TUTORIAL REVIEW

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Combined approach using capillary electrophoresis and NMR spectroscopy for an understanding of enantioselective recognition mechanisms by cyclodextrins

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Received 2nd February 2004
First published as an Advance Article on the web 5th July 2004

This *tutorial review* describes the contribution of chiral capillary electrophoresis in combination with other instrumental techniques, especially nuclear magnetic resonance spectroscopy, to a better understanding of the chiral recognition mechanisms by cyclodextrins. Aspects such as affinity pattern of enantiomers towards various cyclodextrins as well as the stoichiometry of the resulting complexes, the equilibrium constants and the structure of complexes are addressed. In addition to the aforementioned techniques, the usefulness of complementary instrumental and molecular modeling techniques for an understanding of the chiral recognition mechanisms of cyclodextrins is also illustrated.

1 Introduction

Cyclodextrins (CDs) are amongst the most remarkable of macrocyclic molecules with significant theoretical and practical impact in chemistry. The multi-ton scale production and widespread application in chemical, pharmaceutical, food, *etc*. technologies as enzyme mimics, enantioselective catalysts, drug carriers, odor and taste-masking compounds, as well as in analytical fields, especially separation science,[1] is primarily due to two properties—complex formation and chiral recognition ability. At present, CDs are used as chiral selectors in high-performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), capillary electrophoresis (CE), capillary electrochromatography (CEC) and most recently in lab-on-chip enantioseparations.[2] It must be noted that among presently applied chiral selectors only CDs are effectively used in all enantioseparation techniques. The discovery of CDs as probably the most universal chiral selectors contributed enormously to the maturation of instrumental enantioseparation techniques. *Vice versa*, it can be noted during the last few years that enantioseparation techniques advanced cyclodextrin chemistry because recent developments, especially in CE, allow a better understanding of inclusion complex formation and the chiral recognition mechanisms of CDs.

Major developments in cyclodextrin chemistry during the past 100 years are summarized in Table 1.[3–9] The basic mechanisms of the interactions between CDs and other molecules are known,[10] but there are still many open questions.

The most critical questions in CD chemistry addressed in part in this review are the following: a) is the inclusion complex formation a prerequisite for chiral recognition by CDs? b) Does any correlation exist between the binding strength and enantiorecognition power by CDs? c) What are the major forces responsible for binding and what factors are responsible for chiral recognition? e) What sites of the CDs are primarily responsible for binding and enantiorecognition?

The present paper starts with a description of those characteristics that make CE a suitable technique for the aforementioned mechanistic studies. Further, particular examples are described illustrating the power of CE in combination with other instrumental techniques, especially nuclear magnetic resonance (NMR) spectroscopy, for a better understanding of the affinity pattern of enantiomers towards the chiral selector, as well as for describing the stoichiometry, the equilibrium constants and the structures of the selector–selectand transient diastereomeric complexes.

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DOI: 10.1039/b111412n

Table 1 Most important developments in cyclodextrin chemistry over the past 100 years

Year	Event/Property	Reference
1903	Assumption was made regarding the cyclic structure of cycodextrins	3
1911	Intermolecular complex formation ability of cyclodextrins was described	4
1938	Macrocyclic structure of cyclodextrins was experimentally confirmed	5
1939	Assumption was made that cyclodextrin complexes are of inclusion type	6
1951	Chiral recognition ability of cyclodextrins in intermolecular complexes was described	7
1965	Experimental evidence of inclusion complex formation in the solid state was provided	8
1970	Experimental evidence of inclusion complex formation in the solution was provided	9

2 CE *vs.* other techniques

In this section CE is compared to other instrumental techniques as a tool which may provide information about enantioselective interactions between the two counterparts of the transient diastereomeric complex. One of these counterparts is arbitrary called selector (receptor, host) and the other one is called selectand (ligand, guest).

The magnitude of enantiorecognition in selector–selectand interactions can be characterized by enantioselectivity. Enantioselectivity of recognition is a thermodynamic quantity correlating in some way with the separation factor of enantiomers (α) in separation techniques. This correlation is more straightforward in the case of chromatographic techniques with stationary phases while it is quite complex in the case of CE.

In the majority of cases, enantioseparations in CE rely on the same principle (enantioselective interaction between a chiral selector and the chiral analyte) as in chromatographic techniques.[11,12] In spite of this fact, there are significant differences in the results achieved with these techniques. Responsible for the most striking differences between chromatographic and electrophoretic enantioseparations is the property of the electrophoretic mobility to be selective for charged species residing in the same phase.[12] Another important point is that in chromatographic techniques, except for the case with a chiral mobile phase additive (CMPA), the analyte is virtually immobile when associated with a chiral selector. In CE the analyte selector complex is usually mobile.[12]

Some differences between chromatographic and electrophoretic enantioseparations can be derived analyzing the equation for the electrophoretic mobility difference $\Delta\mu$ between enantiomers:[13]

$$\Delta\mu = \mu_1 - \mu_2 = \frac{\mu_f + \mu_{c_1} K_1[C]}{1 + K_1[C]} - \frac{\mu_f + \mu_{c_2} K_2[C]}{1 + K_2[C]} \qquad (1)$$

where μ_1 and μ_2 are the mobilities of the first and the second migrating enantiomer, respectively, K_1 and K_2 are the binding constants of enantiomers 1 and 2, respectively with the chiral selector, μ_f and μ_c are the mobilities of the free and complexed analyte and [*C*] is the concentration of a chiral selector.

One important point obviously seen from eqn. (1) is a crucial role of the mobilities in enantioseparations in CE. This parameter is absent in the major chromatographic techniques except the above mentioned mode with a CMPA. The contribution of the mobilities in chiral CE separations results in the following remarkable effects:

1. It is feasible in chiral CE but not in chromatographic techniques that the selectivity of an enantioseparation exceeds the thermodynamic selectivity of chiral recognition and approaches an infinitely high value.[11,12,14]

2. It is possible in chiral CE to adjust the enantiomer migration order without changing the affinity pattern of the analyte enantiomers with a chiral selector. This is impossible in chromatographic techniques unless using a chiral selector as a CMPA.[11]

3. The most striking difference between these two techniques seems to be the fact that CE allows, in principle, the separation of enantiomers in the case when the equilibrium constants of both enantiomers with the chiral selector are equal.[11,12]

As already mentioned, in chromatographic techniques the selectivity of an enantioseparation is primarily dependent on the difference between the affinities of the enantiomers towards the chiral selector. Therefore, the selectivity of enantioseparations in common chromatographic techniques may in the best case approach the thermodynamic selectivity of the chiral recognition (ratio of the binding constants) but will never exceed it. One major consequence of the mobility contribution in CE separations is the fact that the separation selectivity may exceed the thermodynamic selectivity of the recognition. This is experimentally illustrated in Fig. 1.[14] In all separations of the chlorpheniramine maleate

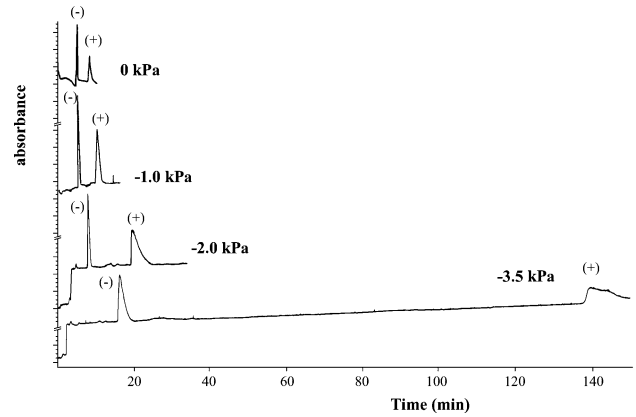

Fig. 1 Effect of increasing counterpressure on the separation of (±)-chlorpheniramine in the presence of 2 mg ml^{-1} CM-β-CD. (Reproduced with permission of Wiley-VCH from Ref. 14.)

