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Use of vancomycin silica stationary phase in packed capillary electrochromatography
II. Enantiomer separation of venlafaxine and *O*-desmethylvenlafaxine in human plasma

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Abstract

A capillary electrochromatography method, using vancomycin chiral stationary phase packed capillary, was optimized for the simultaneous chiral separation of the antidepressant drug venlafaxine and its main active metabolite *O*-desmethylvenlafaxine. Simultaneous baseline enantiomeric separation of the two compounds was obtained using a mobile phase composed of 100 m*M* ammonium acetate buffer pH 6/water/acetonitrile (5:5:90, v/v). The electrokinetic injection for sample introduction provided a limit of quantitation for both the compounds of 0.05 μg/ml racemate concentration suitable for the analysis of venlafaxine and metabolite in biological samples. The acetonitrile mobile phase concentration was found to modulate the analytes elution times, the enantiomeric resolution and the efficiency of the separation. The column was tested for repeatability and linearity showing RSD values (%) in the range of 0.13–0.24, 2.47–3.66 and 1.35–2.50 for migration time, sample/internal standard peak area ratio and enantiomeric resolution, respectively and correlation coefficients higher than 0.9990. The method was applied to the analysis of clinical samples of patients under depression therapy showing a stereoselective metabolism for venlafaxine.

Keywords: Stationary phases, CEC; Enantiomer separation; Electrochromatography; Vancomycin; Venlafaxine; *O*-Desmethylvenlafaxine

1. Introduction

In pharmaceutical and biomedical research fields the availability of analytical separation methods with high efficiency and resolution capability is of relevant importance in studying and understanding the pharmacological activity and the pharmacokinetics of newly developed drugs. Moreover, several drugs, besides containing a chiral molecule as active principle, are generally administered as a racemic mixture. The availability of chiral analytical methods is important in understanding how a new chiral drug exerts its pharmaceutical activity and the contribution of each enantiomer to this. In fact, only very

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rarely do the two enantiomers exhibit both the same pharmaceutical activity and/or potency and can be discriminated in the biological systems resulting in diverse absorption, metabolism and elimination rates. Furthermore, in some cases the non-active enantiomer can be responsible for side effects of a different degree of toxicology [1,2].

Recent regulations on the development of new stereoisomeric drugs [3] pressed the research activity towards the optimization and validation of new, fast and feasible analytical methods for the determination of the chiral compounds of interest present in pharmaceutical formulation or in complex matrices such as the biological fluids.

Venlafaxine (1[-2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol hydrochloride) is a particularly effective second generation antidepressant chiral drug, administered as a racemic mixture, exerting a dual mechanism of action on the monoaminergic system [4–6]. The two enantiomeric forms exhibit different activity: the *R*(−) enantiomer inhibits both the noradrenaline and serotonine synaptic re-uptake whereas the *S*(+) enantiomer inhibits only the serotonine one [7]. *O*-desmethylvenlafaxine is the main metabolite produced by biotransformation in humans presenting a pharmaceutical activity similar to that of venlafaxine [8,9].

The determination of the enantiomeric concentration of venlafaxine and *O*-desmethylvenlafaxine in human plasma is important to understand the mechanism of action of each enantiomer and their pharmacokinetic and pharmacodynamic relations.

Analytical methods used so far for the stereoselective analysis of venlafaxine include high-performance liquid chromatography (HPLC) [10] and capillary zone electrophoresis (CZE) [11–13].

In the specific field of chiral separation Capillary Electrophoresis (CE) is recognized as a challenging and powerful analytical tool allowing the use of very few quantities of expensive chiral selectors mainly using the simple and feasible direct method of enantiomeric separation [14–16]. Moreover CE has been successfully used for chiral separation of compounds in biological fluids demonstrating high resolution power, short analysis time, reduced sample pretreatment and use of very low (few microlitres) quantities of real samples [2,17–19]. The last aspect is particularly important when biological samples of, e.g. newborn origin or low volume available (lachrymal fluids, etc.) are to be analysed.

More recently, capillary electrochromatography (CEC) using several kinds of chiral selectors immobilized on the capillary wall (open tubular CEC) [20], simply dissolved as additives in the mobile phase [21] or packed into the electrophoretic capillary (chiral stationary phase, CSP) [22–27] were successfully used for enantiomer separation [28].

