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Separation of vinca alkaloid enantiomers by capillary electrophoresis applying cyclodextrin derivatives and characterization of cyclodextrin complexes by nuclear magnetic resonance spectroscopy

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ABSTRACT

In this work, the enantiomeric separation of three vinca alkaloid enantiomers (vincamine, vinpocetine and vincadifformine) has been investigated in an aqueous capillary electrophoresis (CE) system using cyclodextrins (CDs). The investigated CDs were the native α-, β-, and γ-CDs and their hydroxypropylated, randomly methylated, carboxymethylated and sulfobutylated derivatives. The first part of this study consisted of the determination of the apparent averaged complex stability constants with the selected CDs. Several parameters, such as the nature and the concentration of the CD, were studied and were found to have a significant effect on the enantiomeric resolution for all studied compounds. All three vinca alkaloids were successfully enantioseparated with CDs where different migration orders were observed in case of several CDs depending on the cavity size or substituent of the host. Chiral separation and determination of the stability constants were also performed with NMR spectroscopy which confirmed the CE results. Averaged stoichiometries of the complexes were determined using the Job plot method resulting in a 1:1 complex irrespective of the alkaloid enantiomers or cyclodextrin derivative. The structures of the inclusion complexes were elucidated using 2D ROESY NMR spectroscopy. On the basis of NMR results reversal of enantiomer migration order was clarified in various cases.

1. Introduction

Vinca alkaloids (e.g. vinpocetine, vincamine, see Fig. 1) are widely marketed as supplements for vasodilatation and nootropics or neuroprotective agents [1]. Although these alkaloids can be extracted from plants and used as semisynthetic precursors for the preparation of related structures [2,3], they are industrially produced by stereoselective total synthesis. The members of the enantiomeric pairs frequently show rather different biological effects, so their pharmacological study and analysis is necessary. Capillary electrophoresis (CE) has been increasingly employed for the chiral separation of pharmaceutical agents and drugs. To resolve enantiomers, two methods are employed: indirect and direct separation [4]. Indirect chiral separation is a method in which the enantiomers are derivatized with an optically pure reagent into diastereomers. Diastereomers have different chemical properties, and thus can be separated using an achiral method. The other method is the direct chiral separation, wherein a chiral selector can be added to the background electrolyte (BGE), bound to the capillary wall, or included in a gel matrix. The direct separation of enantiomers by CE is often more facile than the indirect separation. The most powerful and widely used chiral selectors for CE are cyclodextrins (CDs) and their derivatives [5,6]. CD derivatives can be synthesized with varying functional groups, degree of substitution, and position of the modification. Native CDs and their neutral and charged derivatives are currently available as chiral selectors for CE. All the CDs used in this study are listed with the applied abbreviations in the materials section. Derivatized CDs can be randomly substituted or selectively derivatized, termed multicomponent mixtures or single-isomer CD, respectively. Randomly substituted mixtures offer the advantages of being readily available and less expensive, but are less suitable for mechanistic studies and method validation. Single-isomer derivatives are more expensive, however, these chiral selectors are better suited to mechanistic studies and method validation.

Capillary electrophoresis is a powerful technique for the analysis of chiral compounds, still, few studies are known concerning the

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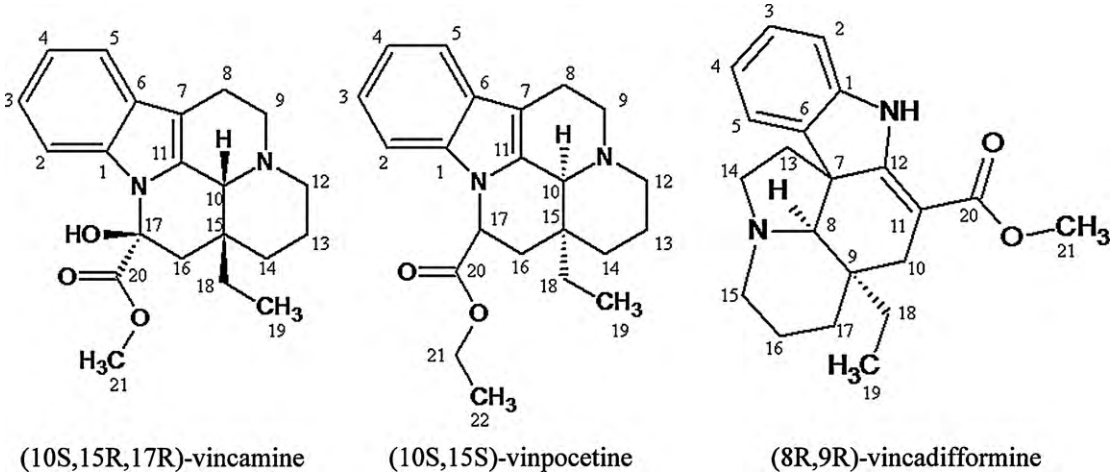

Fig. 1. Structure and numbering of vincamine, vinpocetine and vincadifformine.

CE analysis of vinca alkaloids [7,8]. In contrast with CE, numerous chromatographic methods have been developed for their analysis [9–11].

In consideration of the various methods to study inclusion complexation, ^{1}H NMR spectroscopy is one of the most powerful. This analytical technique allows the quantitation of equilibria between CD hosts and various guests [12] in terms of their stability constant with a dynamic range from 10 to 10^4 [13]. Incorporation of datasets of several nuclei into the simultaneous equation results in more robust estimates of stability constants. At the same time, the observed chemical shift changes ($\Delta\delta$) may provide insight into the structure of the complexes [12]. The averaged stoichiometry and binding constant of the host/guest complex can be readily obtained from ^{1}H NMR titration data [13–15].