enantiomers with CM-β-CD shown here, the components involved in chiral recognition on the molecular level are invariant. This means that chiral recognition itself is not significantly different in the various runs shown in this figure. However, an enormous (in principle unlimited) enhancement of the separation selectivity becomes possible when transforming the chiral recognition into a chiral separation. In this particular example it was achieved by applying a counterbalancing pressure to the separation capillary in the opposite direction to the analyte migration.[12,14]

As shown schematically in Fig. 2 this concept may allow the design of a separation system in such a way that two enantiomers possessing electric charge of the same sign will migrate towards opposite electrodes which means that the enantioseparation factor becomes infinitely large. By analogy with electrolysis, this phenomenon was named "enantiolysis".[14] The technique proposed in ref. 14 can also be applied for micro preparative purposes[15] as well as for separations of achiral analytes not only in a binary but also in multicomponent mixtures.[14]

Another important point is that a manipulation of the mobility terms in CE allows not only an easy adjustment of the selectivity of enantioseparations but also a reversal of the enantiomer migration order without changing the affinity pattern of the enantiomers towards chiral selector.[11,12] This is again impossible in chromatographic techniques. This significant difference between chromatographic and electrophoretic separations from the viewpoint of the enantiomer migration order has been noted in previous studies.[11,12,16]

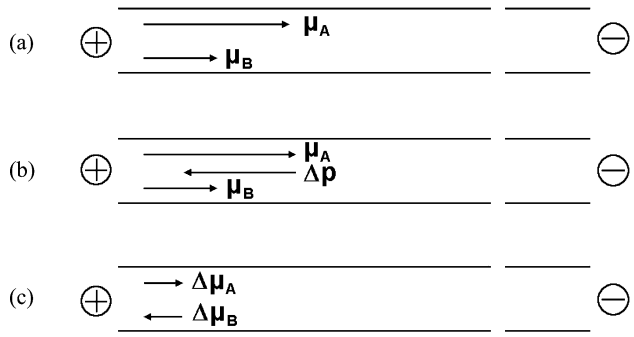

Fig. 2 Schematic representation of flow-counterbalanced separation principle in CE: a) without counterbalanced flow; b) with counterbalanced flow; c) final mobilities. (Reproduced with permission of Wiley-VCH from Ref. 14.)

The feasibility of enantioseparations even in the case when the binding constants of both enantiomers with a chiral selector are equal[11,12] can be derived from eqn. (1). According to this equation for the generation of a mobility difference between the enantiomers, *e.g.* an enantioseparation in CE, the following is required:

a) Formation of transient diastereomeric complexes between the analyte and the chiral selector. This means that enantioseparation is impossible in CE without a chiral selector.

b) The effective mobilities must be different for the free and complexed analyte ($\mu_f \neq \mu_c$).

If both of the above mentioned prerequisites apply, then enantiomers may be resolved with equal success by the following two alternative mechanisms or a combination thereof:

1) The residence time in the free and complexed forms is not equal for both enantiomers. The period of time in which the enantiomers reside in the free and complexed form is defined by the binding constants between the analytes and the chiral selector. In this case a difference in the binding constants is required but the difference in the mobilities of the diastereomeric complexes is not necessarily required for an enantioseparation. Thus, the enantioseparation will be based on the same principle (difference in the binding constants) as in chromatographic techniques. Under these conditions eqn. (1) may be rewritten in the following form:[13]

$$\Delta\mu = \frac{C(\mu_f - \mu_c)(K_1 - K_2)}{1 + C[K_1 + K_2] + C^2 K_1 K_2} \qquad (2)$$

2) Alternatively, both enantiomers may reside the same time in the free and complexed form, *e.g.* $K_1 = K_2 = K$ but the mobilities of the diastereomeric complexes may be different. Under these conditions eqn. (1) may be rewritten in the following form:[12]

$$\Delta\mu = \mu_1 - \mu_2 = \frac{K[C](\mu_{C1} - \mu_{C2})}{1 + K[C]} \qquad (3)$$

From eqn. (3) it is obvious that the prerequisite for the enantioseparation in this case is the formation of the transient diastereomeric complexes of both enantiomers with different mobilities μ_{C1} and μ_{C2}, *e.g.* $\mu_{C1} \neq \mu_{C2}$.

Either the binding constant difference or the mobility difference of the corresponding diastereomeric complexes may both result in enantioseparations in CE. Rather common is the first case ($K_1 \neq K_2$ or a combination of both.

Thus, as summarized in this section, there are significant differences between enantioseparations in pressure-driven and electrically-driven systems. These differences make both techniques complimentary. The success of CE for enantiomer separations is based on these differences between chromatographic and electrophoretic techniques.

The enantioseparation can be decoupled into two processes: chiral recognition and the transformation of a chiral recognition into a chiral separation, *e.g.* the generation of an enantioseparation from enantioselective recognition.[12]

As mentioned above, the correlation between chiral recognition and a separation is less complex in pressure-driven techniques compared to CE. A binding constant difference of the enantiomers with a chiral selector is required and may result in enantioseparation in chromatographic techniques. In contrast to this, a binding constant difference is neither required nor necessarily leads to enantioseparations in CE.

Despite the complex relationships between chiral recognition and enantioseparations in CE, this technique offers significant advantages for the investigation of enantioselective CD–analyte interactions.

The major advantages of CE compared to chromatographic techniques from the viewpoint of molecular recognition science are the following:

1. CE allows very fast screening of analyte–CD interactions in order to prevail the most interesting pairs from the huge number of chiral analytes and numerous CDs. There is no other instrumental separation or non-separation technique that may compete with CE from this point of view.

2. The high peak efficiency in CE allows the observation of (enantio)selective effects in selector–selectand interactions which are invisible by other (separation) techniques.

3. A small thermodynamic selectivity of recognition can be transformed into a high separation factor in CE.

4. CE is more flexible than chromatographic techniques from the viewpoint of the adjustment of the (enantio)separation factor.

3 Analyte–CD binding interactions

CE allows the observation of the enantioselective effects and in this way a determination of the enantioselective apparent equilibrium constants for those intermolecular interactions where other techniques are unsuitable. This is the major advantage of CE for binding studies between chiral analytes and CDs.

Another advantage of CE which was not adequately addressed in earlier studies is the technically easier possibility of studying the binding of a given solute to multiple hosts and *vice versa*, the competitive binding of multiple guests to a single host or even a combination of both. This potential seems especially challenging with the increasing activity in the fields of combinatorial synthesis, high throughput screening and functional proteomics.

The equation for the description of equilibrium in electrophoresis was introduced by Tiselius.[17] A few years ago, Rundlett and Armstrong[18] provided a critical treatment of the subject. Together with the advantages of CE for the binding studies, such as high efficiency, ease of automation, short analysis time, small sample size and buffer volume, limitations of the technique and possible error sources in CE binding constant determinations were also addressed in this work.

The following two points seem worthwhile mentioning. As in most other techniques, concentrations are used instead of activities in CE. Therefore, the binding constants are not true thermodynamic equilibrium constants but apparent constants. Second, almost all equations used for equilibrium constant determinations in CE assume a 1 : 1 stoichiometry while actually complexes with other stoichiometries are also common.

As shown in interesting studies by Rizzi and Kremser,[19] and by Scriba and co-workers[20] the determination of the binding constants, in spite of the aforementioned limitations, may allow a clear differentiation between binding- and mobility-dependent enantioseparations in CE.

Although CE offers certain advantages for the detection of very fine enantioselective effects in analyte–CD interactions, this technique also suffers from some severe disadvantages for studies of molecular recognition phenomena. In particular, CE does not provide any direct information regarding chemical and structural mechanisms of analyte–CD interactions. In order to overcome this disadvantage several instrumental techniques can be applied in

combination with CE. Among these, nuclear magnetic resonance (NMR) spectroscopy appears to be the most powerful one.