CEC combines the high resolution capability and efficiency of CE with the selectivity of HPLC. Analytes can be separated on the basis of the chromatographic principle of different partition between the stationary and the mobile phase. When charged compounds are analysed, the electrophoretic mobility will also play an important role in the separation process. The electroosmotic flow, generated at the application of the separation voltage, acts as a pump transporting both the analytes and the mobile phase through the column. The plug profile of the electroosmotic flow is responsible for the high efficiency that can be obtained in CEC.

Very few papers report the use of CEC techniques for the analysis of biological fluids and most of them were recently published. Stead and coworkers [29] compared CEC and HPLC techniques for the analysis of steroids in plasma demonstrating the superior separation capability of CEC also in the analysis of complex biological matrices. Reversed-phase packed capillaries were successfully utilized for analysis of pharmaceutical and biomedical interest [30,31] also in combination with visible diode laser induced fluorescence (VDLIF) [32] and mass spectrometry [29,33–36]. The coupling of isotachophoresis with CEC was successfully used to provide a strong enhancement in low molecular mass cations detection in plasma and urine samples by mass spectrometry [36]. To our knowledge, no papers dealing with CEC application for chiral determination of drugs and metabolites in biological fluids have been published yet.

Macrocyclic antibiotics, e.g. vancomycin [22–25] and teicoplanin [37,38] chiral stationary phases, were recently successfully used in CEC packed capillary columns for the separation of enantiomers of pharmaceutical interest using the reversed-phase or the polar organic modes. Vancomycin possesses several stereogenic centres and functional groups responsible

for stereoselective interactions (mainly based on electrostatic, dipole–dipole, π–π, hydrophobic interactions and hydrogen bonds) [39].

Vancomycin chiral stationary phase was recently used by us [25] for the enantioseparation of venlafaxine and other basic compounds using ammonium acetate pH 6.0/acetonitrile mobile phase mixtures.

In this paper the selectivity and the enantioresolution capability of vancomycin CSP column in CEC was investigated for the simultaneous separation of venlafaxine and its main active *O*-desmethylated metabolite in biological samples studying the optimum experimental conditions offering the highest resolution and speed of analysis. In order to demonstrate the real applicability of chiral CEC to pharmaceutical and biomedical analysis the method was tested for a quantitative performance and applied to clinical cases.

2. Experimental

2.1. Apparatus

A Hewlett-Packard HP^{3D} Capillary Electrophoresis automated apparatus (Waldbronn, Germany) equipped with diode array UV detector and external nitrogen pressure (up to 12 bar) was used for capillary electrochromatography experiments using a 75 μm I.D. capillary packed with silica immobilized vancomycin chiral stationary phase (CSP) prepared in the laboratory following the procedure and the packing process described in a previous paper [25]. The capillary was packed for all the length using a combination of different packing materials, the silica–diol phase and the vancomycin–CSP. Both phases were mixed with Lichrospher silica 5 μm (3:1, w/w) particles. The packed capillary used for separation was of 35 cm total length, composed of 11 cm diol/silica, 23 cm vancomycin CSP/silica and 1 cm diol/silica.

During the run the capillary was pressurized (10 bar) at both inlet and outlet ends and air maintained at 20°C. The applied separation voltage was 27 kV or otherwise stated.

Samples were injected electrokinetically by applying 15 kV for 10 s, or otherwise stated, followed by an injection of the mobile phase by pressure at 12 bar for 0.5 min.

Capillary pre-run conditioning was made by pressure applying 12 bar for 5 min of the mobile phase before the injection step.

Mixtures of acetonitrile, water and 100 m*M* ammonium acetate buffer pH 6.0 were used as mobile phase in order to have a final ammonium acetate concentration of 5 m*M* in presence of different organic solvent concentrations. 100 m*M* ammonium acetate buffer at pH 6.0 was prepared by titrating an appropriate volume of acetic acid (99.9%), corresponding to 10 mmol, with ammonium hydroxide solution (15%) and diluting to the final volume of 100 ml with water. The buffer/acetonitrile mobile phase was prepared daily.

2.2. Chemicals

Acetic acid, methanol and acetonitrile pure reagents grade were from BDH (Poole, England). Imipramine was purchased from Sigma (MO, USA). Ammonium hydroxide 37% was from Riedel de Haën (Seelze, Germany). Venlafaxine enantiomers, racemic venlafaxine and racemic *O*-desmethylvenlafaxine were kindly provided by Wyeth-Ayerst Research (Princeton, NJ, USA). Double distilled water was used for the preparation of the solutions. Venlafaxine and *O*-desmethylvenlafaxine and imipramine concentrated solutions (1 mg/ml) were prepared in methanol and further diluted in water at the desired concentration.