The averaged stoichiometries of the complexes were investigated with the continuous variation method (Job plot) in case of vinpocetine/ME-β-CD (randomly methylated β-CD) and vincadifformine/HP-γ-CD ((2-hydroxy)propyl-γ-CD) complexes with both alkaloid enantiomers [16]. Information about the geometry of the complex and direction of inclusion into the CD cavity was obtained from 2D ROESY NMR data. In the case of inclusion complex formation, the alkaloid penetrates the cavity of the cyclodextrin derivative where spatial proximities can be detected between the inner cavity cyclodextrin protons (H-3 and H-5) and the appropriate protons of the guest using NMR. In 2D rotating frame Overhauser effect spectroscopy (ROESY), proximities between protons at distances <5 Å are detected as cross-peaks, identifying a real inclusion and the moiety of the guest involved in the complexation [12,15,17].

Cyclodextrin complexation has already been investigated by Nie et al. [18], Ribeiro et al. [19] and Kata and Selmeczi [20] between vinpocetine and β-CD (K = 75 mM^{-1}), γ-CD, HP-β-CD ((2-hydroxy)propyl-β-CD) (K = 282 mM^{-1}), and SBE-β-CDs (sulfobutyl-β-CD) (K = 340 mM^{-1}). The Job plot analysis demonstrated a 1:1 stoichiometry between vinpocetine and both β-CD and SBE-β-CD. Structural analyses based on ^{1}H and 2D ROESY NMR experiments indicate that the aromatic ring of the alkaloid enters the CD cavity from the wider rim, and, in case of SBE-β-CD, the side-chain of the CD takes part in the complexation.

The aims of this study are to report the complexation properties of three vinca alkaloids with a wide variety of CDs and the chiral separation of their enantiomers. This will afford deeper insight into the molecular interactions between the guest and host molecules focusing on the effect of various CD cavity sizes and side-chains.

2. Experimental/materials and methods

2.1. Materials

Buffer components of CE background electrolyte are sodium hydroxide (NaOH), phosphoric acid (H_3PO_4), purchased from Merck GmbH (Darmstadt, Germany). Cyclodextrins [α-cyclodextrin (α-CD), β-CD (β-cyclodextrin), γ-cyclodextrin (γ-CD), carboxymethyl-α-CD (DS ~ 3) (CM-α-CD), carboxymethyl-β-CD (DS ~ 3.5) (CM-β-CD), carboxymethyl-γ-CD (DS ~ 3) (CM-γ-CD), sulfobutyl-β-CD (DS ~ 4) (SBE-β-CD), sulfobutyl-γ-CD (DS ~ 4) (SBE-γ-CD), (2-hydroxy)propyl-α-CD (DS~3) (HP-α-CD), (2-hydroxy)propyl-β-CD (DS ~ 3) (HP-β-CD), (2-hydroxy)propyl-γ-CD (DS ~ 3) (HP-γ-CD), methylated α-CD (DS~12) (ME-α-CD), methylated β-CD (DS ~ 12) (ME-β-CD) and methylated γ-CD (DS~12) (ME-γ-CD)] were produced by CycloLab R&D Ltd. (Budapest, Hungary). As both an NMR solvent and to adjust pH, D_2O (>99.9 atom% D) and DCl (20% m/m) from Sigma were used. Methanol (MeOH) used in NMR titrations as the inner reference and H_3PO_4 as the main buffer component in Job plot experiments and NMR titrations were purchased from commercial suppliers. 2D ROESY spectra were processed with MestreNova 5.3.1-4825 software.

2.2. Capillary electrophoresis

CE experiments were carried out on a Hewlett Packard ^{3D}CE system (Hewlett Packard, Waldbronn, Germany) with a diode array UV detector at 25 °C. Uncoated fused-silica capillary (FSOT) 33.5 cm (25 cm effective length) and 50 μm I.D. (375 μm O.D.) (Composite Metal Services Ltd., Worcestershire, UK) were used throughout the study. On-line UV detection was set to 200 nm. 15 mM NaOH (pH adjusted to 2.5 with 10% (w/w) aqueous H_3PO_4) was used as a background electrolyte, to which the CDs were added in appropriate concentrations (ranging from 0.25 to 50 mM). All buffer solutions were filtered with a 0.2 μm membrane. Between measurements, the capillary was subsequently rinsed with 1 M NaOH, 0.1 M NaOH, and MilliQ-water for 2 min each followed by BGE for 5 min. The vinca alkaloid sample solutions were prepared by dissolving each analyte at a concentration of 1 mg/ml in MeOH, and after a tenfold dilution, introduced hydrodynamically (50 mbar pressure for 4 s). CE experiments were performed in positive-polarity mode with 15–30 kV voltage depending on the actual cyclodextrin concentration.

Table 1
Apparent averaged complex stability constants of vinpocetine, vincamine and vincadifformine formed with α-, β- and γ – CD (second column) and their derivatives, with the resolution of the enantiomers (K_1 – stabilitiy constant of first migrating enantiomer, K_2 – stabilitiy constant of second migrating enantiomer, R_S – resolution of enantiomers).

