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Quantitative approach for the screening of cyclodextrins by nuclear magnetic resonance spectroscopy in support of chiral separations in liquid chromatography and capillary electrophoresis Enantioseparation of norgestrel with α-, β- and γ-cyclodextrins

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Abstract

A quantitative NMR approach is proposed for the screening of cyclodextrins with regard to their enantioselectivity as chiral mobile phase additives in column reversed-phase chromatography and capillary electrophoresis. Similarities and differences between the mechanism of enantiomeric peak-separation in NMR and HPLC and CE are interpreted. The affinity of *d*-norgestrel to bind to (α-, β-, γ-) cyclodextrins in aqueous solution was quantified and compared by determining the association constants from chemical shift data. The association constant of *l*-norgestrel was estimated from titration of the racemate. Differences between the apparent association constants of the enantiomerically pure drug and the racemate are discussed from the point of view of enantiomeric competition for the cyclodextrin. The apparent association constants and chiral selectivities determined by ^{1}H NMR for *dl*-norgestrel/γ-CD system at various water–methanol ratios are correlated with the corresponding chromatographic results found in the literature. The pitfalls of previously proposed screening methods based on comparison of chemical shift differences with separation parameters are discussed. © 2002 Published by Elsevier Science B.V.

Keywords: Enantiomer separation; Nuclear magnetic resonance spectrometry; Association constants; Non-linear fitting; Cyclodextrins; Norgestrel

1. Introduction

With a growing demand for the exploration of the different toxicities and metabolic pathways of drug enantiomers, pharmaceutical companies are putting increasing efforts into the characterisation of optically active drugs with respect to their optical purity. Although the basics of the NMR methods developed for the determination of enantiomeric excess (ee) have long been established [1–3], in industrial laboratories the majority of separations are traditionally handled mainly by high-performance liquid chromatography (HPLC) [4] and capillary electrophoresis (CE) [4–6]. However, by the widespread application of high-field NMR instruments this area is experiencing a breakthrough. In pharmaceutical chiral separations, NMR spectroscopy plays a multiple role. Unlike RP-HPLC or CE, NMR is an analytical method which allows the measurement of

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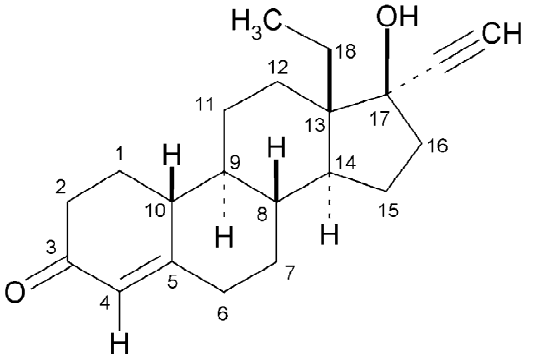

Fig. 1. The structural formula of norgestrel.

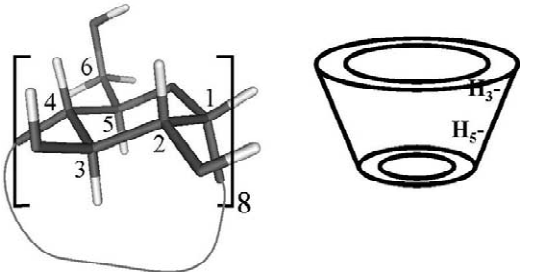

Fig. 2. The conical conformation of γ-CD with the approximate positions of the H_3- and H_5-hydrogens located at the interior surface of the CD.

the enantiomeric ratio without spatial separation of the enantiomers [7], while simultaneously providing direct structural information about the nature, conformation and dynamics of the diastereomeric complexes of chiral molecules in a way that is not accessible from separation methods. Many workers recognised the importance of NMR in the exploration of the nature of cyclodextrin complexes [8–15]; however, the number of systematic studies on correlations between enantiomeric separations in NMR and in HPLC or CE is limited [12,16,17].

Norgestrel (Fig. 1), an important progestogen component of oral steroid contraceptives, is either used in its racemic form (*dl*-norgestrel) or as one of its two enantiomers (*d*-norgestrel or levonorgestrel) (**1**). It was reported that norgestrel enantiomers are very well separable in reversed-phase liquid chromatography (RP-HPLC) by γ-cyclodextrin (γ-CD) as the chiral mobile-phase additive [18–22]. CDs [23] [cyclic oligosaccharides composed of six, seven or eight α-D-glucopyranose units (α-, β-, γ-CD, respectively)] form a family of excellent chiral selectors in NMR spectroscopy as well [24]. Although we previously described that in ^{1}H NMR the ethynyl moiety of *dl*-norgestrel shows a considerably larger chemical shift non-equivalence (ca. 0.16 ppm) when complexed with γ-CD (Fig. 2) [25] than in the presence of chiral shift reagents [26], these results were not correlated with the success of chromatographic separations.