3. Results and discussion

The knowledge of the pharmacokinetics and pharmacodynamics of a newly developed drug is strongly related to the availability of analytical tools to determine the drug and active metabolite concentrations in short time with good precision and high efficiency. This is particularly important when the active principle of the drug is a chiral molecule where the enantiomers possesses different activities and/or when a stereoselective metabolism is suspected.

Venlafaxine (Vx) is a second generation antide-

pressant drug that exhibits variation in the stereoselectivity of its metabolism [8,13].

The important inter-individual differences in the formation of *O*-desmethylated metabolite (O-Vx) stimulated researches into developing analytical methods, possessing the above mentioned characteristics, capable of performing the simultaneous chiral determination of Vx and its main active metabolite in complex matrices.

Fig. 1 shows the chemical structures of the analysed racemic compounds.

The high enantiomeric resolution capability exhibited by vancomycin chiral stationary phase towards venlafaxine and other basic compounds [25] encouraged us to test this CSP in packed capillaries for the simultaneous separation of Vx and O-Vx by capillary electrochromatography (CEC) in plasma samples of clinical provenence.

As reported in our previous paper [25], and described in the experimental section, the capillary used for the separation was packed for all the length using the combination of different stationary phases: the vancomycin CSP and the underivatized diol–silica phase. The diol–silica phase was used for both the production of the inlet and outlet frits at the capillary ends and for the packing of the capillary part including the detection cell. This packing method was sort of the CZE partial filling method applied to CEC packed capillary to avoid the presence of the UV absorbing vancomycin in the detection path. In this way it was possible to combine a sensitive detection with the robustness and the lifetime typical of complete packed capillaries. Furthermore both the stationary phases were mixed 3:1 with silica particles in order to (i) easily prepare the frits from diol phase in water (the same delivering solvent used for packing) and (ii) to provide a stable electrophoretic current ensuring a homogenous conductivity for all the capillary length.

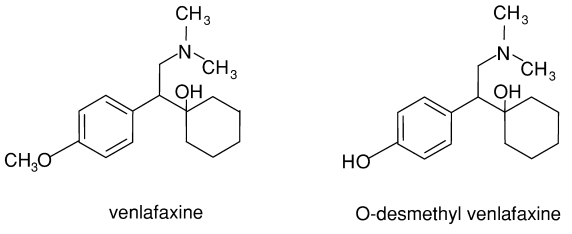

Fig. 1. Chemical structures of venlafaxine (Vx) and *O*-desmethyl-venlafaxine (O-Vx).

Because venlafaxine enantiomers were successfully resolved with vancomycin CSP column in CEC using a mobile phase composed of 5 m*M* ammonium acetate buffer pH 6.0 (final concentration) containing 90% of acetonitrile as organic modifier [25], the same experimental conditions were applied for the simultaneous separation of both couples of enantiomers of Vx and its main active metabolite O-Vx. The resulting electropherogram is shown in Fig. 2.

Although the chemical structures of the compounds were very similar, differing for only one methyl group in para position of the phenolic moiety, vancomycin–CSP column provided the simultaneous baseline separation of the four optical isomers in less than 12 min. Therefore, in addition to the high chiral discrimination vancomycin–CSP demonstrated also a very high separation capability for structurally related compounds, which is sometimes difficult to obtain in aqueous CZE.

The O-Vx metabolite enantiomers were eluted after the Vx ones probably due to a stronger interaction with the stationary phase. However the enantiomeric resolutions of the compounds were almost comparable showing enantiomeric resolution factors (R_s) of