Alkaloid	Substituent	–	HP	ME	SBE	CM
Vinpocetine	α-CD	K_1 = 33.7 (3)	K_1 = 36 (1)	K_1 = 115 (2)	–	K_1 = 41 (5)
		K_2 = 33.7 (3)	K_2 = 38 (1)	K_2 = 115 (2)	–	K_2 = 41 (5)
		R_S < 0.5	R_S = 1.16	R_S < 0.5	–	R_S < 0.5
	β-CD	K_1 < 0.5	K_1 = 13 (1)	K_1 = 35 (1)	K_1 = 1130 (2)	K_1 = 162.0 (1)
		K_2 < 0.5	K_2 = 14 (2)	K_2 = 52 (1)	K_2 = 1130 (2)	K_2 = 163.7 (1)
		R_S < 0.5	R_S = 1.51	R_S = 2.84	R_S < 0.5	R_S = 1.01
	γ-CD	K_1 = 7 (2)	K_1 = 6.2 (2)	K_1 = 7.1 (2)	K_1 = 8500 (5)	K_1 = 127 (1)
		K_2 = 13 (1)	K_2 = 6.9 (4)	K_2 = 8.8 (3)	K_2 = 9500 (4)	K_2 = 137 (1)
		R_S = 1.84	R_S = 0.91	R_S = 0.91	R_S = 4.08	R_S = 3.81
Vincamine	α-CD	K_1 = 9.0 (2)	K_1 = 6 (2)	K_1 = 41 (1)	–	K_1 = 32 (1)
		K_2 = 11.3 (1)	K_2 = 9 (3)	K_2 = 49 (1)	–	K_2 = 40 (1)
		R_S = 0.97	R_S = 1.78	R_S = 1.11	–	R_S = 7.78
	β-CD	K_1 < 0.5	K_1 = 7 (1)	K_1 = 14 (1)	K_1 = 880 (4)	K_1 = 191 (1)
		K_2 < 0.5	K_2 = 7 (1)	K_2 = 14 (1)	K_2 = 1110 (5)	K_2 = 198 (1)
		R_S < 0.5	R_S < 0.5	R_S < 0.5	R_S = 3.86	R_S = 2.05
	γ-CD	K_1 = 8 (1)	K_1 < 0.5	K_1 < 0.5	K_1 = 1600 (4)	K_1 = 275 (3)
		K_2 = 8 (1)	K_2 < 0.5	K_2 < 0.5	K_2 = 2800 (6)	K_2 = 292 (2)
		R_S < 0.5	R_S < 0.5	R_S < 0.5	R_S = 7.84	R_S = 3.39
Vincadifformine	α-CD	K_1 = 18.2 (2)	K_1 = 35 (1)	K_1 = 72 (1)	–	K_1 = 21 (5)
		K_2 = 18.2 (2)	K_2 = 35 (1)	K_2 = 72 (1)	–	K_2 = 26 (6)
		R_S < 0.5	R_S < 0.5	R_S < 0.5	–	R_S = 3.3
	β-CD	K_1 < 0.5	K_1 = 27 (2)	K_1 = 16 (1)	K_1 = 1360 (2)	K_1 = 72 (1)
		K_2 < 0.5	K_2 = 28 (1)	K_2 = 23 (1)	K_2 = 1360 (2)	K_2 = 79 (1)
		R_S < 0.5	R_S ~ 0.5	R_S = 2.09	R_S < 0.5	R_S = 4.05
	γ-CD	K_1 = 20 (1)	K_1 = 12.7 (4)	K_1 = 10.2 (2)	K_1 = 4300 (1)	K_1 = 94 (2)
		K_2 = 38 (1)	K_2 = 51 (1)	K_2 = 21.2 (5)	K_2 = 7500 (1)	K_2 = 128 (1)
		R_S = 2.22	R_S = 8.54	R_S = 4.08	R_S = 5.39	R_S = 25.1

Uncertainties in parentheses are estimated standard deviations of the last significant digit.

2.3. Nuclear magnetic resonance spectroscopy

All NMR experiments were carried out on a 600 MHz Varian VNMRS spectrometer equipped with a dual 5-mm inverse-detection gradient (IDPFG) probehead. For structure identifications, standard pulse sequences and processing routines available in VnmrJ 2.2C/Chempack 4.0 were used. In all experiments, the probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used. The NMR titrations and Job plot experiments were carried out in H_2O/D_2O 9/1 (15 mM phosphate buffer), as solvent suppression presaturation sequence was used. ROESY experiments were carried out in D_2O (pD adjusted to 2.5 with DCl). The NMR titrations were carried out in 0.1 M NaCl solution to ensure constant ionic strength. Identification of the enantiomers was achieved by spiking the samples with the single *S* enantiomer. For Job plot analysis stock solutions of 2 mM alkaloid (single isomer) and 2 mM CD derivative were prepared and mixed in varying ratios. 32–128 scans (depending on the experiment) with a spectral window of 4800 Hz were collected into 32,000 data points, giving a digital resolution of 0.3 Hz/point. ROESY spectra of a 5 mM alkaloid single enantiomer, 3 mM CD derivative sample were recorded using a mixing time of 300 ms during a spin-lock of 2.2 kHz. 320 increments were collected with 32 repetitions and the measured data matrix was processed as a matrix of 4K (F2) and 1K (F1) data points. Intermolecular NOEs between alkaloid and CD protons involved in the host-guest interaction were detected as cross-peaks. The samples were equilibrated for 12 h before all NMR experiments.

3. Results and discussion

3.1. Determination of the stability constants by capillary electrophoresis

The apparent averaged complex stability constants assisted in optimizing several of the separation parameters including peak symmetry and the number of theoretical plates, so their determination was necessary to achieve desired enantioseparations. Assuming 1:1 averaged stoichiometry for the complex between the host CD and the guest, the effective mobility then depends on the CD concentration according to Eq. (1) [21]:

$$\mu_{\text{eff}} = \frac{\mu_{\text{free}} + \mu_{\text{cplx}} K[\text{CD}]}{1 + K[\text{CD}]} \quad (1)$$

where μ_{eff} is the effective mobility, μ_{free} and μ_{cplx} are the effective mobilities of the free and fully complexed guest, respectively, K is the apparent averaged complex stability constant, and [CD] is the molar concentration of the CD type selector. Apparent complex stability constants were determined according to the *x*-reciprocal method by plotting the data in the form $(\mu^i_{\text{eff}} - \mu_{\text{free}})/[\text{CD}]$ vs. $(\mu^i_{\text{eff}} - \mu_{\text{free}})$, yielding $-K$ as the slope [21]. The optimal selector concentration for the separation of enantiomers in CE can be expressed using the following Eq. (2) [22]:

$$c_{\text{opt}} = \frac{1}{\sqrt{K_1 \cdot K_2}} \quad (2)$$

where K_1 and K_2 are the apparent stability constants of complexes for the two enantiomers.