The aim of the present article is to provide a general basis for the prediction of enantioselectivity of CDs in RP-HPLC and CE on the grounds of analysing NMR chemical shift data measured in the racemate. We also make an attempt to emphasize the similarities and the possible differences between the interpretation of enantiomeric separations in NMR and other separation techniques (RP-HPLC and CE using CDs as mobile phase additives), which is necessary for the correct insertion of NMR results into chromatographic or electrophoretic work. In connection with the complexation of racemic norgestrel with native α-, β-, γ-CDs, a method is proposed for the interpretation of the titration results in NMR.

2. Theory

2.1. Relationship between NMR and RP-HPLC and CE

The issue of screening cyclodextrins by NMR spectroscopy with the aim of predicting the success of chiral separations in RP-HPLC and CE systems with CDs as mobile phase additives became a field of interest in a few pharmaceutical research groups in the past few years [8,12,14]. Owens et al. [12] were first to make an attempt to describe a ''possible correlation'' between enantioseparation in CE and the chemical shift differences induced by the CD in NMR. Since their method, as well as others [27,28] lacks the theoretical basis for the quantification of the binding equilibrium between the enantiomers and the CD, it cannot describe properly the disagreement—occasionally occurring—between NMR and CE.

By using a simplified model, the theoretical grounds of the quantitative correlation between NMR

and HPLC or CE will be discussed below. In order to explore the problem, we take the simplest (1:1 stoichiometry) complexation equilibrium for a chiral guest (G) molecule and CD

$$G + CD \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} GCD; \qquad G = d,l$$

where G represents either of the enantiomers (*d* or *l*). The equilibrium association constant is then defined as

$$K_a^G = \frac{k_{+1}}{k_{-1}} = \frac{[GCD]}{[G][CD]} \tag{1}$$

where [G], [CD] and [GCD] are the equilibrium (or analytical) concentrations of the free (uncomplexed) analyte, the free cyclodextrin and that of the complex, respectively, whereas k_{+1} and k_{-1} are the rate constants of the formation and dissociation processes.

By assuming that the observed spectroscopic, chromatographic or electrophoretic property ξ_{obs}^G for the analyte is such that it can be written as the population-weighted average of the properties of the two individual states (the uncomplexed ξ_{free} and complexed state ξ_{GCD}), the observed property is related to K_a^G by

$$\frac{1}{(\xi_{obs}^G - \xi_{free})} = \frac{1}{(\xi_{GCD} - \xi_{free})K_a^G[CD]} + \frac{1}{(\xi_{GCD} - \xi_{free})} \tag{2}$$

Since its first derivation [29] in connection with UV spectroscopy, Eq. (2)—called the Benesi–Hildebrand equation—has gained importance in other analytical methods (NMR [30], RP-HPLC [31,32] and CE [33]) as well. The measurement of ξ_{obs}^G at large excess of the chiral selector, allows us to replace [CD] in Eq. (2) by the total concentration of the analyte c_{CD} since in

$$c_{CD} = [CD] + [GCD] \tag{3}$$

[GCD] can be neglected relative to the concentration of the uncomplexed host. This commonly used approximation is useful for the determination of the association constant K_a^G from the slope and intersection of the linear plot $(\xi_{obs}^G - \xi_{free})^{-1}$ versus $[c_{CD}]^{-1}$.

In our treatment, Eq. (2) is the simplest and most fundamental equation by which the measured spectroscopic, chromatographic and electrophoretic properties are connected to each other via the value of the association constant K_a^G. We note that the measured quantity corresponding to the CD-free state of the analyte (ξ_{free}) is inherently identical for the enantiomers, whereas the quantities ξ_{dCD} and ξ_{lCD} for the complexed state of the analyte will either be identical (RP-HPLC and CE) or will differ (NMR) according to the applied analytical method. In the following, a RP-HPLC and CE system is described where the knowledge of the association constants (K_a^d, K_a^l) determined by NMR is especially useful for the prediction of the enantioselectivity of the chiral selector.