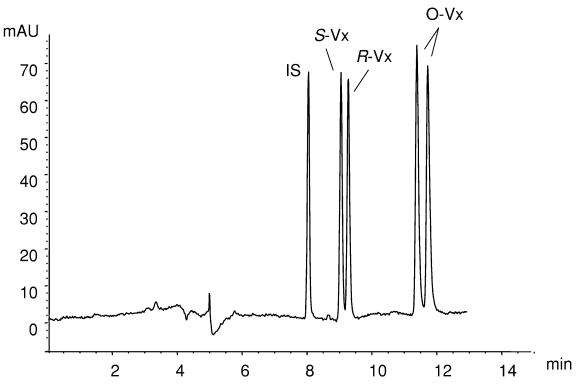

Fig. 2. Enantiomeric separation of Vx and O-Vx standard mixture. Mobile phase composition: 100 m*M* ammonium acetate buffer pH 6.0/water/acetonitrile (5:5:90, v/v); applied voltage, 27 kV. Capillary: 75 μm I.D.×35 cm total length, packed as following: 11 cm diol/silica, 23 cm vancomycin CSP/silica and 1 cm diol/silica. Both the inlet and the outlet ends were pressurized at 10 bar. Capillary temperature 20°C. Sample injection: 12 kV for 10 s of Vx, O-Vx and IS (imipramine) each at 2.5 μg/ml racemic concentration followed by a mobile phase plug (12 bar for 0.2 min).

1.68 and 1.57 for Vx and O-Vx, respectively. The enantiomeric resolution (R_s), the number of theoretical plates (N) and the plate height (H) were calculated using Eqs. (1)–(3):

$$R_S = 1.18 \frac{(t_{R_2} - t_{R_1})}{w_2 + w_1} \quad (1)$$

$$N = 5.54\left(\frac{t_R}{w}\right)^2 \quad (2)$$

$$H = \frac{l}{N} \quad (3)$$

where t_{R_2} and t_{R_1} are the retention times of the second and first eluting enantiomers, respectively, w is the peak width at half height and l is the effective length of the capillary.

The availability of pure Vx enantiomers allowed the characterization of their enantiomeric migration order finding the S-isomer migrating first.

It is noteworthy that the peak efficiency data obtained under these experimental conditions for Vx and O-Vx enantiomers were in the range 236 094–255 324 number of theoretical plates meter^{-1} (N/m) with a plate height value (H) between 3.92 and 4.23 μm.

3.1. Influence of different sample injection methods on sensitivity

In order to obtain good method sensitivity suitable for the analysis of Vx and O-Vx in the biological fluids different sample injection methods were studied. Sample injection was performed by using only high pressure (hydrodynamic injection) or only voltage application (electrokinetic injection) or a combination of both methods and the analyte signal, enantiomeric resolution and peak efficiency data obtained were compared. Fig. 3 shows the comparison of the analysis of Vx and O-Vx mixture (each 5 μg/ml racemic concentration) obtained injecting for 0.5 min the sample by (a) hydrodynamic (12 bar), (b) hydrodynamic (12 bar) and electrokinetic (4 kV) and (c) electrokinetic only (4 kV) injection.

The use of only pressure produced a very low signal (Fig. 3a) that was strongly increased when the pressure was supplemented by the voltage application (Fig. 3b).

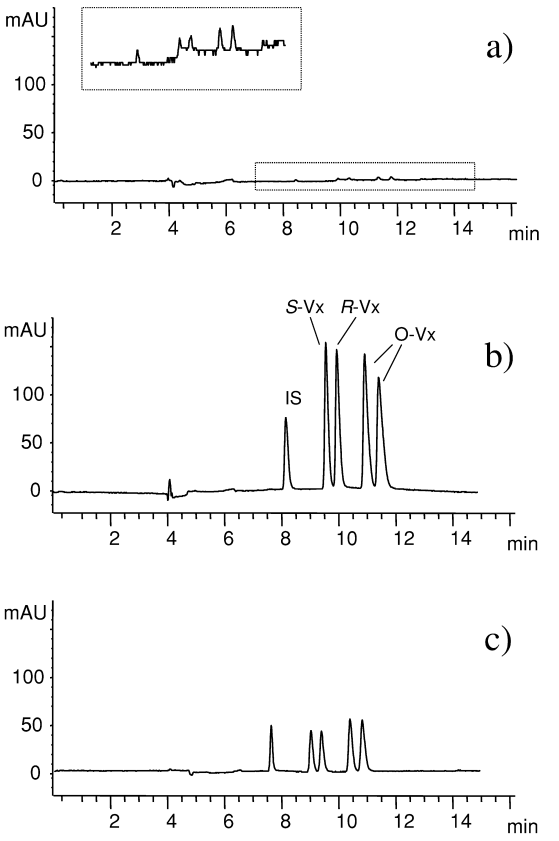

Fig. 3. Effect of different injection methods on Vx and O-Vx enantiomeric separation. Sample injection: (a) hydrodynamic (12 bar), (b) hydrodynamic (12 bar) and electrokinetic (4 kV) and (c) electrokinetic only (4 kV). All the injections were followed by a post plug of mobile phase (12 bar for 0.2 min). Sample concentration: Vx and O-Vx 5 μg/ml racemic concentration, IS (imipramine) 2.5 μg/ml. Applied voltage 25 kV. Other experimental conditions as in Fig. 2.