4 Combination of CE and NMR spectroscopy for studies of analyte–CD interactions

The most distinct advantage of NMR spectroscopy compared to other spectrometric techniques is the fact that NMR spectroscopy may in principle provide separate resonance signals for non-covalent diastereomeric complexes between the selector and both enantiomers of the selectand. Thus, NMR spectroscopy allows the application of racemic samples or nonracemic mixtures of enantiomers for the stereoselective determination of the stoichiometry and equilibrium binding constants of selector–selectand complexes. Besides the easier availability of racemic analytes compared to pure enantiomers, NMR spectroscopy offers the possibility of competitive binding studies. This means, that the interaction of one of the enantiomers of an analyte with a chiral selector may be studied in the presence of the other enantiomer which mimics closely the real conditions in chiral CE separations. An additional advantage of NMR spectroscopy is the fact that it provides a multiple set of data based on a single set of experiments. Most other instrumental techniques provide a change of averaged molecular characteristics (specific molar absorbance, shift of absorption maximum, solubility, *etc*.) while NMR spectroscopy provides distinct signals for each proton, ^{13}C, *etc*. belonging to different fragments and functional groups of a selectand and a selector molecule. Thus, NMR spectroscopy may provide specific information about the involvement of the different moieties of a selectand and a selector in the intermolecular interactions, as well as statistically more reliable data for the characterization of selector–selectand complexes.

The application of NMR spectroscopy for a better understanding of CD–guest interactions has quite a long history. The technique provided the very first evidence of inclusion complex formation between CDs and guest molecules in solution in 1970[9] as well as very useful information on the stoichiometry, binding constants, structure, free energy, enthalpy, entropy and dynamics of CD guest complexes during the last 30 years.[10–12,21–26] Studies on the combined application of CE and NMR spectroscopy for a better understanding of the binding and enantiomer recognition ability of chiral analytes by CDs have been published since 1992.[11,12,20,27–56] These studies include a correlation between the signal splitting pattern and extent in ^{1}H-,[28–30,32–47] ^{13}C-,[30] and ^{19}F-NMR[9,48,49] spectra on the one hand and enantioseparations in CE on the other hand, as well as stoichiometry,[28,31–33,39–41,45] binding constants,[31,32,39] structure[34,44,45,50–56] and dynamics of CD–guest complexes related to CE enantioseparations.

The NMR spectroscopic pattern of CD–guest complexes may provide preliminary information on the extent of chiral recognition, as well as on the involvement of different groups and moieties of the guest and host molecules in the intermolecular complex formation. Branch *et al*. performed comparative ^{1}H-NMR spectroscopic and CE studies on the chiral recognition ability of native β-CD and heptakis(2,3-di-*O*-acetyl) β-CD towards some chiral phenylethylamine derivatives of pharmaceutical interest.[28] It was shown based on NMR data that the chiral recognition was much better in the case of heptakis(2,3-di-*O*-acetyl) β-CD. These results correlated well with the data obtained by CE enantioseparations. Good correlation between the signal splitting pattern in NMR spectroscopy and enantioseparation results in CE were also observed for different neutral and charged cyclodextrins several studies.[29–41,43,48,49] In some cases in which there is overlap of critical signals between the host and the guest molecule, absence of measurable signal splitting or any other reasons make the application of ^{1}H-NMR spectra impossible, ^{13}C-NMR or ^{19}F-NMR spectra can be used as illustrated in several studies.[9,30,48,49]

Very interesting observations regarding the stoichiometry of CD complexes with chiral guests, in particular, a dependence of the complex stoichiometry on the absolute stereochemical configuration of some chiral guests, as well as on the size of the CD cavity and the nature and position of the substituents on CD rim, illustrate the relevance of such work. Several studies[28,30–32,39–41,45] report the stoichiometry of CD–analyte complexes in combination with CE enantioseparations, but a distinct difference in the stoichiometry of the complexes was observed in only a few cases. Thus for example, Kano and co-workers[57,58] observed different stoichiometries for the complexes of the *S*- and *R*-1,1′-binaphthyl-2,2′-diol with heptakis (2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD). The stoichiometry of *S*-guest/TM-β-CD complex was 2 : 1 while for the *R*-guest/TM-β-CD a 1 : 1 complex was found. According to ^{1}H-NMR data the antihistaminic drug dimethindene may form mixed complexes of different stoichiometries with native β-CD (a mixture of 1 : 1 and 1 : 2 CD-guest complexes) as well as with randomly derivatized carboxymethyl-β-CD.[53] The antihistaminic drug brompheniramine may form complexes of different stoichiometry with native β-CD and TM-β-CD (at least in the solid state).[54] The stoichiometric differences observed in the latter two studies may be the reason for the opposite affinity pattern of the related enantiomers towards the CDs involved in these studies.

NMR spectroscopy may also provide interesting information regarding the strength of CD–analyte complexes. Rather few examples of the binding constant determination using NMR spectroscopy in CE related studies have been published. The reason for this could be the fact that CE itself is considered as a very useful technique for the determination of binding constants. In addition, the migration time of the samples under certain conditions may provide preliminary information on the strength of the CD–analyte complexes. Some interesting results in which the binding constants determined by NMR spectroscopy allowed "unusual" results observed in CE to be explained are reported in the literature.[31,32,40]

In the above discussed articles NMR spectroscopy may complement the results obtained by CE on CD–analyte interactions. However, in studies dealing with the structure of intermolecular CD–analyte complexes NMR spectroscopy holds a unique position. CE cannot provide any structural information. A very elegant article on the structure of analyte–CD complex determined using NMR spectroscopy in relation to enantioselective CE was published by Yamashoji *et al*. in 1992.[27] Subsequently, several studies were published on this topic.[34,44,45,50–56] In many of the above mentioned works the authors tried to explain quantitative differences observed in the behavior of chiral selectors based on their structure with the guest molecules. All of these studies contributed significantly to a better understanding of CD–analyte interactions. However, at present it is difficult even with very sophisticated powerful NMR techniques to define the structure and dynamics of the complexes on a level providing the key for the explanation of sometimes very fine quantitative differences in the enantioselective recognition by different cyclodextrins. Therefore, in our studies the main emphasis was the elucidation of possible structural mechanisms of qualitatively different behavior of the CD-type chiral selectors in CE, in particular, for examples in which the affinity of the enantiomers is opposite towards given chiral selectors.

The reversal of the affinity pattern of enantiomers is the most dramatic change that may appear due to any chemical or structural modification of a chiral selector. 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 CD.[60] Some recent examples are shown in Tables 2–4.[60]

When differences in the enantiomer affinity pattern towards different CDs are observed, the following questions arise:

1. Are there any detectable structural differences present, which may be responsible for the affinity reversal?

Table 2 Enantiomer affinity pattern of selected chiral analytes towards native CDs having different cavity sizes. (Reproduced with permission of Wiley-VCH from Ref. 60.)

Analyte	Chiral selector and the first migrating enantiomer		
	α-CD	β-CD	γ-CD
Aminoglutethimide	*R*	*S*	*R*
Ephedrine	(−)	(+)	No separation
Ketamine	*S*	*R*	*R*
Ketoprofen	*S*	*R*	*R*
Mefloquine	(−)	(−)	(−)
Metharaminol	(+)	(+)	(+)
Norephedrine	(+)	(−)	No separation
Promethazine	(−)	(−)	(−)
Tetramisole	*S*	*R*	*R*
AlaPheOMe	*RR*	*SS*	*SS*
Ala–Tyr	*RR*	*SS*	*SS*
Asp–PheOMe	*RR*	*SS*	*SS*

Table 3 Enantiomer affinity pattern of selected chiral analytes towards native and selectively methylated CDs. (Reproduced with permission of Wiley-VCH from Ref. 60.)

Analyte	Chiral selector and the first migrating enantiomer		
	β-CD	DM-β-CD	TM-β-CD
Aminoglutethimide	*S*	*S*	*R*
Brompheniramine	(−)	No separation	(+)
Chlorpheniramine	(−)	(+)	(+)
Dimethindene	*S*	*R*	*R*
Ephedrine	(+)	(−)	(−)
Ketoprofen	*R*	No separation	*S*
Metharaminol	(+)	(−)	(+)
Tetramisole	*R*	*R*	*S*
Verapamil	(−)	(−)	(+)

2. Are there instrumental techniques available, or is the selected technique adequate one in order to detect these changes on the molecular level?