The apparent averaged complex stability constants of the vinca alkaloid complexes formed with 14 different CDs were determined from CE data, summarized in Table 1. The complex stability constants with the native CDs show that the most suitable cavity size for vinpocetine and vincamine is that of the α-CD, while vincadifformine prefers that of γ-CD. The derivatization of native CDs increased the stability constant in almost all cases, where vinca alkaloids often formed stronger complexes with derivatized CDs than with the native CDs.

The most stable complexes were formed with sulfobutylated derivatives. Under the experimental conditions these CDs are anionic therefore the interaction with the cationic guests is fortified by ionic interactions. The CM-CDs are also negatively charged, however, their degree of substitution (DS) is not as high as that

of SBE-CDs and thus the charge density is lower. While CM-α-CD yields lower stability constants than ME-α-CD, the CM-β-CD and CM-γ-CD demonstrate higher affinities toward all three vinca alkaloids than toward the proper randomly methylated derivatives. Comparing the neutral derivatives, the superiority of random methyl substitution is evident except in the case of vincadifformine, which forms a complex of higher stability with HP-β-CD and HP-γ-CD than with ME-β-CD and ME-γ-CD, respectively. Compared to the previously reported data by Nie et al. [18] and Ribeiro et al. [19], the stability constant of vinpocetine determined by CE was significantly lower in the case of β-CD ($K = 75\ \mathrm{mM}^{-1}$ vs. $K < 0.5$) and HP-β-CD ($K = 286\ \mathrm{mM}^{-1}$ vs. $K = 14\ \mathrm{mM}^{-1}$) and higher with SBE-β-CD ($K = 340\ \mathrm{mM}^{-1}$ vs. $K = 1130\ \mathrm{mM}^{-1}$). These differences can be attributed to the different experimental conditions (e.g. pH), different methods applied (capillary electrophoresis vs. solubility studies), and in case of the substituted CDs, the varying degree of substitution.

3.2. Enantioseparation of vinca alkaloids

The separation of the two enantiomers can take place only if the observed mobility of the enantiomers differ ($\Delta\mu \neq 0$), causing the two analytes to migrate with different velocities [6,23]. Mobility differences between two enantiomers ($\Delta\mu$) can be expressed by the following equation in chiral selective CE [6,22,24]:

$$\Delta\mu = \mu_R - \mu_S = \frac{\mu_f + \mu_{cR}K_R[\mathrm{C}]}{1 + K_R[\mathrm{C}]} + \frac{\mu_f + \mu_{cS}K_S[\mathrm{C}]}{1 + K_S[\mathrm{C}]} \quad (3)$$

where μ_R and μ_S are the observed mobilities of the two enantiomers, μ_f is the mobility of the analyte in free form, K_R and K_S are the complex stability constants of the enantiomers with the cyclodextrin, μ_{cR} and μ_{cS} are the mobilities of the temporary diastereomeric complexes, and [C] is the concentration of the chiral selector. Based on the preceding equation the separation can take place in one of two cases: either the stability constants of the diastereomeric complexes of the enantiomers or the mobilities of the complexes must differ. A combination of both scenarios may apply as well.

The separation of enantiomers was characterized by resolution (R_s), which can be calculated by the following equation:

$$R_s = 1.18\left(\frac{t_2 - t_1}{w_1 + w_2}\right) \quad (4)$$

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

Most of CDs studied showed enantioselectivity, however, the best enantioseparations were achieved with ionizable carboxymethylated and sulfobutylated derivatives. The highest R_S values were obtained by SBE-γ-CD, SBE-γ-CD, and CM-β-CD and CM-γ-CD for vinpocetine, vincamine and vincadifformine, respectively. Among the native CDs only γ-CD could resolve the enantiomers of vinpocetine and vincadifformine, whereas none of the native CDs were selective for vincamine enantiomers. α-CD and γ-CD derivatives proved to be principally selective for vincamine and vincadifformine, respectively. The R_S values are presented in Table 1.

In almost all cases the resolution increased with the concentration of the CD (Fig. 2), showing that the optimal concentration of the host has not been reached yet [22].

3.3. Reversal of migration order

The reversal of the affinity pattern of enantiomers is the most dramatic change that may appear due to any chemical or structural modification of the chiral selector. In many cases even minor

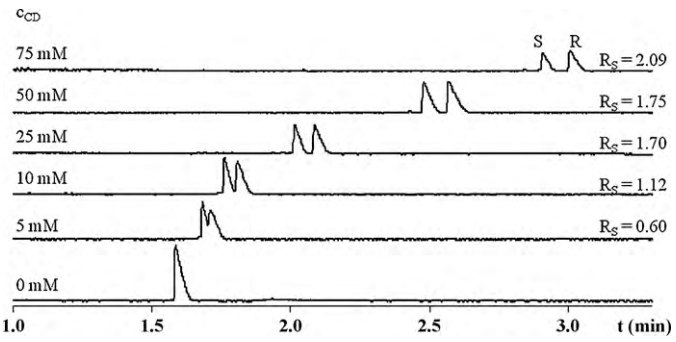

Fig. 2. Cyclodextrin concentration-dependent resolution in the enantioseparation of vincadifformine with ME-β-CD. BGE: 15 mM NaOH (pH 2.5-H_3PO_4) containing 0–75 mM selector.

structural or chemical modification of a chiral selector may dramatically affect its chiral recognition properties and revert the affinity pattern of analytes towards it. The screening of the affinity pattern of a wide range of chiral analytes towards CD-type hosts using CE revealed that the affinity pattern may change depending on the type and position of the substituent on the CD rim and even depending on the cavity size of the CD [25,26]. Examples of cavity size dependent, substituent dependent and substituent location dependent reversals of the molecular recognition are widely known in the literature. In case of cavity size-dependent reversals intermolecular selector–selectand interactions are the same and the distance between noncovalently interacting groups may change from CD to CD [26–29]. Introduction of substituents on the CD rims affects the cavity size, as well as the nature of intermolecular forces involved in host-guest interactions and possibly leads to the reversal of the migration order [15,26,30–36]. The observation that not only the nature of substituents but also their location on the CD rim may determine the enantiomer affinity pattern of some chiral analytes was made by Tanaka et al. [37–39] and the phenomenon was confirmed later for acetyl and sulfate groups as substituents by Chankvetadze et al. [40,41]. The potential for reversal of migration order holds great significance in chiral analysis since the main component can disturb the analysis of the minor component via system overloading or because of heading/tailing peaks [42].

