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Venlafaxine Chiral Separation by Capillary Electrophoresis Using Cyclodextrin Derivatives as Chiral Selector and Experimental Design Method Optimization

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Abstract: Venlafaxine (VFX) is a modern antidepressant from the serotonin and norepinephrine reuptake inhibitor (SNRI) class. It is a chiral substance used in therapy as a racemate, but differences between the pharmacological properties of the two enantiomers have been reported. The current article presents the development of a simple capillary electrophoresis (CE) method for the rapid chiral separation of VFX enantiomers. A complex cyclodextrin (CD) screening at four different pH levels was carried out to establish the optimum chiral selector; carboxymethyl-β-CD (CM-β-CD) at pH 2.5 was selected for further method development. An initial "one factor at time" (OFAT) screening strategy was used to establish the influence of analytical parameters on the separation, followed by a face centered central composite design (FCCD) for the optimization process. The analytical performances of the newly developed method were verified in terms of accuracy, linearity, precision, repeatability, and sensitivity. The method was used for the determination of VFX enantiomer ratio in pharmaceutical forms. Finally, computer modelling of VFX-CD complexes was undertaken to characterize host–guest chiral recognition.

Keywords: venlafaxine; chiral separation; capillary electrophoresis; cyclodextrins; experimental design

1. Introduction

Chirality plays an important role in modern drug research, as chiral drug enantiomers can exhibit different pharmacokinetic and pharmacodynamic properties. In the case of a racemic mixture the desired pharmacological effect is usually limited to only one of the enantiomers, called eutomer whereas the other enantiomer known as distomer may be inactive, less active, or sometimes can be even responsible for unwanted side effects [1].

Examples of chiral pharmaceuticals with differences between enantiomers activity can be found in many pharmaceutical classes: β-blockers, calcium channel blockers, proton pump inhibitors, H1 antihistamines, anticoagulants, angiotensin converting enzyme inhibitors, and others [2–4]. Among these substances one of the most interesting and intriguing class of chiral pharmaceuticals are the one of modern antidepressants, represented by selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs) [5].

Venlafaxine (1-[2-(dimethylamino)-1-(4-methoxy-phenyl)ethyl]cyclohexanol) (VFX) is a modern antidepressant from the SNRI class used in the treatment of major depressive, generalized anxiety, and panic disorders. VFX is a bicyclic phenylethylamine derivative with an asymmetric carbon atom in its structure, which leads to the existence of two enantiomers, *S*-VFX and *R*-VFX. The stereochemical structures of VFX enantiomers are presented in Figure 1.

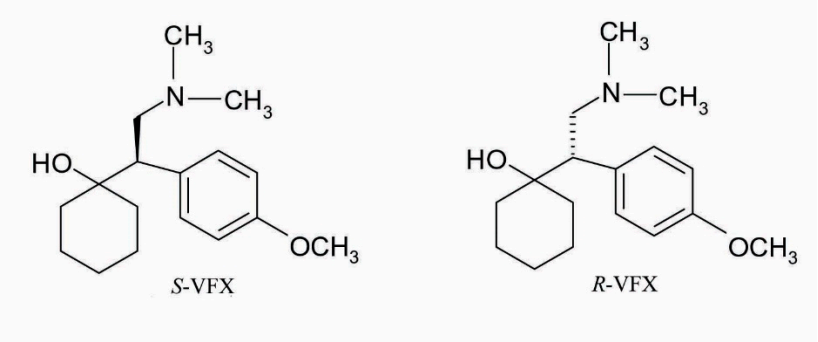

Figure 1. Chemical structures of venlafaxine (VFX) enantiomers.

VFX is used in therapy as a racemic mixture of *R*- and *S*-VFX, however differences have been reported between the pharmacological profiles of the two enantiomers. *R*-VFX is a strong inhibitor for both serotonin and norepinephrine reuptake (SNRI), while *S*-VFX has higher selectivity for serotonin inhibition reuptake (SSRI) [6,7].