In Fig. 3b, where both hydrodynamic and electrokinetic injections were combined for sample introduction, Vx and O-Vx showed R_s values of 1.68 and 1.57, respectively, with N of 117 411 and 92 124 for the first migrating enantiomers. In Fig. 3c analytes were injected using only the electrokinetic method and the recorded R_s and N values (R_s = 1.69 and 1.67 for Vx and O-Vx, respectively, with N values of 110 634 and 110 958 for the first migrating enantiomers) were almost comparable exhibiting only slightly higher values.

It is interesting to remark that, although a strong increase in analyte signal was obtained when hydro-

dynamic and electrokinetic injections were combined, no changes in the migration times were observed for all studied enantiomers.

Pressure can be therefore advantageously used in combination with the electrokinetic injection to increase the injected amount of sample not remarkably affecting the characteristics of the separation (R_s and N). This procedure can be useful especially if both charged and uncharged analytes are present in the sample. However for injection of charged analytes the use of electrokinetic injection is advantageous considering that it is a more selective sample introduction method and higher peak efficiency can be obtained. In this case a strong increase in analytes detectability can be obtained by slightly increasing the injection voltage applied and/or using longer injection times. It was however observed that the use of higher voltages for shorter migration time provided higher peak efficiency (data not shown) also if relatively high sample loading was obtained.

3.2. Effect of acetonitrile concentration on enantiomeric resolution

Besides electrokinetic injection resulted to be the most selective and effective sample introduction method, the effect of acetonitrile mobile phase content was studied injecting the analytes by pressure in order to avoid additional effects due to mobile phase composition and conductivity changes.

Fig. 4 shows the effect of acetonitrile mobile phase content in pressure injected racemic Vx and O-Vx analytes mixture. As can be observed in panel (a) and (b) the IS peak is disturbed by a baseline interference. By adjusting the acetonitrile concentration the analytes elution times can be modified in order to have the analytes of interest eluting in an interference free electropherogram window.

It is also interesting to observe that by increasing the acetonitrile content longer migration times were observed together with different enantiomeric resolutions and peak efficiency values. In accordance with previously published data [25] high acetonitrile mobile phase content produced higher efficiency of Vx enantiomeric peaks and consequently higher analytes detectability, important for biological applications of the method.

The efficiency values (N/m) for the first migrating

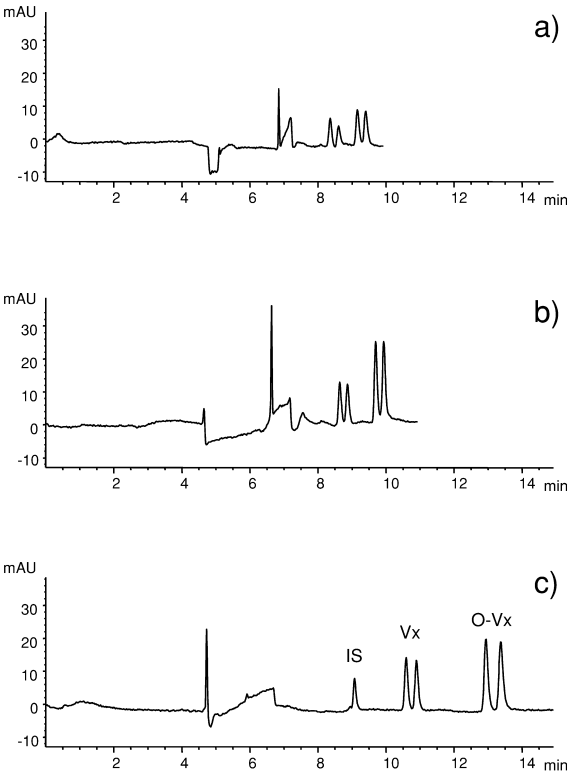

Fig. 4. Effect of acetonitrile mobile phase content on the enantiomeric separation of the studied analytes. Mobile phase composition: 100 m*M* ammonium acetate buffer pH 6.0/water/acetonitrile; (a) (5:25:70, v/v); (b) (5:15:80, v/v); (c) (5:5:90, v/v). Sample injection: hydrodynamic at 12 bar for 0.5 min followed by a post plug of mobile phase (12 bar for 0.2 min). Other experimental conditions as in Fig. 3.

enantiomer of Vx were 154 943, 172 539 and 206 370 at 70, 80 and 90% of acetonitrile, respectively. For the metabolite an increment in efficiency was observed from 70 to 80% of acetonitrile but a comparable or slightly lower value was recorded from 80 to 90%.