Cavity size-dependent reversal of migration order was observed in three cases: vinpocetine with HP-β-CD and HP-γ-CD; vincadifformine with HP-β-CD and HP-γ-CD; vincadifformine with ME-β-CD and ME-γ-CD (Fig. 3).

In the case of vinpocetine the fastest migrating enantiomers were the (−)-enantiomer with HP-β-CD and the (+)-enantiomer with HP-γ-CD, indicating that the chiral recognition of the two CDs is different. HP-β-CD formed a more stable complex with the (+)-enantiomer, while HP-γ-CD with (−)-enantiomer. The more stable complex resides in the capillary longer and thus migrates more

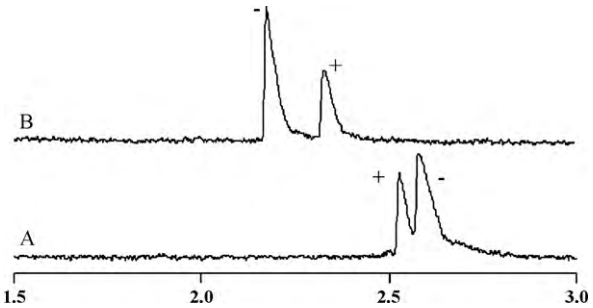

Fig. 3. Enantioseparation of vincadifformine enantiomers and reversal of migration order in the presence of 75 mM ME-β-CD (A) and 50 mM ME-γ-CD (B). BGE: 15 mM NaOH (pH 2.5-H_3PO_4).

Table 2
^{1}H and ^{13}C NMR assignments of the alkaloids in D_2O (pD set to 2.5 with DCl) referenced to MeOH signal (3.300 ppm).

No.	Vincamine			Vinpocetine			Vincadifformine		
	^{13}C	^{1}H	m, *J* (Hz), int.	^{13}C	^{1}H	m, *J* (Hz), int.	^{13}C	^{1}H	m, *J* (Hz), int.
1	134.5			134.5			134.2		
2	111.4	7.15	d, (6.1), 1H	112.7	7.17–7.21	d, 1H	129.2	7.18	t, (7.7) 1H
3	123.5	7.19	dd, (2.0, 6.1), 1H	123.5	7.16–7.20	dd, 1H	110.6	6.89–6.93	m, 1H
4	121.4	7.16	dd, (2.0, 6.4), 1H	121.3	7.14–7.18	dd, 1H	120.4	6.90–6.94	m, 1H
5	119.2	7.53	d, (6.4), 1H	119.1	7.47	d, (7.2) 1H	121.7	7.33	d, (6.9) 1H
6	127.1			124.6			132.8		
7	105.2			107.8			110.1		
8	45.5	3.05–3.07	m, 2H	45.1	3.02–3.06	m, 2H	69.6	3.90	s, 1H
9	51.2	3.61–3.63	m, 2H	51.4	3.57–3.60	m, 2H	34.7		
10	59.6	3.92	s, 1H	56.7	3.88	s, 1H	29.3	2.38–2.40	m, 2H
11	124.4			128.3			37.7		
12	45.0	2.95	ddd, (5.2, 5.6, 11.1), 1H	45.1	2.82	ddd, (4.0, 5.4, 12.3), 1H	91.3		
		3.15	ddd, (5.2, 5.6, 11.1), 1H		3.11	ddd, (4.0, 5.4, 12.3), 1H			
13	27.9	1.57–1.59	m, 1H	25.9	1.52–1.55	m, 1H	28.3	2.36–2.39	m, 2H
		1.78–1.80	m, 1H		1.72–1.74	m, 1H			
14	22.8	1.59–1.61	m, 2H	25.8	1.73–1.76	m, 2H	42.4	2.12–2.19	m, 1H
								2.49–2.56	m, 1H
15	35.3			38.0			47.7	3.34–3.39	m, 2H
16	42.0	2.22	d, (14.5), 1H	44.2	2.81	d (15.0), 1H	24.0	1.72–1.77	m, 1H
		2.35	d, (14.5), 1H		2.96	d (15.0), 1H		1.90–1.96	m, 1H
17	82.2			87.4	4.32	dd, (6.7, 14.1), 1H	20.3	1.46–1.53	m, 1H
								1.93–1.98	m, 1H
18	27.3	1.42	dd, (7.2, 14.4) 1H	17.3	1.53	dd, (7.2, 14.6) 1H	31.7	0.79	dd, (7.0, 14.2), 1H
		1.9	dd, (7.2, 14.4) 1H		1.73	dd, (7.2, 14.6) 1H		0.90	dd, (7.0, 14.2), 1H
19	6.3	0.83	dd, (7.2, 7.2), 3H	7.4	0.90	dd, (7.2, 7.2), 3H	5.7	0.46	dd, (7.0, 7.0), 3H
20	173.1			164.0			169.6		
21	71.7	3.73	s, 3H	63.4	4.27–4.37	m, 2H	51.6	3.66	s, 3H
22				15.3	1.24	t, (7.2), 3H			

slowly. For vincadifformine, the chiral recognition was the opposite. The migration order was (+), (−) with HP-β-CD and ME-β-CD and (−), (+) with HP-γ-CD and ME-γ-CD. HP-β-CD and ME-β-CD formed a more stable complex with the (−)-enantiomer, while HP-γ-CD and ME-γ-CD with the (+)-enantiomer.