3. Do we possess reliable techniques at present in order to accurately determine the intermolecular forces based on the structure of a complex? Do these techniques allow a correct identification of the force(s) responsible for affinity reversal? Can these calculations be performed accurately enough in order to distinguish very fine free energy differences (commonly less than 1 kcal mol^{-1}) which are responsible for the affinity reversal of enantiomers?

Table 4 Enantiomer affinity pattern of some chiral analytes towards β-CD, HDA-β-CD and randomly acetylated β-CD. (Reproduced with permission of Wiley-VCH from Ref. 60.)

Chiral analyte	β-CD	HDA-β-CD	Acetylated β-CD
Aminoglutethimide	(−)	No separation	(+)
Clenbuterol	(−)	(+)	(−)
DNS-Phenylalanine	L	D	not studied
Ephedrine	(+)	(−)	(+)
Mefloquine	(−)	(+)	(−)
Metaraminol	(+)	(+)	(−)
Tetramisole	(+)	(−)	(+)

A few approaches to dealing with some of aforementioned questions are described below.

4.1 Analyte–CD interaction pattern

In general, for a given pair of an analyte and a CD, one may expect a higher enantioseparation power in CE compared to the enantiorecognition ability in NMR spectrometry. In particular cases however, NMR spectroscopy may provide an indication for a chiral recognition for those selector–selectand pairs which have been considered to be unsuccessful based on the CE experiment alone. For instance, native β-CD has been suggested to be an unsuitable chiral selector for the enantioseparation of the cationic form of chiral cholinergic drug aminoglutethimide (AGT) in contrast to α- and γ-CD which allowed baseline enantioseparations of AGT.[61,62] In contrast to the CE data, NMR studies indicated the most pronounced interactions between AGT and β-CD among the three native CDs (Fig. 3).[50] Together with the signal splitting pattern due to the non-equivalence of the complexation-induced chemical shifts (CICS) of AGT protons, interesting effects were also observed for CD protons. In particular, the resonance signals due to the H-5 protons which are located inside the cavity close to the narrower primary ring of the CD were strongly shifted upfield in the case of β-CD whereas only moderate effects were observed in the case of α- and γ-CD. These data were also supported by electrospray ionization mass spectrometry (ESI-MS) studies on the comparative affinity of AGT enantiomers towards these CDs.[50] Careful optimization of the separation in CE also allowed the resolution of the enantiomers of AGT with β-CD. The migration times in the presence of β-CD were the longest which also indicates the strongest interaction in this case. In addition, the enantiomer migration order was opposite compared to two other CDs (Fig. 4).[50]

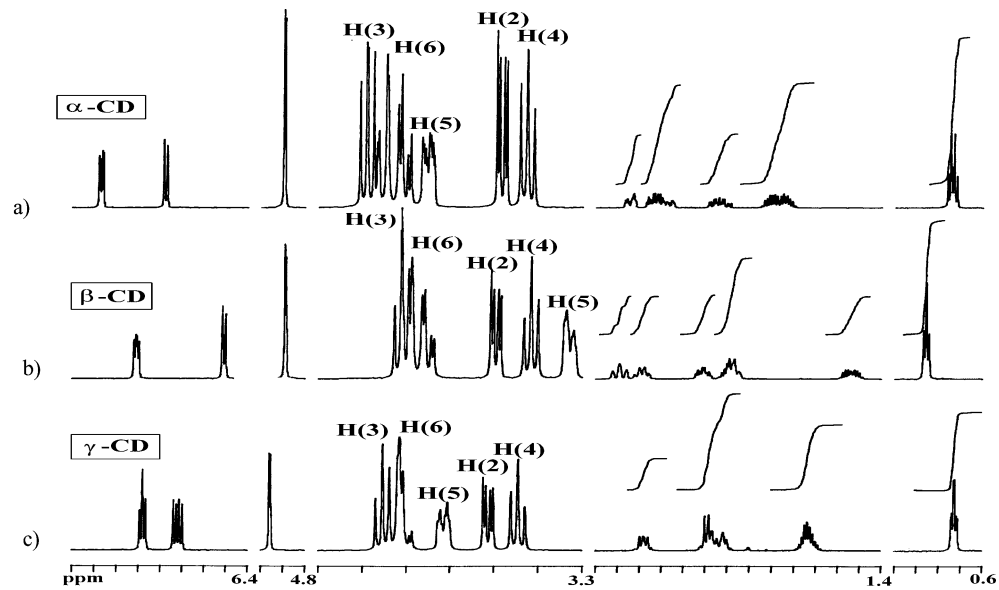

Fig. 3 The 600 MHz ^{1}H-NMR spectra of (±)-AGT in the presence of two equivalents of α-CD (a), β-CD (b) and γ-CD (c). (Reproduced from Ref. 50.)

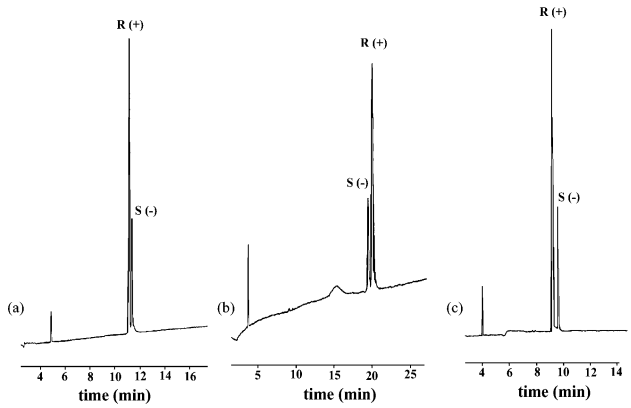

Fig. 4 Electropherograms of AGT enantiomers [(+)/(−) = 2 : 1] in the presence of 10 mg ml^{-1} α-CD (a), β-CD (b) and γ-CD (c). (Reproduced from Ref. 50.)

Thus, in this particular case NMR and ESI-MS studies allowed the optimization of the enantioseparation in CE. The combination of these techniques allowed an example of opposite affinity of the AGT enantiomers towards native CDs to be found. Examples of an affinity reversal of enantiomers depending just on the size of the CD cavity are rather few (see Table 2).[60]

Another interesting example when NMR spectroscopy provides interesting information regarding the involvement of different parts of an analyte in intermolecular complex formation with CDs was observed for the chiral β-blocker drug clenbuterol (CL). CE experiments revealed that the enantiomers of CL display opposite affinity patterns towards native β-CD and heptakis-(2,3-*O*-diacetyl)-β-CD (HDA-β-CD, Fig. 5).[51] The splitting of the resonance

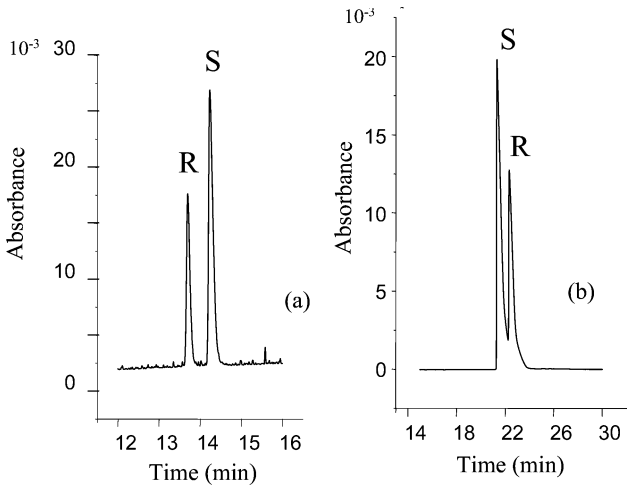

Fig. 5 Electropherograms of CL enantiomers [(*R*)/(*S*) = 2 : 1] in the presence of 18 mg ml^{-1} β-CD (a), and 12 mg ml^{-1} HAD-β-CD (b). (Reproduced with permission of Wiley-VCH from Ref. 51.)

