DETERMINATION OF VENLAFAXINE TOXIC LEVELS FROM HUMAN SERUM BY NON-AQUEOUS CAPILLARY ELECTROPHORESIS

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Abstract
A rapid and sensitive procedure using nonaqueous capillary electrophoresis (NACE) for the assay of venlafaxine has been developed and validated. Optimum detection of venlafaxine was obtained on a 56 cm (effective length) x 50 μm capillary, using a nonaqueous electrolyte solution system consisting of 7:3 methanol-acetonitrile with 15 mM ammonium acetate, capillary temperature 25°C and hydrodynamic injection, detection at 230 nm. The method is simple, accurate and reproducible and was successfully applied for the assay of toxic levels of venlafaxine in biological samples (human serum).

Keywords: venlafaxine; biological sample; nonaqueous capillary electrophoresis

Introduction
Venlafaxine hydrochloride, (1-[2-dimethylamino]-1-(4-methoxy phenyl) ethyl] cyclohexanol) hydrochloride (Fig. 1) is a third generation antidepressant [5]. The drug is a potent inhibitor of neuronal serotonin and norepinephrine reuptake, but a weak inhibitor of dopamine reuptake. Venlafaxine is well absorbed, with peak plasma concentrations occurring approximately 2 hours after dosing. It is extensively metabolised, to O-desmethylvenlafaxine, the only major active metabolite [3].
The most commonly used analytical methods for the determination of venlafaxine in biological matrices, such as blood and urine, are HPLC methods with UV detection [8, 11].

On the other hand, capillary electrophoretic techniques have been successfully applied in toxicological analysis [2, 6].

![Chemical structure of venlafaxine hydrochloride](image)

**Figure 1**

The purpose of the paper is to present a simple and fast analysis method for venlafaxine at toxic levels in human serum samples by non-aqueous capillary electrophoresis with diode-array detection. The major factors affecting the electrophoretic separation were studied, including the mobile phase composition and ionic strength of electrolyte, the applied voltage, capillary temperature and injection time.

**Materials and methods**

**Reagents**

Methanol (99.8% containing max.0.1% water) and acetonitrile (99.9%) containing 0.02% water) were purchased from Scharlau Chemie SA.

Venlafaxine hydrochloride standard substance was provided by Sigma. A 0.1 mg/mL stock standard solution was prepared in methanol and stored at 4°C for 10 days. Working standard solutions were prepared daily by dilution of the stock standard solutions with methanol.

**Equipment**

Analysis was performed on a G1600A Agilent capillary electrophoresis equipment with diode-array detector (DAD) and controlled by Agilent ChemStation ver. B.0 2.0x capillary electrophoresis software. The 64.5 cm (effective length 56 cm) x 50 μm i.d. fused-silica separation capillary was thermostated at 25°C. The use of photodiode detector allowed us to confirm the identity of the peaks, not only by their migration time, but also by the overlay of the UV–Vis spectra of the samples with a standard.

For the UV spectrum of venlafaxine, a Cary 100 Bio Varian Inc. UV-Vis absorption spectrophotometer was used.
Treatment of the biological samples

Fresh 1 mL of human serum samples spiked with different amounts of venlafaxine were brought at pH =10 created by 0.5 M Na₂CO₃, placed in separatory funnels and extracted with 5 ml of n-hexane: iso-amylc alcohol (93:7 v/v) by shaking for 15 mins. After the separation, the organic phase was evaporated to dryness under N₂ flow. The residue was dissolved in the nonaqueous electrolyte solution system, filtered through a 0.45 μm microfilter and immediately injected into the capillary.

Method

Before the first use the capillary was conditioned by flushing with 0.1M NaOH for 60 min, then with water for 30 min, and finally with the background nonaqueous electrolyte solution for 20 min.

Electrophoretic separation was performed using a nonaqueous electrolyte system consisting in a solution of 7:3 (v/v) methanol-acetonitrile with 15mM ammonium acetate. Before use, the electrolyte solutions and samples were filtered through a 0.45 μm microfilter and degassed for 10 min. At the start of each analysis the capillary was washed for 5 min with 0.1M NaOH, 5 min with water and 15 min with the nonaqueous electrolyte solution.

The capillary cassette was thermostated at 25°C, the injection was performed in the hydrodynamic way at 35 mB for 5s, and the applied voltage was 25 kV.

Results and discussion

In order to design a reliable NACE method for the venlafaxine assay, we studied the effect of certain modified electrophoretic parameters, such as: the nonaqueous phase composition and ionic strength of the electrolyte, the applied voltage, the capillary temperature and injection time.

Running buffer composition

Acetonitrile/methanol (ACN/MeOH) mixtures are widely used in NACE. The selectivity of the separation systems changed significantly with the ratio of ACN/MeOH, and the electrophoretic mobility varied according to the ACN/MeOH composition, with a maximal value at 75% [4,7]. The zeta potential in ACN is higher than in MeOH if both solvents do not contain supporting electrolytes [10]. Therefore, changes in viscosity and dielectric constant predict a steadily increase of electroosmotic flow (EOF) with the ACN concentration [9, 12].

Several ACN/MeOH mixtures (10, 30, 50 and 70% assay of acetonitrile (v/v)) containing 15mM ammonium acetate were tested. The results obtained (Fig. 2) show a decrease of the migration time with the
increased acetonitrile percentage, from 18.21 minutes (for 10 % acetonitrile) to 2.08 minutes (for 70 % acetonitrile). Acetonitrile levels over 30% were not satisfactory as they resulted in uncontrollable increase of the current. Therefore a composition with 30% ACN was chosen as optimum, which corresponds to a convenient migration time of 9.25 minutes.