VFX main metabolite, *O*-desmethylvenlafaxine (ODVFX) has equal potency to VFX in inhibiting neurotransmitters reuptake and adds to the overall pharmacological effect [8].

Capillary electrophoresis (CE) is a modern analytical technique with wide applications in the chiral analysis of pharmaceuticals; its advantages being attributed to its high separation efficiency, relatively short time of analysis, and low use of analytes and reagents. By comparing it with the more frequently used high performance liquid chromatographic (HPLC) technique, CE has the benefit of lower operating costs. Additionally, CE can be considered a more environmentally friendly method due to the use of small quantities of organic solvents, simple instrumentation set-up, and low energy consumption. In CE, a direct separation approach is almost always used, by simply adding the appropriate chiral selector to the background electrolyte (BGE) [9].

Due to their commercial availability, UV transparency, and excellent complexation capacity, cyclodextrin (CD) derivatives are by far the most widely applied chiral selectors in CE. Nowadays there are a wide variety of CDs available on the market: native and derivatized, neutral and ionized [10].

In the last two decades several CE methods have been published for the chiral separation of VFX enantiomers from different matrices. Simultaneous chiral determination of VFX and ODVFX from clinical samples was resolved using a phosphate-Tris buffer at pH 2.5 and a dual CD system containing an anionic CD, 7.5 mM carboxymethyl-β-CD (CM-β-CD) and a neutral CD, 10 mM α-CD [11] or a 50 mM phosphate buffer at pH 2.5 and 20 mM phosphated-γ-CD (P-γ-CD) as chiral selector [12]. In both cases the disadvantage of the methods is represented by the long migration times, of over 20 min. A capillary electrochromatography (CEC) technique, using vancomycin chiral stationary phase packed capillary, was developed for the simultaneous chiral separation of VFX and ODVFX in plasma samples [13]. A micellar electrokinetic chromatography-electrospray ionization-tandem mass spectrometry (MEKC-ESI-MS-MS) method was used for the enantiodetermination of VFX and ODVFX in plasma, employing a polymeric chiral surfactant [14].

Molecular modelling methods are valuable tools for obtaining information on the interaction energy and geometry of the analyte-CD inclusion complexes; to provide a deeper insight into the interactions between the chiral selector and the enantiomers [15].

The objective of the current study was the development of a new CE method for the chiral separation of VFX, using an experimental design approach for method optimization, molecular

modelling to characterize chiral interactions and to verify the application of the optimized method on pharmaceutical preparations.

2. Materials and Methods

2.1. Chemicals

R,S-Venlafaxine hydrochloride was acquired from Ercros Pharmaceuticals (Barcelona, Spain).

Alpha-CD (α-CD), beta-CD (β-CD), gamma-CD (γ-CD), hydroxypropyl-β-CD (HP-β-CD), randomly methylated-β-CD (RAMEB) were acquired from Cyclolab (Budapest, Hungary); heptakis(2,6-di-o-methyl)-β-CD (DIMEB), heptakis(2,4,6-tri-o-methyl)- β-CD (TRIMEB), carboxymethyl)- β-CD (CM-β-CD) sodium salt (DS≈3) were obtained from Sigma (St Louis, MO, USA). Sulfobuthyl ether β-CD sodium salt (SBE-β-CD) (Captisol®) was donated by Cydex Pharmaceuticals (Kansas, MO, USA).

Phosphoric acid (85%) (Merck, Germany), sodium dihydrogenophosphate (Alfa Aesar, Germany), disodium hydrogenophosphate (Merck, Germany), sodium hydroxide (Lach-Ner, Czech Republic) were used to prepare buffers. Methanol (Merck, Germany) was used to prepare stock and standard solutions. All buffers were prepared using analytical grade reagents. Purified water was prepared using a Milli-Q purification system (Millipore, Burlington, MA, USA).

Alventa (Krka, Slovenia) capsules containing 75 mg *R,S*-VFX were acquired from a local pharmacy.