signals due to the CICS of the protons of the enantiomers was primarily observed for the aromatic protons in the case of β-CD (Fig. 6a) and for the protons of the *tert*-butyl moiety of CL in the case of HDA-β-CD (Fig. 6b). These data indicate indirectly that the aromatic part of CL is mainly involved in the interactions with β-CD while the *tert*-butyl moiety is involved in the interactions with HDA-β-CD.[51]

4.2 NMR spectroscopic studies on structure elucidation of selector–selectand complexes in solution

As emphasized above, the reversal of the enantiomer affinity pattern is the most remarkable change that may occur due to any

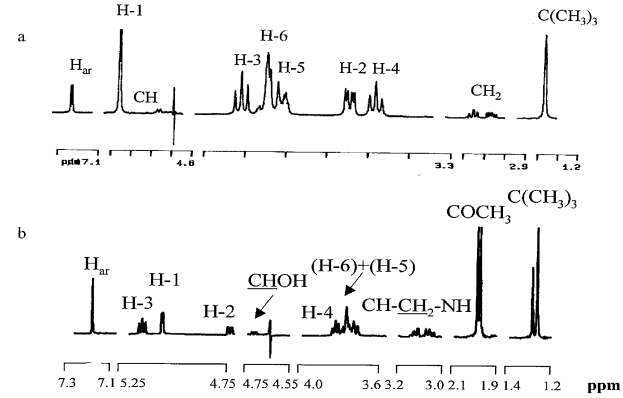

Fig. 6 The 600 MHz ^{1}H-NMR spectra of (±)-CL in the presence of two equivalents of β-CD (a) and HAD-β-CD (b).

chemical or structural modification of a chiral selector. However, the structural changes of the intermolecular complexes responsible for a significant change in the chiral recognition pattern may be minor or even invisible by the applied techniques. Another critical issue of our current understanding of molecular recognition phenomena is to correctly assign the changes observed in the molecular recognition to the structural variation of an intermolecular complex. In other words, even when the structural changes are seen, it does not *a priori* mean that they are responsible for the affinity reversal. Due to multiple forces acting between a CD and the analytes it may easily happen that the major structural changes observed by one or another technique have nothing in common with the change observed in molecular recognition. Therefore, the results described below require very careful interpretation.

The enantiomers of the anesthetic drug ketamine (KT) possess an opposite affinity pattern towards native α- and β-CD.[52] Rotating frame nuclear Overhauser effect spectroscopy (ROESY) does not indicate dramatic differences in the structures of the corresponding intermolecular complexes. The enantiomers are just more deeply included in the cavity of β-CD compared to α-CD.[52] Similar to this example no clear differences besides the extent of intermolecular inclusion could be observed between the structures of complexes of dimethindene,[53] brompheniramine[54] and chlorpheniramine[55] with β-CD and heptakis-(2,3,6-tri-*O*-methyl)- β-CD (TM-β-CD) although the enantiomer affinity patterns were opposite for all three analytes towards these two CDs.[53–55]

In other examples however, significant differences in the structures of intermolecular complexes could be observed. The structural reasons of the above mentioned opposite affinity of the enantiomers of AGT towards native β- and γ-CD were investigated in ref. 50. The nuclear Overhauser effect (NOE) data shown in Fig. 7 allow the structure of the complexes depicted in Fig. 8 to be deduced. Thus, by selective saturation of the aromatic protons in the *ortho* position of (±)-AGT equally strong intermolecular NOE-effects were observed for both H-3 and H-5 protons of β-CD (Fig. 7a). However, by irradiation of the aromatic protons in the *meta* position only a minor effect was observed for the H-3 protons of β-CD and the NOE-effect appeared instead at the H-6 protons. These data support a deep inclusion of the *p*-aminophenyl moiety of AGT into the cavity of β-CD entering it from the wider secondary side (Fig. 8a). The deep inclusion of the aromatic moiety of the AGT molecule into the cavity of β-CD on the secondary side is supported also by a significant NOE effect observed between the H-3 protons of CD and the ethyl moiety of AGT. Rather strong "NOE-like" effects observed on the external CD protons in this experiment make it questionable whether the structure represented in Fig. 8a is the only possible structural element of this complex or if the alternative structures are also present. In contrast to the AGT/β-CD complex, the NOE effect decreased for the H-5 protons and remained almost unchanged for H-3 protons when irradiating the protons in the *meta* position instead of the protons in the *ortho*

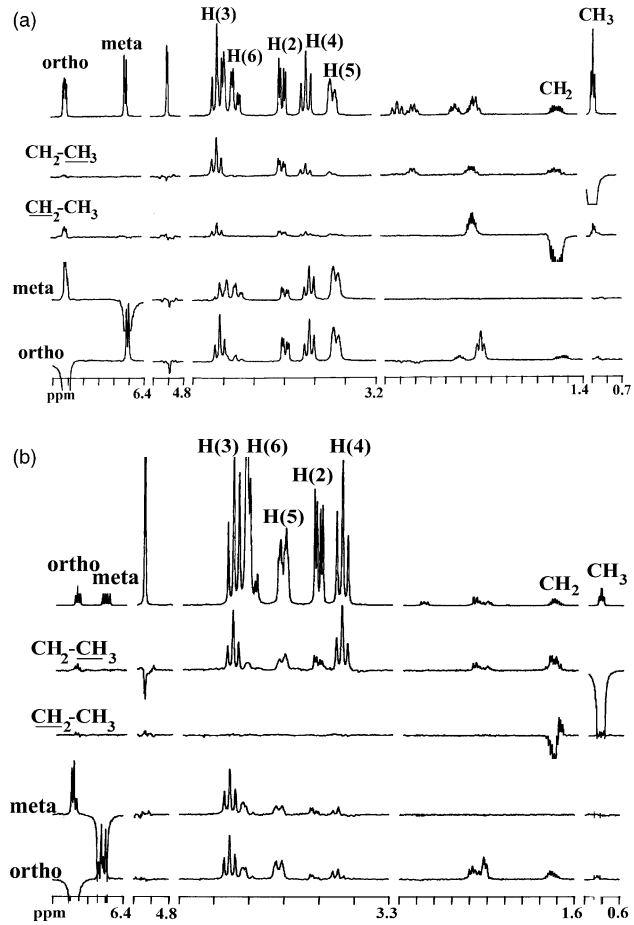

Fig. 7 1D-ROESY spectra of (±)-AGT in the presence of two equivalents of β-CD (a) and γ-CD (b). (Reproduced with permission of Wiley-VCH from Ref. 50.)

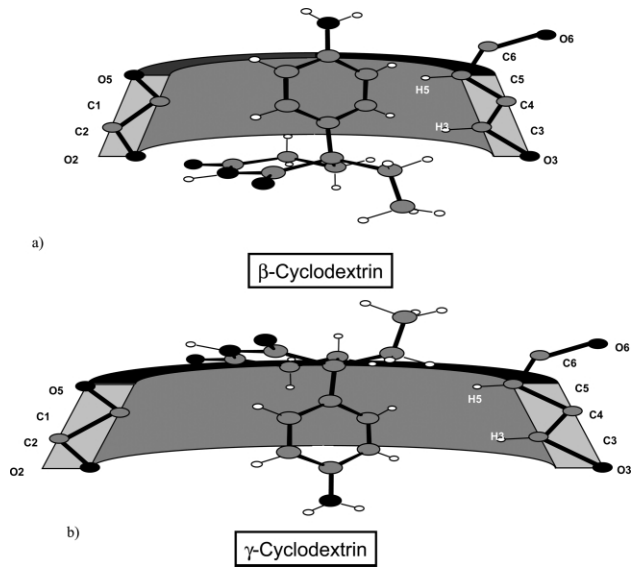

Fig. 8 Structure of AGT complexes with β-CD (a) and γ-CD (b). (Reproduced with permission of Wiley-VCH from Ref. 50.)

position of the aromatic ring of AGT in the (±)-AGT/γ-CD complex (Fig. 7b). These data support a complex formation from the narrower primary side of γ-CD with amino group ahead (Fig. 8b). The glutarimide ring is apparently less involved in the complex formation in this case. However, the involvement of the methyl group in complex formation by a still unknown mechanism can not be completely excluded. Thus, as shown with this example, ROESY experiments may reveal principal differences in the structure of analyte–CD complexes in solution (see also ref. 22 and the references cited herein).

