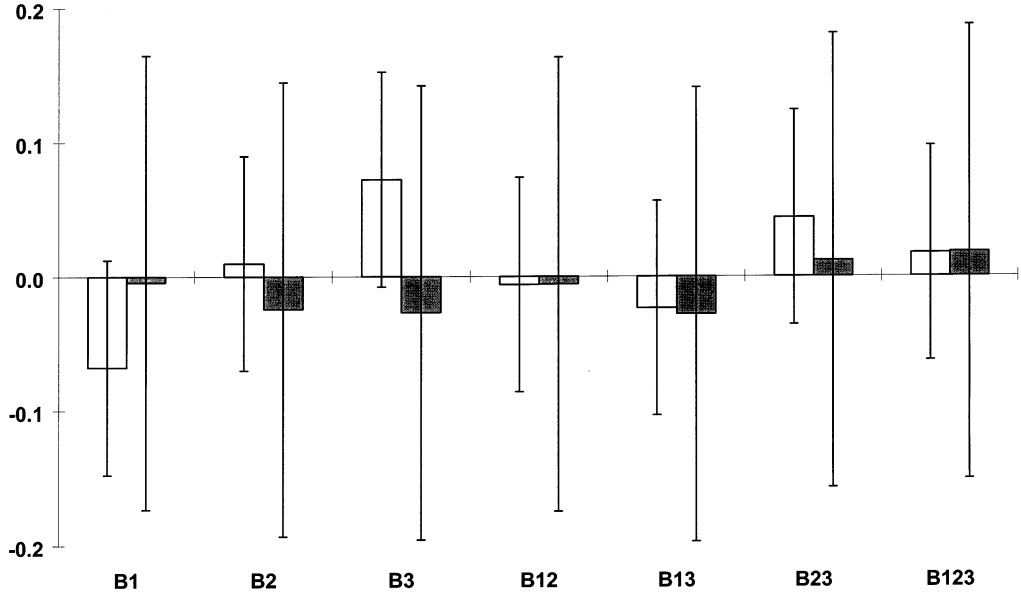

Fig. 3. Effects of main factors and interactions calculated for the resolution of Vx (white) and ODV (grey) obtained by full factorial design.

tion procedures were tested. The one-step LLE procedure, described in the experimental section, was the best to recover sufficient amounts of both analytes in a serum sample, without a co-extraction of endogenous interfering substances. Absolute extraction recovery was determined by comparing the normalised peak areas of Vx and its metabolite, extracted from serum to standard solutions in HCl 0.01 M at the expected concentration ($n = 6$). On three selected concentrations (50, 250 and 500 ng/ml), recoveries were superior to 75.0% with a relative standard deviation (R.S.D.) inferior to 6.0%, and to 73.0% with a R.S.D. inferior to 5.0% for Vx and ODV, respectively.

In order to prevent any variability in the extraction and injection processes, T was used as IS (Fig. 1). Absolute recovery of T was 81.1% with a R.S.D. of 4.6%, showing its appropriate use as IS.

HCl 0.01 M was chosen, instead of water or diluted BGE, to improve the dissolution of the analytes from the dried extracted serum.

Linearity was verified by analysing spiked serum samples of known concentration of racemic Vx and ODV. Five points calibration ($k = 5$) with three analyses ($n = 3$) at each concentration level were performed over the range of 25–500 ng/ml for each enantiomer. Normalised peak-area ratio of analytes/IS were plotted against the theoretical concentration of the spiked standards. Least square linear regression analyses were performed to determine correlation coefficients, slopes and intercepts. As shown in Table 1, linearity assays showed correlation coefficients superior to 0.996 for the four enantiomers. Furthermore, intercepts were not significantly different from 0 (Student's t-test, $P = 0.05$). For each enantiomer, the quantification limit was determined according to the literature on bioanalytical method validation [37,38] and was of 25 ng/ml.

Within-day accuracy and precision were assessed by performing six replicate determinations of three spiked standards (50, 250 and 500 ng/ml). Within-day accuracy was expressed as the mean of the assays relative to the theoretical values (%). For both analytes, accuracies were superior to 95% and, as reported in Table 2, the confidence interval ($t = 0.05$) included the theoretical value. The within-day precision, expressed as the relative standard deviation (%) of the accuracy assays, was satisfactory (R.S.D. $< 10.0\%$) for all compounds at the three tested concentrations.