2.2. Instrumentation

CE measurements were made using an Agilent 1600 CE system (Agilent, Germany) equipped with a diode array (DAD) detector. Data registration was carried out with Chemstation 7.01 software (Agilent, Germany).

A short silica capillary of 30 cm length (effective length 22 cm) × 50 μm internal diameter (Agilent Technologies, Germany) was used in the measurements.

Design Expert 7.0 statistical software (Stat-Ease, Minneapolis, USA) was used to develop experimental design matrix and to analyze results during method optimization. Microsoft Excel Office 365 (Microsoft, USA) was used for statistical analysis during analytical performance testing.

For molecular modelling of inclusion complexes Schrödinger Suite (version 2019-1) tools were used. The crystal structures of β-CD (CCDC number: 181781) [16] and R-VFX (CCDC number: 678316) [17] were obtained from Cambridge Structural Database (CSD) [18] and used as starting geometries. The CM-β-CD structure was prepared by adding carboxymethyl substituents to β-CD. The *S*-VFX was created by inversion of substituents on chiral carbon of the molecule. The starting geometries of the inclusion complexes were build using Maestro, a graphical user interface of Schrödinger Suite. The semiempirical method RM1 was exploited for geometry optimization of all prepared structures. Calculation of interaction energy of optimized complexes was accomplished through dispersion-corrected DFT (density functional theory) method via functional M06-2X-D3 with 6-31** basis set and solvation energy by SM8 method.

2.3. Electrophoretic Procedure

The capillary was conditioned for 30 min with 0.1 N sodium hydroxide, 15 min with deionized water, and 15 min with the BGE, before first use. Between every injection, capillary was preconditioned for 2 min with 0.1 N sodium hydroxide, 1 min with water, and 2 min with the BGE.

Stock solutions of 0.5 mg/mL VFX were prepared in methanol and later diluted to the appropriate concentration. Both buffer and sample solutions were filtered through a 0.45 μm pore size membrane filter and degassed in an ultrasonic bath, prior to use.

UV detection was set at 210, 220, and 240 nm; hydrodynamic injection was performed at the anodic end of the capillary, the detection taking place at the cathode.

A complex CD screening was made to establish the optimum chiral selector. We assessed the use of both neutral (α-CD, β-CD, γ-CD, HP-β-CD, RAMEB, DIMEB, TRIMEB) and anionic CD (CM-β-CD,

SBE-β-CD) as chiral additives; 5 mM of ionized CD derivatives and 10 mM of neutral CD derivatives were added to the BGE at four pH levels (2.5, 5.0, 7.0, 9.0).

Chiral resolution, migration times, and peak symmetry were calculated to determine which CD provides the best enantiomeric separation. Resolution (R) was assessed with the formula:

$$R = 2 \times (t_2 - t_1)/(w_1 + w_2) \text{ at baseline}$$

where t_1 and t_2 are the migration times, and w_1 and w_2 are the peak widths of the two enantiomers.

2.4. Pharmaceutical Sample Preparation

10 capsules were weighed and an average mass was calculated; the powder was mixed in a mortar. An amount of powder equal with the average weight of one capsule was moved to a 20 mL volumetric flask and dissolved in methanol, sonicated for 5 min, and filtered through a 0.45 μm cellulose membrane filter. Before use, the solutions were further diluted to the desired concentration with methanol. The same analytical conditions were applied as for the determination of standard solutions.

3. Results

3.1. Preliminary Analysis

VFX is a basic substance (an amine) (pKa 9.4) and will be ionized in an acidic environment and can be detected over the entire studied pH range (2.5–9.0). Migration times of VFX decreased in an achiral system with the increase of the BGE pH value; at pH above 5.0 where electroosmotic flow (EOF) becomes relevant, VFX migrated in front but close to the EOF.

Chiral interactions were observed with CM-β-CD at all four pH levels and with SBE-β-CD at pH 9.0. Based on the initial results CM-β-CD was chosen as the optimum chiral selector in an acidic BGE (pH 2.5). CM-β-CD (pKa 4.0) is an anionic CD derivative that can be neutral or negatively charged, depending on the pH of the BGE [19].