Substituent dependent reversal of migration order was observed in the case of vinpocetine with native and hydroxypropylated γ-CD. With γ-CD the migration order was (−), (+) and with HP-γ-CD (+), (−), suggesting that (−)-vinpocetine formed a more stable complex with HP-γ-CD than γ-CD.

The migration order of enantiomers with sulfobutylated CD derivatives could not be determined experimentally because their high stability constants demanded the use of low concentration of chiral selectors (in concert with Wren mobility difference theory) resulting in significant distortions of peaks [22].

3.4. ^{1}H NMR titrations

For the determination of stability constants and detection of the separated enantiomers ^{1}H NMR spectroscopy is another suitable method. NMR spectra were referenced to the residual methanol signal (3.300 ppm), with the advantage of a reference without any significant interaction with CDs in minute concentrations [43,44]. Full assignment of the alkaloid protons is presented in Table 2. Samples of racemic alkaloids and different CDs were prepared with a molar ratio [CD]/[alkaloid] ranging from 1.2 to 166 while the ionic strength was kept constant. The observed chemical shift (δ^{obs}) of the carbon bonded protons of the host and guest molecules at various CD concentration ratios are the weighted average of the free (δ_L) and bound ($\delta_{L\text{-}CD}$) species (5) [45]:

$$\delta^{obs} = \delta_L x_L + \delta_{L\text{-}CD} x_{L\text{-}CD} = \frac{\delta_L + \delta_{L\text{-}CD} K_{L\text{-}CD}[CD]}{1 + K_{L\text{-}CD}[CD]} \quad (5)$$

Based on the observed chemical shifts and the concentration of the alkaloid and CD, the binding constants were calculated with the OPIUM program [46].

The stability constants determined from NMR datasets are collected in Table 3.

These experiments served as a confirmation of only the CE results, as a significant number of the constants are out of NMR's dynamic range. As the stability constants frequently differ for the enantiomers, higher CD concentrations cause the splitting of the signals resulting in two sets of signals. Representative ^{1}H NMR spectra of the resolution of vincadifformine enantiomers upon increasing HP-γ-CD concentrations are depicted in Fig. 4.

3.5. The Job's method

The continuous variation method was adopted to verify the stoichiometry of the complexes. The ^{1}H chemical shifts (δ) were measured at different ratios of [alkaloid]/[CD], while keeping the total [alkaloid] + [CD] constant. The calculated quantities ($\Delta\delta$ [alkaloid] or $\Delta\delta$ [CD]) were plotted as a function of molar ratio. The resulting plots have a maximum at 0.5 indicating 1:1 binding stoichiometry (independent of alkaloid or CD derivative). These results confirm the previously reported findings of Ribeiro et al. [19] Representative Job plot curves of the two vinpocetine enantiomers and the ME-β-CD are shown in Fig. 5.

3.6. 2D ROESY NMR studies

Deeper insights into the geometries of alkaloid–CD complexes can be derived from the spatial proximities of protons on the host and guest molecules. ROESY experiments were used to provide use-

Table 3
Complex stability constants determined by ^{1}H NMR chemical shift titrations.

Alkaloid	CD derivative	*K* (+)	*K* (−)
Vincadifformine	CM-γ-CD	85 (5)	10 (1)
Vincadifformine	HP-γ-CD	74 (9)	19 (4)

Uncertainties in parentheses are estimated standard deviations of the last significant digit.

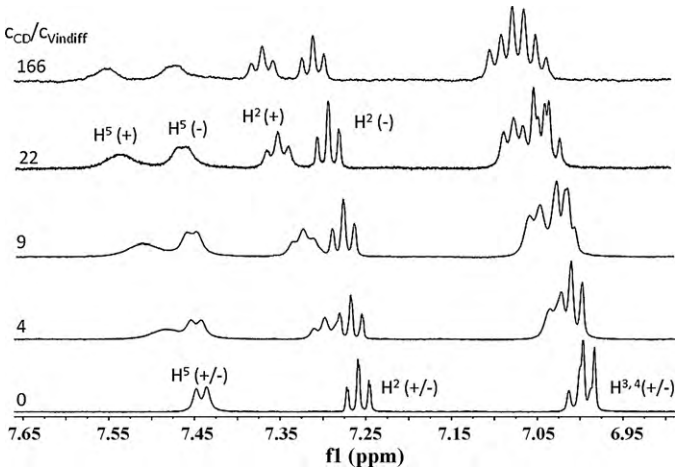

Fig. 4. ^{1}H NMR titration of racemic vincadifformine with HP-γ-CD (D_2O, pD = 2.5).

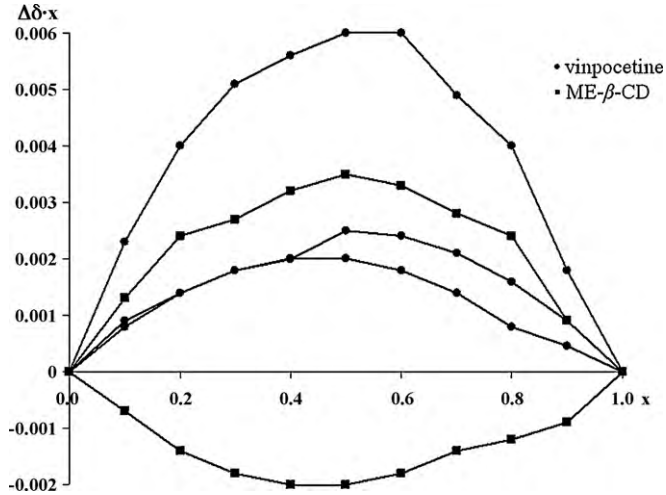

Fig. 5. Representative Job plot curves of the two vinpocetine enantiomers with ME-β-CD.