Effect of the electrolyte ionic strength

As in aqueous capillary electrophoresis, the nature of the electrolyte plays an important role during method optimization. Considering the pKₐ value 9.40 for venlafaxine [3], it is necessary to have an acidic pH value of the solvent to maintain it in its protonated form. Initially, ammonium chloride was tested but without any success, as it is not soluble in the organic solvents mixture used. Therefore, ammonium acetate (NH₄OAc) was preferred, because it produces a low and constant current (< 20 μA) which avoids current courts.

The effect of the concentration of NH₄OAc (10, 15, 20 and 25 mM) on the migration time of the investigated compound was further studied. As expected (Fig. 3), when the concentration of NH₄OAc increases, the migration time of venlafaxine also increases. A concentration of 25 mM NH₄OAc resulted in a migration time of 18.46 minutes, while a concentration of 10 mM NH₄OAc corresponds to a lower migration time (2.74 minutes) but also a lower current (7 μA). Therefore a concentration of 15mM NH₄OAc buffer was selected as optimum since this value maintains good peak shapes, low currents (25 μA) and a reasonable migration time.
**Optimisation of the injection time**

In order to decrease the detection limits, the injection time was varied between 5 and 11 s (in steps of 2 s). The injection pressure was always kept at 35 mb.

As expected, when the injection time increases, the peak area of the compound also increases, but the peak symmetry decreases. Therefore, 5 s of injection time was chosen as optimum value.

**Effect of the applied voltage**

The effect of the voltage applied, from 10 to 30 kV (in steps of 5 kV), was also investigated (Fig. 4). Voltages higher than 25 kV generate currents higher than 40 μA, unsuitable due to bubble formation and consequently current courts. Lower voltages (as 10 to 20 kV) generate lower currents, but also longer migration times (more than 20 minutes). A voltage of 25 kV seemed to be the best compromise in terms of lower migration time and higher current level.

**Effect of temperature**

As temperature increases, the viscosity and the resistance of the buffer decrease, so the intensity of the current increases while the migration time decreases. In NACE it is suggested to work with a current level lower
than 20–25 μA to avoid the current courts. Because a convenient migration time with a current level of 25 μA was obtained at 25°C, this value of temperature was selected as optimum.

**Detection wavelength**

The detection wavelength was selected based on UV-Vis absorption spectra (Fig. 5) recorded between 200 and 500 nm. The analysis of the UV spectra (Fig. 5) revealed that venlafaxine exhibits two peaks, at 230 nm and 266 nm, respectively. Because the signal intensity is higher at 230 nm, this wavelength, corresponding to the first maximum in the venlafaxine absorption spectrum, was selected for detection.

The validation of the newly established method was performed according to bioanalytical guidelines[1], and the results obtained are summarized in Table I.

The selectivity of the method against the biological matrix studied was proved by checking the peak purity of venlafaxine (Fig.6). Data obtained showed no interference, proving the selectivity of the method.
Table I
Summary of validation data obtained for the new NACE method for the assay of venlafaxine

<table>
<thead>
<tr>
<th>Validation criterion</th>
<th>Parameter</th>
<th>Results</th>
</tr>
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<tbody>
<tr>
<td>Precision*</td>
<td>RSD = 3.02 %</td>
<td></td>
</tr>
<tr>
<td>Intermediate precision**</td>
<td>RSD = 1.118%</td>
<td></td>
</tr>
<tr>
<td>Linearity</td>
<td>Regression parameters</td>
<td>Y = 15.605X – 3.7385</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9913</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>3.17 – 10.56 µg/mL</td>
<td></td>
</tr>
<tr>
<td>Detection limit***</td>
<td>0.79 µg/mL</td>
<td></td>
</tr>
<tr>
<td>Quantification limit***</td>
<td>2.39 µg/mL</td>
<td></td>
</tr>
</tbody>
</table>

*from 5 replicate for 3 different concentrations corresponding to 3.17 µg/mL; 5.28 µg/mL and 10.56 µg/mL venlafaxine
**in two different days
***based on the standard deviation of the response and slope

Analysis of biological samples
The proposed method was successfully applied for the determination of venlafaxine toxic levels in human serum samples, taking into consideration that venlafaxine-related deaths occurred at blood concentration higher than 7.27 mg/L, while toxic effects appeared at blood concentration greater that 1.0 mg/L [3]. The results obtained are shown in Table II.

Table II
Extractive recovery of venlafaxine from human serum

<table>
<thead>
<tr>
<th>Nominal (µg/mL)</th>
<th>Recovery %</th>
<th>SD</th>
<th>RSD %</th>
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<tbody>
<tr>
<td>3.17</td>
<td>59.71</td>
<td>3.7301</td>
<td>6.25</td>
</tr>
<tr>
<td>5.28</td>
<td>64.87</td>
<td>3.7301</td>
<td>5.75</td>
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<tr>
<td>10.56</td>
<td>71.33</td>
<td>3.2488</td>
<td>4.55</td>
</tr>
</tbody>
</table>

*average of 5 measurements

Conclusions
A simple NACE method for venlafaxine assay was developed. The optimum experimental conditions for this NACE method are: 64.5 cm (56 to the detector) x 50 µm i.d. fused-silica capillary, nonaqueous electrolyte solution of 7:3 methanol-acetonitrile (v/v) containing 15mM ammonium acetate, capillary temperature of 25°C, 35 mb hydrodynamic injection for 5 s,
25 kV applied on the electrodes during the electrophoretic migration, UV detection at 230 nm. The method is simple, accurate and reproducible. It was successfully applied for the assay of toxic levels of venlafaxine in biological samples (human serum).

References

1. ***Guidance for Industry. Bioanalytical Method Validation, U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2001

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