Another example when ROESY experiments may provide information about distinct differences between the structures of analyte–CD complexes is the complex of CL with β-CD and HDA-β-CD. Again it was noted that the enantiomers of CL exhibit an opposite affinity pattern towards these two CDs (Fig. 4).[51]

A 1D ROESY spectrum of the (±)-CL complex with β-CD is shown in Fig. 9a. Upon saturation of the aromatic protons of CL a

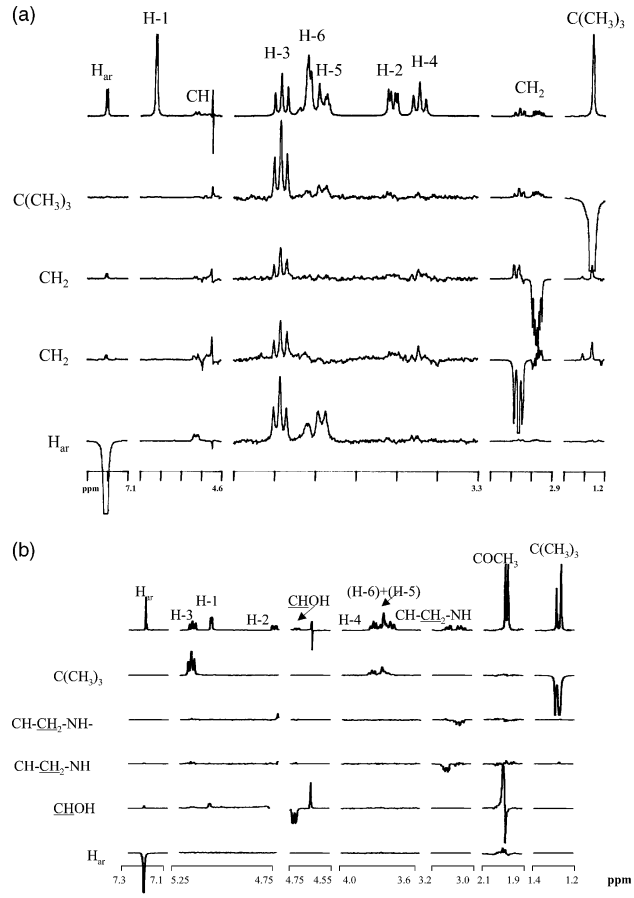

Fig. 9 1D-ROESY spectra of CL [(*R*)/(*S*) = 2 : 1] in the presence of one equivalent of β-CD (a) and HAD-β-CD (b). (Reproduced with permission of Wiley-VCH from Ref. 51.)

significant response was observed for the H-3 protons and a rather weak but measurable response for the H-5 protons of β-CD. Both, the H-3 and H-5 protons, are located inside the cavity of β-CD close to the secondary and primary CD rims, respectively. Thus, these data clearly indicate that the CL molecule forms an inclusion complex with β-CD entering the cavity from the secondary wider side with substituted phenyl moiety ahead. An additional indication for the analyte approaching the cavity of β-CD from the secondary side is the following: when the protons of CH_2 and $C(CH_3)$ groups of CL were saturated, the NOE-response was observed on H-3 protons which are located inside the cavity of β-CD close to the secondary rim. At the same time almost no effect was observed on the H-5 and H-6 protons which are located close to the primary narrower rim of β-CD. Thus, based on the 1D ROESY spectrum shown in Fig. 9a the structure of the (±)-CL/β-CD complex can be proposed as shown in Fig. 10a. A rather strong NOE effect was observed between the *tert*-butyl moiety of CL and the H-3 protons of β-CD. The structure shown in Fig. 10a allows some spatial proximity between these groups. However, at present it is impossible to conclude whether this proximity or the formation of a complex with an alternative structure (*tert*-butyl moiety inside the cavity of β-CD) takes place for a significant population. The structure represented in Fig. 10a was unambiguously supported by a 1D ROESY experiment in which the protons were saturated in the

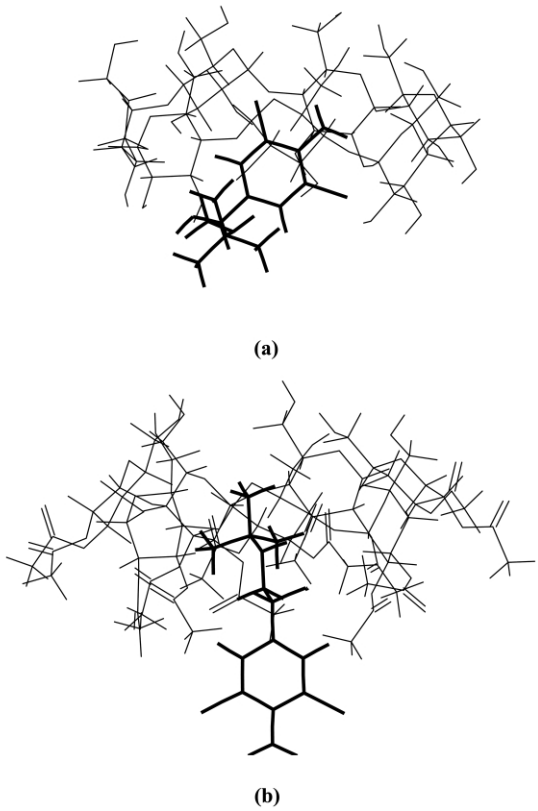

Fig. 10 Structure of CL complexes with β-CD (a) and HAD-β-CD (b). (Reproduced with permission of Wiley-VCH from Ref. 51.)

β-CD molecule and the response was observed on the protons of CL.[51] Thus, the response was found for the protons of CL only when H-3 and H-5 protons of β-CD were saturated. In addition, upon saturation of the H-3 protons both the aromatic and alkyl parts of CL responded equally whereas the rather weak effect was observed only for the aromatic protons of CL and no effect at all on the alkyl part upon saturation of H-5 protons of β-CD.[51]

The information regarding the structure of the (*R*,*S*)-CL complexes with HDA-β-CD was obtained using ROESY experiments, which were performed with three different pulse sequence for the complex between CDs and a 3 :1 (w/w) mixture of (*S*)- and (*R*)-CL.

The 1D ROESY spectrum of the (*R*,*S*)-CL/HDA-β-CD complex was significantly different from that of the (*R*,*S*)-CL/β-CD complex (Fig. 9b). Thus, upon irradiation of the aromatic protons in CL, no response was observed for the H-3 and H-5 protons located inside the cavity of HDA-β-CD and a measurable effect was observed for the protons of the acetyl groups of HDA-β-CD (Fig. 9b).[51] When the protons of the *tert*-butyl moiety were irradiated, a very strong effect was observed for the H-3 protons located inside the cavity of HDA-β-CD. These data indicate that CL enters the cavity of HDA-β-CD also from the secondary wider side (similar to β-CD) but in contrast to β-CD not with the phenyl moiety but with the *tert*-butyl moiety ahead. The most likely structure of the (*R*,*S*)-CL/HDA-β-CD complex based on these data is shown in Fig. 10b.[51]

This structure was clearly supported by the data obtained when irradiating the protons of HDA-β-CD and observing the response for the protons of CL.[51] Thus, upon irradiation of the H-3 protons of HDA-β-CD an intermolecular NOE was observed only for the protons of the *tert*-butyl moiety of CL. In combination with the NOE response observed for the aromatic protons of CL upon irradiation of the acetyl group of HDA-β-CD these data indicate that the *tert*-butyl moiety is included into the cavity and the phenyl moiety is located outside of the cavity close to the secondary rim of HDA-β-CD. Thus, the ROESY experiment shows a significant difference between the structures of the CL complexes with β-CD and HDA-β-CD. 1D and 2D transversal ROESY (T-ROESY) experiments confirmed that the effect observed in the 1D ROESY spectra were solely of intermolecular origin and that there was no significant contribution due to intramolecular TOCSY (total correlation spectroscopy) magnetization transfer. Thus, all ROESY experiments clearly indicated that CL forms intermolecular inclusion complexes with β-CD and HDA-β-CD. The CL molecule is included into the cavity of both CDs from the secondary wider rim. The most distinct difference between the two complexes is that the phenyl moiety of CL is most likely included into the cavity of β-CD whereas the *tert*-butyl moiety is included into the cavity of HDA-β-CD.