At pH 2.5 VFX will be positively ionized migrating towards the detector as a cation, CM-β-CD will be uncharged and the effect of EOF will be negligible.

3.2. Method Optimization

Traditionally analytical methods were developed and optimized by using an "one factor at time" (OFAT) approach. In OFAT optimizations each factor is varied within an appropriate range while the other factors are kept constant; the drawback of this approach being that it does not allow evaluation of the interaction between the studied factors [20].

Design of experiments (DoE) approaches have been implemented more and more frequently in the development of analytical methods in the last 20 years; this strategy can produce more reliable results using fewer experiments than in OFAT based on a set of statistical tools [21].

An OFAT strategy was applied for screening purposes to identify the influence of analytical parameters on the analytical responses (chiral resolution, migration times of the two enantiomers) and to identify significant parameters followed by a face centered composite design (FCCD) for optimization.

In the OFAT screening the influence of five analytical parameters (BGE concentration, CD concentration, applied voltage, system temperature, injection pressure) on three analytical responses (chiral resolution, migration times of the enantiomers) was studied. The results obtained in the OFAT screening are presented in Table 1.

Table 1. Results obtained in the "one factor at time" (OFAT) screening for the optimization of VFX enantioseparation.

BGE Concentration [mM]	CD Concentration [mM]	Voltage (kV)	Temperature (°C)	Injection Pressure (mbar/s)	R	t_1 (min)	t_2 (min)
25	5	25	20	50	0.59	3.22	3.27
50	5	25	20	50	0.52	4.45	4.51
100	5	25	20	50	0.50	6.42	6.49
25	10	25	20	50	1.03	4.24	4.34
25	15	25	20	50	0.55	7.13	7.19
25	10	20	20	50	0.98	6.28	6.42
25	10	30	20	50	0.87	2.78	2.84
25	10	15	15	50	1.12	4.59	4.71
25	10	25	25	50	0.92	3.91	3.99
25	10	25	15	40	1.20	4.22	4.31
25	10	25	15	30	1.31	4.21	4.29

Migration times increased as the BGE concentration (25–100 mM) increased with no noticeable impact upon chiral resolution.

Migration times increased with an increase in CD concentration (5–15 mM) but the relationship between CD concentration and chiral resolution was not linear, as resolution increased when increasing CD concentration from 5 to 10 and decreased when increasing CD concentration from 10 to 15. CD concentration has an optimum value, as the difference in the apparent electrophoretic mobility between the two enantiomers will reach a plateau at a certain CD concentration and will decrease at higher CD levels.

With the increase of applied voltage (20–30 kV) the migration times decreased with a small decrease in chiral resolution as well.

Migration time and chiral resolution decreased with the increase of system temperature (15–25 °C), due to the BGE lower viscosity.

Injection parameters influenced the shape and amplitude of the peaks and consequently chiral resolution but had an insignificant effect upon migration times.

Based on the results obtained in the OFAT screening three analytical factors were selected for further optimization using a FCCD: CD concentration (factor A), system temperature (factor B), and applied voltage (factor C). Two analytical responses were registered: chiral resolution (response 1) and migration time of the second migrating enantiomer (response 2).

FCCD is an optimization design in which the star points are at the middle of each factorial space face ($\alpha = \pm 1$), requiring three levels for each factor [20,21].

We conducted a total of 15 experiments where the selected parameters were varied on three levels (−1, 0, +1) with five center point injections: CD concentration (8, 10, 12 mM), system temperature (15, 17.5, 20 °C), and voltage applied (20, 25, 30 kV). The other parameters, considered to be less significant based on the initial OFAT screening results, were kept constant in the optimization experiments: buffer concentration 25 mM, buffer pH 2.5, injection 50 mbar/s.

The experimental design plan and the response factors are presented in Table 2.