By rising the acetonitrile mobile phase content from 70 to 90% the enantiomeric resolution increased from 1.46 to 1.64 for Vx and from 1.36 to 2.00 for O-Vx. The increase of acetonitrile content changes the mobile phase viscosity and can influence the analyte interaction with the chiral stationary phase via a competing or a promoting effect. The increase in enantiomeric resolution observed at higher acetonitrile content can be ascribed to a stronger

analyte interaction with the stationary phase stimulated by the acetonitrile presence.

Although a strong increase in migration times was observed at 90% of acetonitrile, this organic solvent concentration produced interference free electropherograms with high separation efficiency and high enantiomeric resolution and was therefore selected for further experiments. In order to shorten the analysis time the running voltage was increased up to 27 kV and the baseline separation of the two couple of enantiomers was achieved in less than 12 min.

3.3. Repeatability and linearity of the method

The optimum experimental conditions were found using a 5 m*M* (final concentration) ammonium acetate buffer pH 6.0 mixed with 90% of acetonitrile, running at 27 kV and using the electrokinetic method for sample injection. Under these operating conditions the method provided a detection (LOD, signal-to-noise ratio 4:1) and quantification (LOQ, signal-to-noise ration 10:1) limits of 0.02 μg/ml and 0.05 μg/ml racemic concentrations, respectively, for both Vx and O-Vx standard compounds in water.

The optimized method was than tested for repeatability and linearity analyzing the Vx and O-Vx standard mixtures and recording the migration times, the peak area ratio and the enantiomeric resolution data.

The repeatability of the method was performed by analyzing ten replicated runs ($n=10$) of a standard mixture containing Vx, O-Vx and IS (imipramine) each at 2.5 μg/ml (racemic concentration for Vx and O-Vx). The RSD% obtained data were in the range 0.13–0.24, 2.47–3.66 and 1.35–2.50 for migration times, IS peak area ratio and enantiomeric resolution, respectively.

Linearity of the method was verified by analysing Vx and O-Vx standard solutions mixture in the range 0.05–10 μg/ml racemic concentrations (ten calibration levels), which covered the expected values of Vx and O-Vx levels in biological samples. The regression equations of the enantiomers were calculated on the basis of the internal standard method using imipramine as reference compound and gave correlation coefficients (R) higher than 0.9990. Table 1 reports the statistical data for linearity including the standard deviations (SD) values.

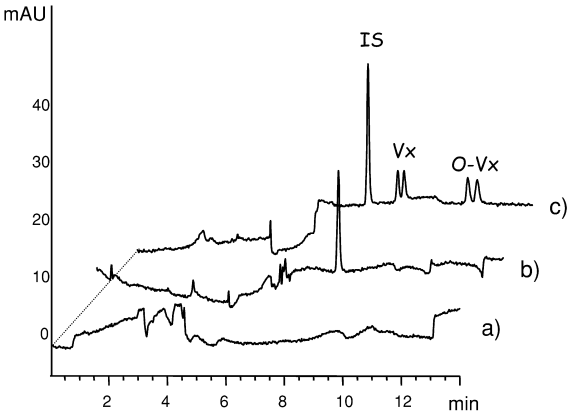

Fig. 5. Electropherograms of extracted (a) blank plasma; (b) blank plasma spiked with the IS (imipramine) only; (c) blank plasma spiked with IS and Vx and O-Vx compounds at 0.05 μg/ml racemic concentration. Sample injection: electrokinetic 15 kV for 10 s followed by 12 bar for 0.2 min of mobile phase injection plug. Other conditions as in Fig. 2.