One "confusing" example of 1D-ROESY studies was described in ref. 54. In this study, the structure of the complex of the antihistaminic drug brompheniramine (BrPh) with β-CD and TM-β-CD was studied by 1D-ROESY experiments in solution. For the complexes of (+)-BrPh with both CDs unambiguous confirmation was obtained indicating the inclusion of the 4-bromophenyl moiety of the analyte into the cavity of the CD. In addition, in the case of the (+)-BrPh complex with β-CD, a weak but positive NOE effect was observed also for the protons of the maleate counter anion when saturating the CD protons H-3 and H-5 located inside the cavity. This observation may indicate the simultaneous inclusion of the 4-bropmophenyl moiety and maleate counter anion into the cavity of β-CD, but this contradicts simple geometric considerations and the assumption that the stoichiometry of the complex is 1 : 1.

An X-ray crystallographic study performed on the monocrystals obtained from a 1 : 1 aqueous solution of (+)-BrPh maleate and β-CD (Fig. 11) provides a plausible explanation for the above

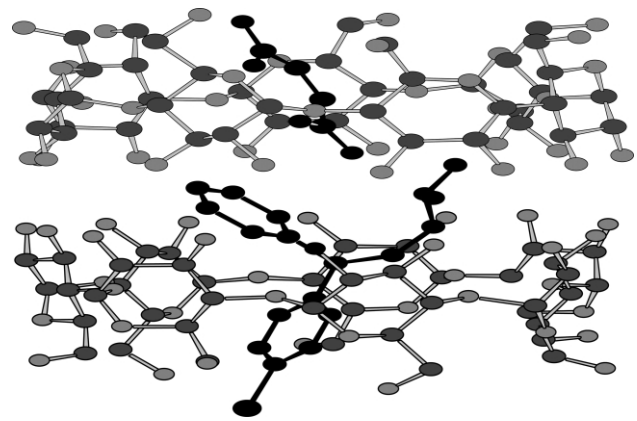

Fig. 11 Structure of (+)-BrPh maleate β-CD complex in the solid state determined by X-ray crystallography. (Reproduced with permission of Elsevier from Ref. 54.)

mentioned contradiction. In particular, as shown in Fig. 11 (+)-BrPh forms with β-CD, at least in the solid state, not a 1 : 1 complex but a complex with 1 : 2 stoichiometry. In this complex the (+)-BrPh maleate is sandwiched between two molecules of β-CD. The 4-bromophenyl moiety of (+)-BrPh enters the cavity of one of the β-CD molecules whereas the cavity of another β-CD molecule is occupied by the maleate counteranion.[54]

One of the interesting questions of CD chemistry is whether the inclusion complexation represents a prerequisite for chiral recognition and, if not, which part of the CDs, external or internal, provides a more favorable environment for enantioselective recognition? Recently the synthesis of highly crowded heptakis-(2-*O*-methyl-3,6-di-*O*-sulfo)-β-CD (HMdiSu-β-CD) with 14 bulky sulfate substituents on both primary and secondary CD rims was reported.[59] The bulky substituents on both sides of the cavity entrance may hinder inclusion complex formation between chiral analytes and HMdiSu-β-CD. In a recent study 27 cationic chiral analytes were resolved in CE using native β-CD and HMdiSu-β-CD.[56] For 12 of 16 chiral analytes resolved with both chiral selectors the enantiomer migration order was opposite. Analysis of the structures of analyte–CD complexes in solution indicated that in

contrast to mainly inclusion-type complexation between chiral analytes and β-CD, external complexes are formed between the chiral analytes and HMdiSu-β-CD.[56]

As can be seen from Fig. 12 the enantiomers of AGT enantioselectively bind to HMdiSu-β-CD and are baseline resolved with this chiral selector in CE. In addition, the significant CICS

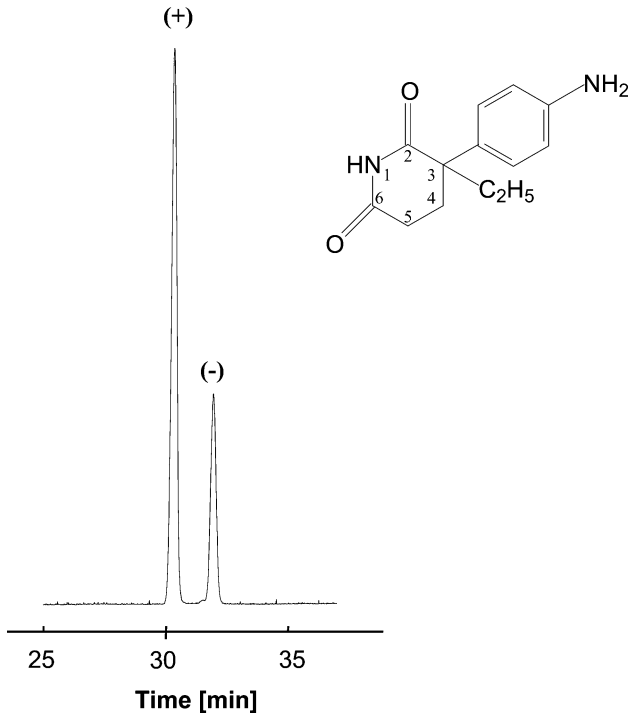

Fig. 12 Enantioseparation of AGT with 20 mg ml^{-1} HMdiSu-β-CD. (Reproduced with permission of Wiley-VCH from Ref. 56.)

differences were observed for the protons of AGT enantiomers in NMR spectrum of the complex (Fig. 13). As the 1D T-ROESY spectra shown in Fig. 13 indicate, AGT most likely does not form an inclusion complex with HMdiSu-β-CD. No intermolecular NOE was observed on any of the HMdiSu-β-CD protons upon irradiation of analyte protons (Fig. 13). Some exception represents the OCH_3 protons of HMdiSu-β-CD which are located on the secondary rim of the CD cavity (Fig. 13). The weak NOE was observed on the protons of OCH_3 group upon saturation of almost all protons of the analyte except those of CH_2–CH_3 group (Fig. 13).

Thus, inclusion complex formation between CDs and their chiral guests does not seem to be a necessary prerequisite for chiral recognition. CDs are able to form quite strong external complexes enantioselectively with some chiral guest molecules. However, the chiral recognition in inclusion type complex, at least in this particular case, appears to be somewhat superior.[56]

As shown from the aforementioned studies, the structural reasons responsible for the affinity reversal between the enantiomers and CDs may vary from analyte to analyte and from CD to CD. Most likely there is no universal structural or chemical reason for affinity reversal either from the side of the chiral analyte or the CD. This means that even significant differences observed in the structures of analyte–CD complexes may not be unambiguously considered to be the reason of affinity reversal. The most likely solution of this dilemma appears to be development of techniques for energy calculations based on the structure, dynamics and statistical weight of given complexes. The contributions of individual intermolecular forces must be assigned and calculated accurately in total energy terms. The methods of molecular modelling and molecular mechanics calculations available at present do not ideally meet this challenge.

5 Molecular modeling of analyte–CD interactions

CDs are rather rigid molecules of medium size and therefore suitable for molecular modeling calculations. In addition, many CDs are well studied by alternative techniques of structure elucidation. Among these, X-ray crystallographic and NMR data are of special interest.