A statistical analysis was performed, in order to include or exclude the linear terms (A, B, C), interaction terms (AB, AC, BC), and the quadratic terms (A^2, B^2, C^2) using the variance of analysis model (ANOVA). Based on ANOVA, the following regression models were obtained:

$$R = +1.39 + 0.030^* A - 0.19^* B - 0.14^* A * C - 0.50^* A^2 + 0.068^* B^2 - 0.052^* C^2$$

$$\text{Analysis time (min)} = +5.84 + 2.41^* A - 0.61^* C + 1.25^* A^2 - 0.47^* C^2$$

Table 2. Face centered composite design (FCCD) experimental matrix and responses for the chiral separation of VFX enantiomers.

Run	Factor A	Factor B	Factor C	Response 1	Response 2
1	10.00	17.50	30.00	1.32	4.29
2	8.00	17.50	25.00	0.87	4.35
3	10.00	17.50	25.00	1.36	5.97
4	10.00	17.50	25.00	1.36	5.94
5	12.00	20.00	20.00	0.90	9.75
6	8.00	20.00	30.00	0.82	3.84
7	10.00	20.00	25.00	1.27	5.58
8	10.00	15.00	25.00	1.65	6.20
9	12.00	15.00	30.00	0.98	8.65
10	10.00	17.50	20.00	1.36	5.77
11	8.00	15.00	20.00	0.93	4.91
12	12.00	17.50	25.00	0.92	9.15
13	10.00	17.50	25.00	1.40	5.95
14	10.00	17.50	25.00	1.40	5.95
15	10.00	17.50	25.00	1.42	5.97

The regression models were evaluated based on the determination of the coefficients R^2 and R^2 -adj; values of 0.9958 and 0.9927 for R and 0.9790 and 0.9707 for analysis time, respectively, were obtained. Pred R^2 values of 0.9902 for R and 0.9330 for analysis time were in a reasonable agreement with Adj R^2 values. The values indicate that our models are suitable for navigation in the design space.

3-D response surface plots for the two analytical responses are presented in Figure 2.

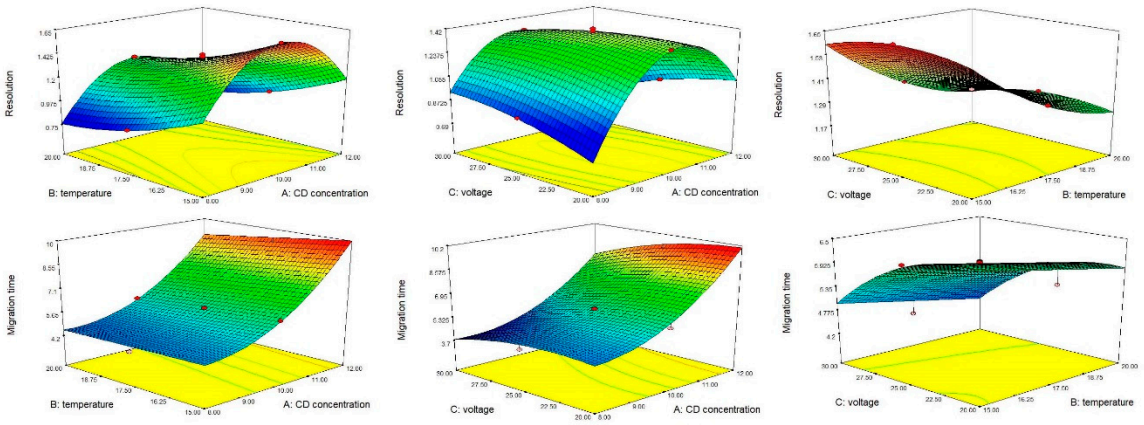

Figure 2. 3-D response surface plots for resolution and migration time.

The error provided by regression model lack-of-fit is significantly smaller than random pure error, indicating that the regression models are fitted.