2.2. The chromatographic model

In an isocratic elution RP-HPLC system using an achiral stationary phase (SP) with cyclodextrin added to the mobile phase, the phenomenon of enantiomeric separation is closely related to the differential stabilities of the diastereomeric complexes *d*CD and *l*CD. When dissolved in water, CDs adopt a conical conformation having a relatively hydrophobic cavity (Fig. 2) which accepts guest molecules by their hydrophobic moiety of proper size and shape [23]. If CD is added to the mobile phase, the retention of the enantiomers of a hydrophobic analyte is reduced differently, which leads to the chromatographic separation of the enantiomers. This phenomenon of enantioseparation can be understood and modelled as follows.

For native CDs under reversed-phase conditions, the interaction of the CD with the C_{18} stationary phase is much weaker than that of a hydrophobic analyte, such as norgestrel. Fujimura et al. proposed [32] that if GCD is predominantly an inclusion-type complex, it is reasonable to assume that the SP⇌GCD interaction is also much weaker than the SP⇌G interaction. It follows that G is adsorbed uncomplexed at the stationary phase and exists both as G and as GCD in the mobile phase. Enantiomeric separation will thus mainly stem from the difference in the stabilities of *d*CD and *l*CD in the mobile phase

rather than any difference between their adsorption properties on the stationary phase. This model became widely accepted in the past two decades [34–38] when describing the retention characteristics in various RP-HPLC systems and will likewise be adopted here in the following discussion.

Using the assumption that the stationary phase concentration of the complexed species is negligible relative to that of the free analyte, it has been derived [38–41] that the retention factors (k'^{G}) of the enantiomers (G=*d*,*l*) are affected by the concentration of the mobile phase additive according to Eq. (4):

$$k'^{G} = \frac{k'}{1 + K_a^G[CD]_M} \quad (4)$$

where the subscript M denotes the mobile phase concentration of the chiral selector. According to Eq. (4), the enantiomer with the larger binding constant (K_a^G) is expected to elute before the one with the smaller K_a^G, giving rise to a smaller retention factor and therefore a shorter retention time. Eq. (4) plays a key role in the theory of the NMR-based prediction of chromatographic behaviour, since it relates a basically chromatographic parameter—the retention factor—to the association constant which is a measurable quantity in NMR spectroscopy [30] (see below). As a further simplification of the theory, we assume no retention difference between the diastereomeric complexes; moreover, the retention times of the complexes are assumed to be identical with that of the unretained solvent.

2.3. The electrophoretic model

Eq. (2) is generally used in CE with the appropriate substitution of the electrophoretic mobilities μ_{GCD}, μ_{free}, μ_{obs}^G for the complex as well as the analyte in the absence and in the presence of the CD, respectively [42–44]. The enantiomers are assumed to have identical electrophoretic mobilities both in their free and their complexed state, and enantiomeric separation is possible when the two enantiomers have different association constants ($K_a^d \neq K_a^l$) [45,46]. If the electrophoretic model uses approximations analogous to the reversed-phase chromatographic model (the relative mobilities are chosen such that $\mu_{free} \ll \mu_{GCD}$), the retention order is identical to that of the chromatographic model (i.e. $\mu_{obs}^l < \mu_{obs}^d$ if $K_a^l < K_a^d$) [47]. In contrast to RP-HPLC however, the application of charged and uncharged CDs together with the possibility to control the direction and magnitude of the electroosmotic flow gives more flexibility to the design of the elution order of chiral separations in CE. By using neutral CDs for the separation of analytes with chargeable groups, the electophoretic mobilities can be reversed $\mu_{free} \gg \mu_{GCD}$ which leads to a reversed retention order [48–50]. It follows that there may exist an intermediate region where the coincidence of separation parameters ($\mu_{free} \approx \mu_{GCD}$) results in poor separation of the enantiomers even if $K_a^d \neq K_a^l$. As the relative electrophoretic mobility of analyte and chiral selector cannot be modelled by NMR, it is left for the chromatographer to select a CD-analyte system where μ_{free} and μ_{CD} are significantly different to avoid the intermediate region ($\mu_{free} \approx \mu_{GCD}$). In the forthcoming comparison of separation mechanisms in RP-HPLC, CE and NMR, the ''electrophoretic model'' will be used under the assumption that either $\mu_{free} \ll \mu_{GCD}$ or $\mu_{free} \gg \mu_{GCD}$ is satisfied.