3.4. Analysis of clinical samples

In order to demonstrate the real applicability of the optimized chiral CEC method blank plasma samples and blank plasma spiked with different concentration levels of Vx and O-Vx were analysed after a simple liquid–liquid extraction [13]. Fig. 5 shows the electropherograms obtained from the analysis of extracted (a) blank plasma, (b) blank plasma spiked with only the internal standard (imipramine) and (c)

Table 1
Statistical data for linearity including standard deviation (SD)

	Vx_1	Vx_2	O-Vx_1	O-Vx_2
R	0.9998	0.9996	0.9991	0.9990
intercept (±SD)	1.43E−02 (±1.10E−02)	2.10E−02 (±1.38E−02)	4.41E−02 (±2.51E−02)	3.51E−02 (±2.73E−02)
slope (±SD)	3.24E−04 (±2.53E−06)	3.27E−04 (±3.17E−06)	3.79E−04 (±5.77E−06)	4.01E−04 (±6.27E−06)

blank plasma spiked with IS, Vx and O-Vx at 0.05 μg/ml concentration level. As can be observed in panel (a), (b) and (c) the extracted plasma showed a very clean profile and both the internal standard and the analytes of interest were eluting in interference free electropherogram windows. The method provided a detection sensitivity suitable for Vx and O-Vx analysis in clinical samples as confirmed by the analysis of the plasma sample in Fig. 5c spiked with a Vx and O-Vx concentration level close to the minimum relevant concentration found in biological samples.

Finally the method was therefore applied to the analysis of real clinical samples of plasma from patients under depression therapy with venlafaxine (Fig. 6a and b). In accordance with previously published data [13], the Vx and O-Vx enantiomeric ratio can be significantly different from patient to patient leading to a difficult interpretation of Vx pharmacokinetic and pharmacodynamic processes. As presented in Fig. 6, a stereoselective Vx metabolism was observed, as confirmed by the S/R enantiomeric ratio data: in the first depicted electropherogram (Fig. 6a) a slower transformation of the S-enantiomeric form was observed while in Fig. 6b the opposite effect occurred. The enantiomeric ratio data for the *O*-desmethylvenlafaxine metabolite were very different showing stereoselectivity of the biotransformation process only in the second electropherogram (Fig. 6b).

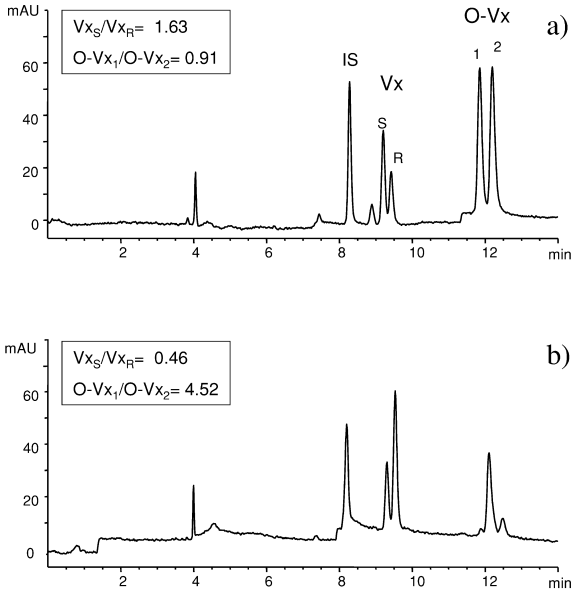

Fig. 6. Analysis of extracted plasma samples of (a) and (b) from different patients under depression therapy with venlafaxine. Experimental conditions as in Fig. 2. Sample injection: 10 kV for 30 s followed by 12 bar for 0.2 min of mobile phase injection plug.

4. Conclusions

Vancomycin chiral stationary phase column used in CEC mode demonstrated to be a powerful analytical tool for the analysis of venlafaxine and *O*-desmethylvenlafaxine providing high separation selectivity and enantiomeric resolution capability.

The optimized method allowed to obtain their simultaneous chiral separation in a very short time (less than 12 min) with good repeatability and linearity. Different mobile phases, organic solvent concentrations and sample injection types can be modulated to increase the method sensitivity and improve the characteristics of the separation (migration time, enantiomeric resolution, and efficiency). The optimized method provided detection sensitivity suitable for the analysis of venlafaxine and *O*-desmethylvenlafaxine in biological fluids. The method was successfully applied to the analysis of clinical samples of patients under depression therapy showing a stereoselective type venlafaxine metabolism and confirming the high variation of its metabolic process.

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