Derringer's desirability functions were used to maximize and optimize the two analytical responses. The statistical software numerical optimization function was used for setting targets for each analytical response to produce optimal conditions: short migration times and high chiral resolution. Numerical optimization feature will search the design, using the models created in the analysis, generating a list of potential factor settings that provide responses that meet the defined criteria.

The optimum solution generated by the software was the following: 10 mM CM-β-CD concentration, system temperature 15 °C, applied voltage 25 kV. Applying the optimum analytical conditions, we succeeded in the chiral separation of VFX enantiomers in about 6 min with a resolution of 1.64.

A typical electropherogram obtained using the optimized conditions is presented in Figure 3.

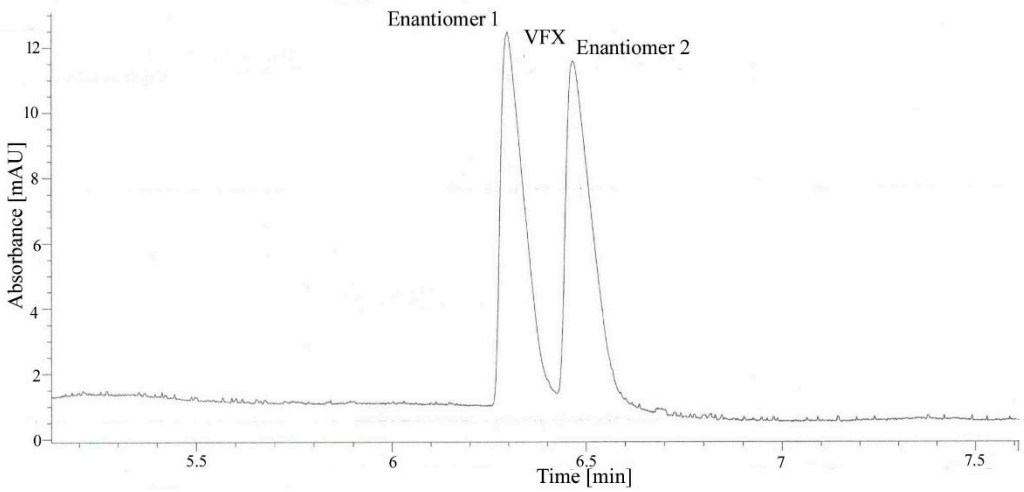

Figure 3. Chiral separation of VFX under optimized condition (analytical conditions: 25 mM phosphate BGE), pH 2.5, 10 mM CM-β-CD, voltage 25 kV, temperature 15 °C, hydrodynamic injection 50 mbar/s, UV detection 230 nm).

Analytical parameters of the optimized method are presented in Table 3.

Table 3. Analytical parameters of VFX enantioseparation (analytical conditions: 25 mM phosphate buffer, pH 2.5, 10 mM CM-β-CD, 15 °C, 25 kV, 50 mbar/s, UV 220 nm).

Enantiomer	Time (min)	Area	Height	Symmetry	Nr Theoretical Plates	Rs	α
1	5.77	48.77	9.60	0.28	36790	-	-
2	5.95	52.61	9.24	0.27	31879	1.64	1.02

The migration order of the enantiomers could not be established by spiking since we did not have pure enantiomers at our disposal.

3.3. Analytical Performance

The analytical performance of the method was evaluated in terms of intra-day and inter-day precision, accuracy, linearity, and limit of detection (LOD) and quantification (LOQ) were calculated.

Intra-day precision was measured by injecting an 0.5 mg/mL racemic VFX standard, six times on the same day; while inter-day precision was checked by injecting an 0.5 mg/mL racemic VFX standard, six times a day for three consecutive days. The precision for migration times and peak area was evaluated through RSD (%) values.

To determine reliability of the method, recovery tests were performed using standard addition method. An appropriate amount of VFX capsule powder was weighed and spiked with a specified amount of the standard and each sample was analyzed in triplicates. The good recovery values are an indication of high accuracy.