ful information concerning the supramolecular system structures present in solution, namely the direction of inclusion of the alkaloid in the host cavity and the moieties involved in the complexation. First, the potential differences between the complexation of the two enantiomers were studied. The cross-peaks in the representative spectrum of (−)-vincadifformine and HP-γ-CD ($K = 13\ mM^{-1}$) indicate, that the guest enters the CD cavity from the wider rim with its aromatic portion (Fig. 6). The aromatic cross-peaks are also observed in the spectrum of the (+)-enantiomer ($K = 51\ mM^{-1}$), where intensive cross-peaks appear between inner cavity CD and alkyl vincadifformine protons (H^{18} and H^{19}). The assignment of vincadifformine validated that these alkyl signals belong to the ethyl moiety of the alkaloid. This suggests that the (+)-enantiomer has a favorable structure enabling it to form complexes with both the aromatic ring and the alkyl side-chain (Fig. 7). The alkyl chain of the molecule can enter the CD cavity from the wider rim as well, based on the intensity of the cross-peaks. This additional complex forming possibility may explain the higher stability of the (+)-enantiomer. According to the Job plot results (1:1 complex stoichiometry), the two possible complex structures coexist in solution where 2:1 (CD:alkaloid) complexes are not formed.

Looking for the structural and chemical reasons of the enantiomer migration order (EMO) i.e., finding the relationships between the structure of a chiral selector, chiral analyte and analyte–selector complex on the one hand, and the EMO on the other hand, may provide important information regarding the nature of intermolecular forces involved in complex formation and enantioselective recognition of analytes by chiral selectors. In the literature several examples were reported on information regarding the involvement of different parts of an analyte in the intermolecular complex formation with CDs [25–27,34,35,47,48]. These data may explain the change of molecular recognition of the analytes. 2D ROESY experiments were carried out to investigate the background of side-chain

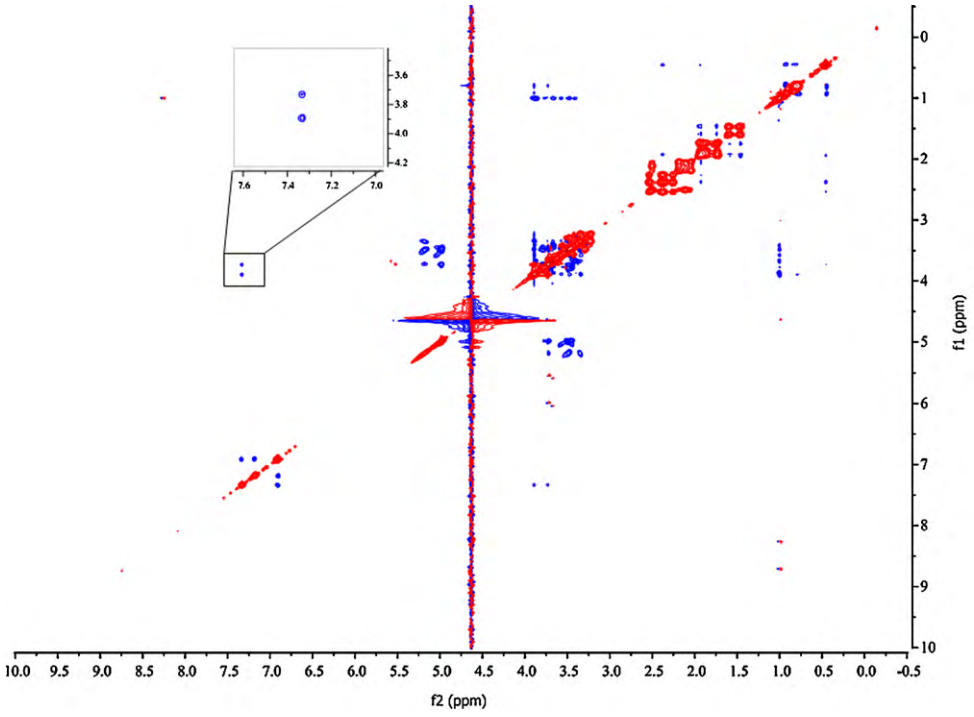

Fig. 6. ROESY spectrum of (−)-vincadifformine/HP-γ-CD complex.

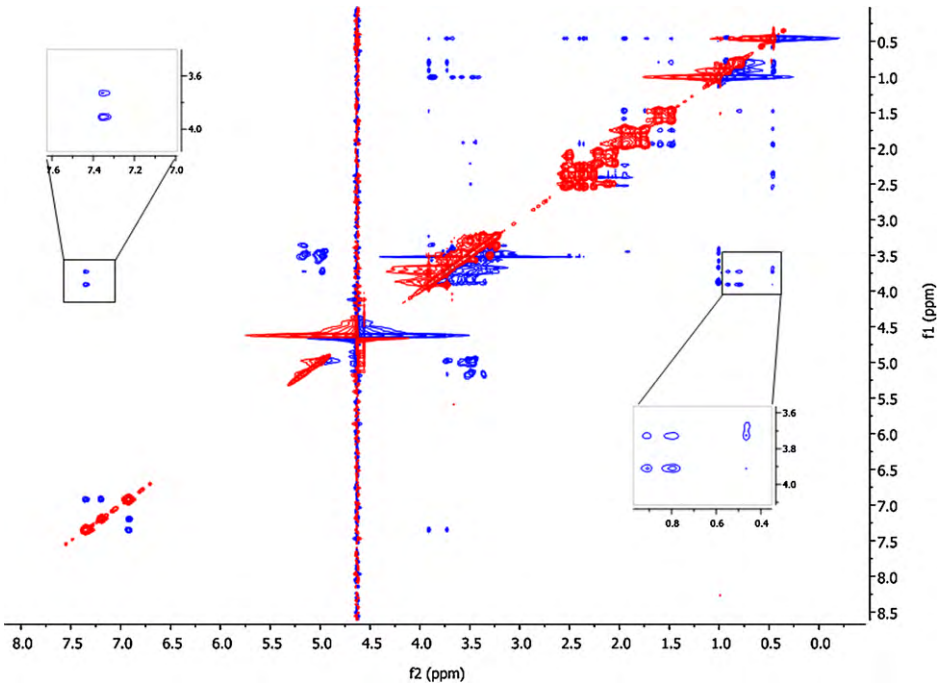

Fig. 7. ROESY spectrum of (+)-vincadifformine/HP-γ-CD complex.