Day-to-day accuracy and precision were assessed by analysing the same standards in replicate on 3 separate days (Table 2). Day-to-day accuracy, for serum spiked samples at a concentration of 50 ng/ml, was superior to 95.0% of the expected value with a precision (R.S.D.) inferior to 15.0%. At 250 ng/ml, day-to-day accuracies were superior to 97.0% with a precision inferior to 5.0%. At the highest tested concentration (500 ng/ml), accuracy did not differ more than 1.0% of expected values with a relative standard deviation inferior to 6.0%. In all cases, the determined confidence interval included the expected value ($t = 0.05$).

Selectivity was assessed by injecting potential clinical interferences, namely chlorazepam, trazodone, acetyl salicylic acid, ibuprofen, zoplicone, and paracetamol. Most of these drugs were chosen following information provided during the clinical study. Furthermore, six blank samples (blood and serum) were tested and, in all cases, no interference with endogenous compounds was observed.

Solution of both (+) and (−) venlafaxine enantiomers were stored for 2 weeks at room temperature and were analysed. No racemisation were observed.

Table 1
Statistical data for linearity including standard deviation (S.D.)

	Vx(+)	Vx(−)	ODV(1)	ODV(2)
r	0.9961	0.9960	0.9982	0.9973
Slope ($\pm$S.D.)	1.01 ± 0.02	1.01 ± 0.02	1.00 ± 0.01	0.99 ± 0.02
Intercept ($\pm$S.D.)	-3.95 ± 11.24	-3.96 ± 5.48	1.74 ± 3.67	2.22 ± 4.45

Table 2
Within and between day variation data from the chiral determination of Vx and ODV, for experimental conditions see text

	50 ng/ml			250 ng/ml			500 ng/ml		
	Concentration found ± IC[a] (ng/ml)	Accuracy (%)	Precision (%)	Concentration found ± IC (ng/ml)	Accuracy (%)	Precision (%)	Concentration found ± IC (ng/ml)	Accuracy (%)	Precision (%)
Within-day									
Vx(+)	49.3 ± 4.5	98.6	3.4	240.2 ± 28.3	96.1	6.8	504.3 ± 21.1	100.9	4.6
Vx(−)	49.5 ± 5.0	99.0	0.9	236.9 ± 25.1	94.8	5.4	505.4 ± 22.4	101.1	5.4
ODV(1)	53.4 ± 2.6	106.8	1.8	247.9 ± 11.4	99.2	3.2	501.4 ± 21.4	100.3	5.8
ODV(2)	54.5 ± 6.6	109.0	7.2	246.2 ± 11.3	98.5	2.7	501.7 ± 26.8	100.3	7.4
Day-to-day									
Vx(+)	49.8 ± 3.1	99.6	9.7	244.8 ± 13.4	97.9	8.7	502.3 ± 12.2	100.5	3.9
Vx(−)	50.0 ± 2.9	100.0	9.0	242.9 ± 11.9	97.2	7.8	503.0 ± 12.9	100.6	4.1
ODV(1)	52.6 ± 3.2	105.2	9.1	244.6 ± 7.9	97.8	5.2	503.8 ± 16.3	100.8	5.1
ODV(2)	52.7 ± 4.4	105.4	12.3	246.2 ± 7.6	98.5	5.1	504.4 ± 17.67	100.9	5.6

[a] Abbreviation: IC, confidence interval ($t = 0.05$).