A calibration curve was constructed by plotting peak area versus concentration of the analyte; eight different concentrations (0.1–2 mg/mL) were used and measurements were performed in triplicate. Correlation coefficients of over 0.99 indicates a good linearity of the method.

LOD and LOQ were calculated as the standard deviation of regression equation divided with the slope of the regression equation multiplied by 3.3 and 10, respectively.

The results obtained during the verification of analytical performance are summarized in Table 4.

Table 4. Analytical performance of the optimized method.

VFX		Enantiomer 1	Enantiomer 2
Precision			
Intra-day precision (sample concentration = 0.5 mg/mL, n = 6)	RSD%, migration time	0.16	0.17
	RSD%, peak area	1.33	1.30
Inter-day precision (sample concentration = 0.5 mg/mL, n = 18)	RSD%, migration time	0.29	0.30
	RSD%, peak area	1.55	1.71
Accuracy (recovery, %)			
0.5 mg/mL (n = 6)		101.06	99.42
0.25 mg/mL (n = 6)		101.35	101.96
Linearity			
Regression equation (0.1–2 mg/mL)		y = 68.837x+ 2.564	y = 70.916x+4.498
Coefficient of correlation		0.998	0.997
LOD (mg/mL)		0.07	0.06
LOQ (mg/mL)		0.21	0.18

The developed method was applied for the determination of VFX enantiomers in pharmaceutical formulation containing 75 mg *R,S*-VFX racemate. Good agreement between the developed method and the values claimed by the manufacturer was obtained, with an enantiomer ratio of approximately 1:1 (Table 5).

Table 5. VFX enantioselective analysis from pharmaceutical formulation.

Pharmaceutical Product	Declared Enantiomers Quantity (mg)		Found Enantiomer Quantity (mg) ± SD (n = 3)	
	Enantiomer 1	Enantiomer 2	Enantiomer 1	Enantiomer 2
Alventa capsule (75 mg VFX)	37.5	37.5	36.65 +/− 0.56	36.63 +/− 0.29

3.4. Molecular Modelling of VFX-CD Complexes

Molecular modelling methods are useful tools to obtain information on the interaction energy as well as preliminary data of the geometry of the inclusion complexes.

The starting geometries of VFX enantiomers and the CM-β-CD structures based on CSD data are presented in Figure 4.

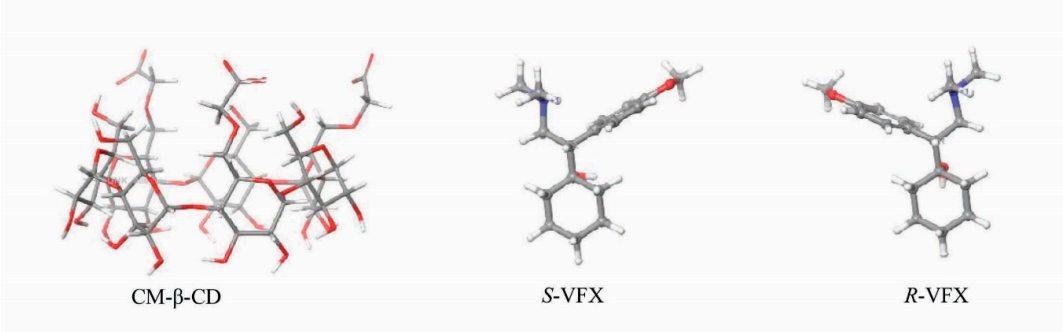

Figure 4. CM-β-CD and *R*-VFX/*S*-VFX structures based on the structures generated from crystallographic parameters provided by CDS.

In the case of CD-analyte complexes, the chiral recognition mechanism is generally based on inclusion complexation where the analyte fits in the CD cavity, so VFX enantiomers were docked in the cavity of the CD, trying to simplify calculations. Two specific inclusion orientations of the guest molecule in the complex were considered, where VFX is inserted at either the wider or the narrower cavity of the CD. To characterize molecular properties of inclusion complexes more accurately, its structure was further optimized using a quantum semiempirical method (RM1).