and CD cavity size-dependent enantiomer reversal phenomena observed in CE experiments. Based on the CE results, four pairs of alkaloid–CD systems were studied as examples: vinpocetine-HP-β-CD/HP-γ-CD, vincadifformine-HP-β-CD/HP-γ-CD, vincadifformine-ME-β-CD/ME-γ-CD (cavity size dependent) and vinpocetine-HP-β-CD/ME-β-CD (side-chain dependent). In each system, the spectrum of both enantiomers was recorded with the appropriate CD.

Experiments with vinpocetine demonstrate that the alkaloid enters the CD cavity from the wider rim with its aromatic ring in each case, confirming previously reported findings [18,19]. Dramatic differences in the structures of the corresponding intermolecular complexes could not be observed. The stronger intensity of the cross-peaks with ME-β-CD suggests that this complex is more stable than the HP-β-CD or HP-γ-CD complexes, confirming the CE results. Presumably the enantiomers are more deeply included in the cavity of randomly methylated CDs compared to hydroxypropylated CDs.

Contrary to vinpocetine, vincadifformine forms two different complexes with CD derivatives based on ROESY data; either the aromatic ring or the ethyl side-chain enters the cavity from the wider rim. The cross-peaks suggested that both complex structures are possible for the vincadifformine enantiomers regardless of the CD derivative except in case of (−)-vincadifformine and HP-γ-CD, where only the aromatic moiety fitting in the cavity is noticeable on the ROESY spectrum.

In the case of the cavity size-dependent investigation of vincadifformine and HP-β-CD/HP-γ-CD the intensity of the cross-peaks indicated that the HP-γ-CD complexation involves different moieties for the enantiomers: only the aromatic part for (−)-vincadifformine and both the aromatic and the alkyl part for (+)-vincadifformine (see Fig. 7). With HP-β-CD the involvement of the alkyl moiety is detectable for both enantiomers. Suggested by the intensity differences the involvement of the alkyl chain is more favorable for (+)-vincadifformine-HP-β-CD complexes than for the (−) enantiomer. The pronounced participation of the alkyl moiety in the complexation may explain the differences of the migration order reversal experienced in CE. Experiments with ME-β-CD/ME-γ-CD led to similar conclusions. The possibility of alkyl complexation is commensurable with the aromatic complexation in the case of ME-γ-CD, while with ME-β-CD the aromatic complexation dominates unambiguously based on the intensity of the

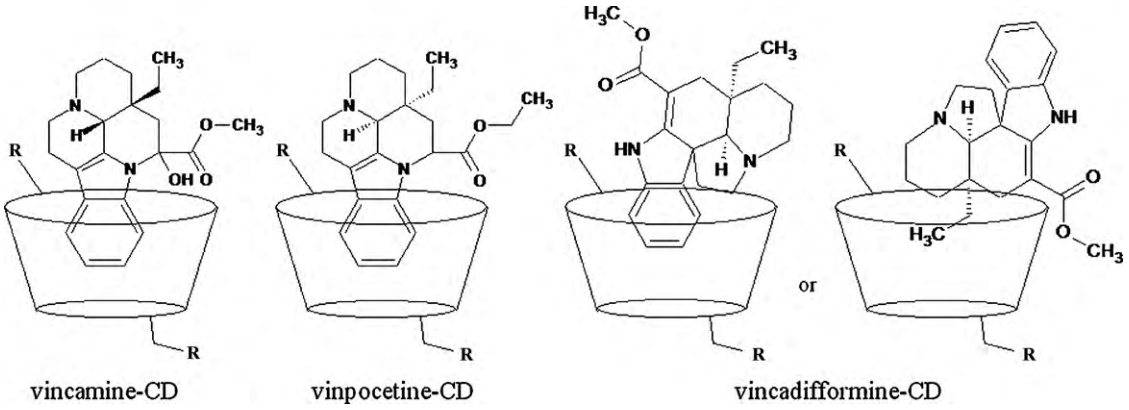

Fig. 8. The proposed structures for the inclusion complexes based on the ROESY experiments.

cross-peaks. These differences likewise support the conclusion that the different complexation may lead to enantiomeric migration order reversal in the case of vincadifformine. Due to multiple interactions acting between the chiral selector and the alkaloid it may happen that the structural changes observed are not responsible for the different molecular recognition. The proposed structures for the inclusion complexes of based on the ROESY experiments are depicted in Fig. 8.

4. Conclusions

In the present work, enantioseparations of three vinca alkaloids were studied using native CDs and their derivatives as chiral selectors. For characterization of the complexes and the enantioseparation, the apparent averaged stability constants, resolution and migration order were determined. The complex stability results determined with capillary electrophoresis were confirmed with NMR titrations. The resolution data correlated with the differences in the apparent stability constants of the enantiomers; the larger the difference between the K values of the enantiomers, the higher the resolution observed. The averaged stoichiometries of the complexes were found to be 1:1 independent of the applied CD and alkaloid. The structures of the complexes of vinpocetine-HP-β-CD/HP-γ-CD, vincadifformine-HP-β-CD/HP-γ-CD, vincadifformine-ME-β-CD/ME-γ-CD and vinpocetine-HP-β-CD/ME-β-CD systems were investigated in detail. Moreover, evidence was found possibly explaining the cavity size enantiomer reversals in case of vincadifformine-CD systems. Differences in the binding modes of vincadifformine enantiomers were revealed.

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