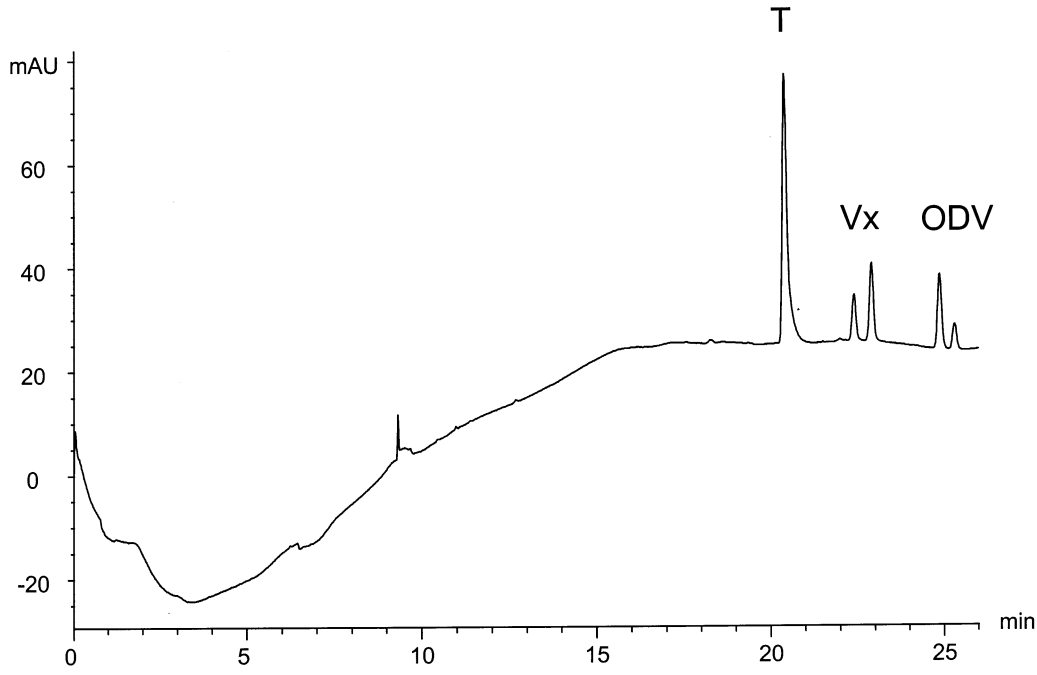

Fig. 4. Electropherogram of a serum (clinical sample) after LLE extraction, containing Vx, ODV and T as IS. Concentration found for Vx and ODV, 294.0 and 281.0 ng/ml, respectively. For experimental conditions, see text.

3.3. Clinical samples

The electropherogram of a clinical serum sample is shown in Fig. 4 where enantiomeric differences are clearly observed for Vx, as well as for ODV. As reported in Table 3, for half the patients the concentrations of the active metabolite, ODV, were higher than the parent drug concentrations. Enantiomeric ratios were calculated as concentration ratios of both enantiomers. As indicated in Table 3, non-racemic ratios were obtained for both analytes in the majority of cases which demonstrates a stereoselective metabolism of Vx. Therefore, and as previously mentioned, this implies to determining Vx and ODV enantiomeric ratios for a better evaluation of the patient's state.

Table 3
Concentration and enantiomeric ratio of Vx and ODV determined in clinical cases

Cases	Vx (ng/ml)	Ratio Vx	ODV (ng/ml)	Ratio ODV
1	79.7	1.29	355.5	0.93
2	272.5	0.48	97.8	1.08
3	190.3	1.37	429.4	0.78
4	273.7	0.69	122.0	1.23
5	294.0	0.68	281.0	1.19
6	390.9	0.92	418.3	0.96
7	421.9	0.87	449.3	1.12
8	95.8	1.71	200.3	0.85
9	246.5	0.93	259.1	1.04
10	98.8	1.15	496.1	0.87
11	63.6	1.48	123.4	1.08
12	305.1	1.00	307.0	1.03
13	155.4	1.51	364.5	1.00
14	228.6	1.39	363.0	0.96
15	108.3	1.15	265.3	1.00
16	260.8	0.96	296.0	0.96

4. Concluding remarks

The simultaneous stereoselective analysis of Vx and its main metabolite, ODV was successfully achieved by CE using a phosphated γ-CD as

chiral selector with a low substitution degree (DS ~ 3). The method was optimised and its robustness verified through a full factorial design.

A liquid–liquid extraction procedure was developed prior to the chiral CE analysis. With recoveries higher than 70% for both analytes, the entire method was validated for the analysis of clinical samples. Results demonstrated important inter-individual differences in the formation of ODV, the active Vx metabolite. Furthermore, the determination of the enantiomeric ratio of both analytes indicated a stereoselective metabolism. This point is actually under study and may open new avenues for a better understanding of the therapeutic effect of Vx.

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