To improve the accuracy of the theoretical calculations, finding the low-lying energy conformation is mandatory. The energy of the complexation is defined as the energy difference between the optimized complex and the isolated host and guest energies, on their complex conformations.

Computational calculations for the inclusion complexes of the two enantiomers with CM-β-CD showed that differences in the stability of these complexes generates chiral stereoselectivity. Through theoretical calculations we can predict the migration order of the enantiomers, based on the establishment of the more stable the inclusion complex. Due to the difference in energy between *S*-VFX and *R*-VFX (13.12 kcal/mol) complexes with CM-β-CD, it was established that the inclusion of *S*-VFX is energetically more favorable by 13.12 kcal/mol (−66.47 kcal/mol for *S*-VFX by comparison with −53.35 kcal/mol for *R*-VFX). This indicates that S-VFX fits more closely into the cavity of the CD and this selective interaction allows chiral discrimination. Taking into consideration these results, we can conclude that the migration order is *R*-CIT followed by *S*-CIT.

Structures of the optimized CM-β-CD inclusion complex with *S*-CIT and *R*-CIT are shown in Figure 5.

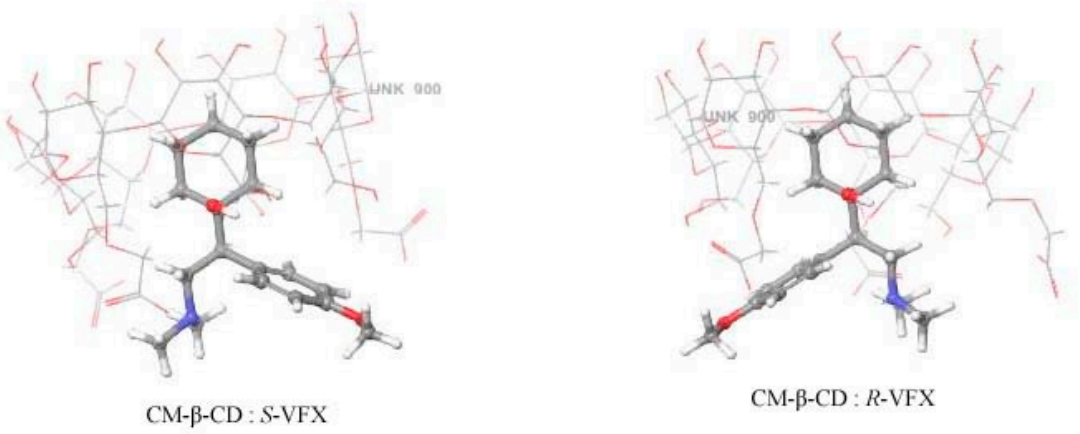

Figure 5. Energy minimized structures obtained from RM1 calculations for the *R*-VFX-CM-β-CD and *S*-VFX-CM-β-CD complexes.

4. Discussion

A fast, cost-effective CE method for the enantioselective analysis of VFX was developed using a simple phosphate buffer electrolyte and normal polarity. The method was developed using a preliminary CD screening at four different pH levels to determine the best chiral selector, an OFAT strategy was used to establish the significant analytical parameters and their influence on the separation and a FCCD for optimization purposes. The analytical performance of the developed method was verified, and the method was applied for the enantioselective evaluation of VFX in pharmaceuticals.

The method also demonstrates the utility of using design of experiments strategies in analytical method development, with advantages related to a high level of accuracy in the estimation of effects for a given number of trials and assessment of interactions between experimental factors.

Our CE method provides a shorter analysis time (6 min) if compared with other CE methods published previously in the literature. When compared to HPLC enantioseparation methods of VFX, our proposed method exhibits less sensitivity, but nevertheless delivers fast analysis time, high separation efficiency, and minimization of analyte and solvent use.

From our molecular modelling calculations, it can be concluded that the stability of the VFX-CD inclusion complexes enables chiral discrimination and leads to differences in the migration times of the complexes.

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