A thermodynamic term describing chiral recognition is determined by the difference between the formation free energies of the transient diastereomeric complexes between the enantiomers and a chiral selector. Therefore, the exact calculation of the absolute energy values is not necessarily required in molecular modeling studies related to enantioseparations. This simplifies the calculations. On the other hand, due to the extremely high efficiency of CE the technique allows enantioseparations to be observed even for those selector–selectand pairs where the difference between the free energies of formation of the diastereomeric complexes is extremely small. The precise calculation of very small energy differences remains a challenging task even for very sophisticated

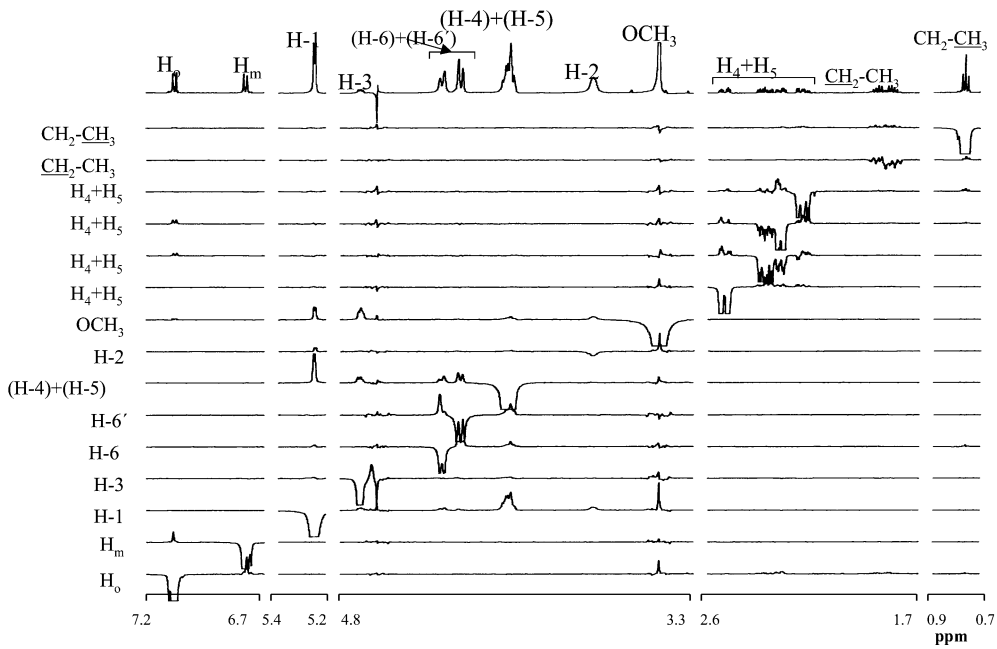

Fig. 13 1D-T-ROESY spectrum of AGT/HMdiSu-β-CD complex. (Reproduced with permission of Wiley-VCH from Ref. 56.)

state of the art energy minimization techniques. Additional care must be taken in order to obtain the maximal approach of the model to the real separation conditions. Thus, for instance, molecular modeling calculations are often performed in vacuum without taking into account the effect of the medium. However, the aqueous medium commonly used in CE, dramatically affects the hydrophobic and hydrogen bonding interactions. Moreover, the ionic strength of the buffer plays a decisive role for electrostatic intermolecular interactions. Another important point is a correct selection of the starting and the boundary conditions for energy minimization. Incorrectly defined conditions may totally confuse the calculations. For instance, when performing the molecular modeling calculations for the complex between TM-β-CD and (+)-BrPh in a neutral form the energy values indicated that the complex formation with the alkyl amino moiety included into the cavity of TM-β-CD would be energetically favorable. The structure with the alkyl amino moiety included into the cavity was also observed by an X-ray experiment performed on the monocrystals obtained from the mixture of an aqueous suspension of deprotonated (+)-BrPh as a free base and TM-β-CD (Fig. 14).[54] These

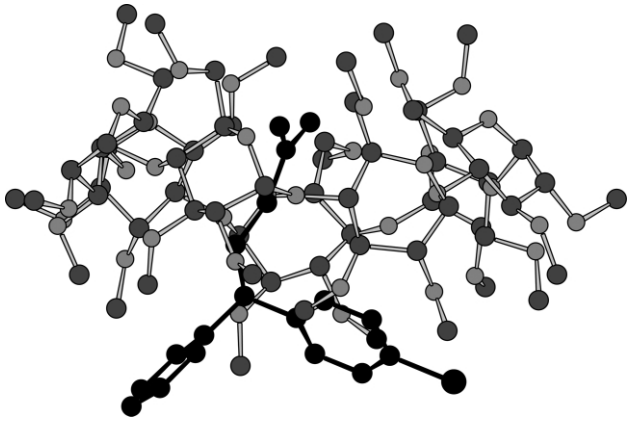

Fig. 14 Structure of (+)-BrPh maleate TM-β-CD complex in the solid state determined by X-ray crystallography. (Reproduced with permission of Elsevier from Ref. 54.)

results are contradictory to the structure derived from the 1D-ROESY experiment in solution. The intermolecular NOE-effects clearly indicated the inclusion of the 4-bromophenyl moiety into the cavity of TM-β-CD as shown in Fig. 15.[54] Taking into consideration that the (+)-BrPh maleate, *e.g*. the protonated form of (+)-BrPh molecule was applied for the 1D-ROESY studies in solution, force-field calculations were performed again for interactions of a single positively charged (+)-BrPh with TM-β-CD. The energy values obtained in this case clearly indicate that the complex formation with the 4-bromophenyl moiety of the (+)-BrPh molecule included into the cavity of TM-β-CD is energetically favorable which is in agreement with the structure observed using 1D-ROESY studies in solution (Fig. 15).[54]

In the authors opinion the most useful application of molecular modeling and molecular mechanics calculations to enantioselective analyte–CD interactions would be a computation of individual intermolecular forces based on the structure, dynamics and population of the complexes determined by instrumental techniques.

6 Conclusions

Multiple forces involved in analyte–CD interactions make an understanding of analyte binding and chiral recognition mechanisms by CDs extremely difficult. CE contributes significantly to a fast screening of the recognition pattern in chiral guest–CD interactions. Molecular modeling studies when used in combination

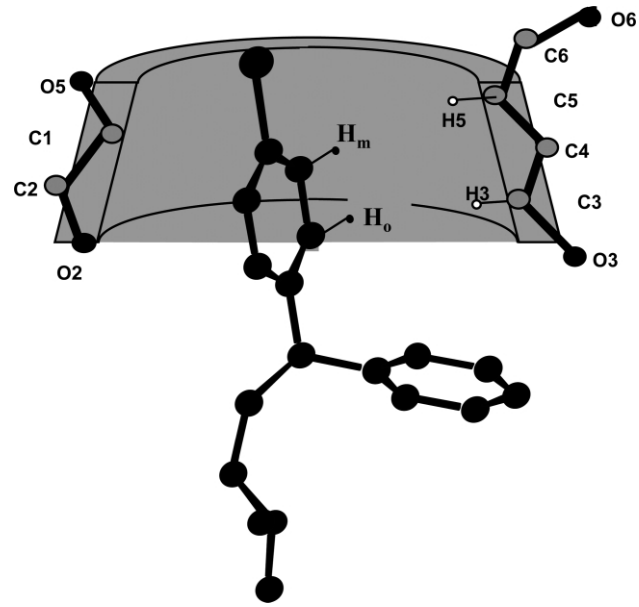

Fig. 15 Structure of (+)-BrPh maleate β-CD complex in solution derived from 1D-ROESY experiment. (Reproduced with permission of Elsevier from Ref. 54.)

with instrumental techniques, especially with ROESY experiments in NMR spectroscopy and X-ray crystallography may significantly contribute to the understanding of the nature of the intermolecular forces responsible for guest–CD interactions and chiral recognition.

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