2.4. Equations used in NMR spectroscopy

As was introduced earlier, Eq. (2) was reported [30] as being generally applicable not only in RP-HPLC and CE but also in NMR, provided the system is in fast exchange on the chemical shift time scale [51]. We can thus write

$$\frac{1}{(\delta_{obs}^{G,i} - \delta_{free}^{i})} = \frac{1}{(\delta_{GCD}^{i} - \delta_{free}^{i})K_a^G c_{CD}} + \frac{1}{(\delta_{GCD}^{i} - \delta_{free}^{i})} \quad (5)$$

where δ_{free}^{i}, δ_{GCD}^{i} denote the chemical shifts of the *i*th nucleus of the free- and complexed analytes, respectively. To avoid the restriction that Eq. (5) is valid only at a large excess of the CD, in NMR, a more generalized equation (Eq. (7))—describing the variation of the chemical shift of the analyte for the whole concentration range of the CD—can be derived by combining Eq. (6):

$$c_G = [G] + [GCD] \quad (6)$$

with Eqs. (1)–(3):

$$\delta^{G,i}_{obs} - \delta^{i}_{free} = \frac{(\delta^{i}_{GCD} - \delta^{i}_{free})\left(c_{CD} + c_G + 1/K^{G}_{a} - \sqrt{(c_{CD} + c_G + 1/K^{G}_{a})^2 - 4c_{CD}c_G}\right)}{2c_G} \quad (7)$$

The difference between the chemical shifts of the analyte measured without (δ^{i}_{free}) and with ($\delta^{G,i}_{obs}$) the chiral additive $\Delta\delta^{G,i} = (\delta^{G,i}_{obs} - \delta^{i}_{free})$ has been termed [7,12] as the *shift displacement*. Analogously, by following the terminology of Owens et al., we will herein term $\Delta\delta^{G,i}_{\infty} = (\delta^{i}_{GCD} - \delta^{i}_{free})$ as the *limiting shift displacement* of the *i*th nucleus.

To obtain the association constant from NMR chemical shift data, one has to perform a series of measurements by following the variation of the chemical shift of a given resonance as a function of the host concentration c_{CD}. Provided that c_G is kept constant throughout the titration, the remaining unknown parameters δ^{i}_{GCD} and K^{G}_{a} can be determined by fitting Eq. (7) to the plot of the experimental data: $\Delta\delta^{G,i}$ versus c_{CD}. The chemical shifts of the nuclei in the uncomplexed state of the ligand (δ^{i}_{free}) are regarded as known parameters from a measurement without the complexing agent.

2.5. Comparison of the mechanisms of enantioseparation in NMR and RP-HPLC and CE

As it was pointed out above, in RP-HPLC and CE, the retention difference and the difference in the electrophoretic mobilities between the diastereomeric complexes has a negligible effect on the degree of enantioseparation (i.e. $\xi_{dCD} \approx \xi_{lCD}$) if compared to the effect arising from the difference between the association constants ($K^{d}_{a} \neq K^{l}_{a}$) in the mobile phase. This holds until the retention or migration behaviour of the chiral modifier is very similar to that of the complexes but drastically different from that of the analyte. For the purposes of quantifying chiral separation, it will prove useful to introduce the parameter *chiral selectivity* (α_c) [52], defined as the ratio of the association constants of the enantiomers

$$\alpha_c = \frac{K^{d}_{a}}{K^{l}_{a}} \quad 1 < \alpha_c \text{ if } K^{l}_{a} < K^{d}_{a} \quad (8)$$

where the parameters are chosen such that $1 \leq \alpha_c$. Ideally, chromatographic or electrophoretic separation of the enantiomers occurs when $1 < \alpha_c$ (this does not imply a successful ''baseline'' separation).

In RP-HPLC, an important measure of separation is the *separation selectivity* (α_s) [32,35] which is independent of the peak width and symmetry and is defined as the ratio of the retention factors:

$$\alpha_s = \frac{k'^{l}}{k'^{d}} = \frac{1 + K^{d}_{a}[CD]_M}{1 + K^{l}_{a}[CD]_M} \quad \text{if } k'^{d} < k'^{l} \quad (9)$$

Using the assumption that the electrophoretic mobilities of the complexes are equal ($\mu_{dCD} \cong \mu_{lCD}$), Wren et al. derived [45] that in CE (for 1:1 stoichiometry), the maximum of the difference between the electrophoretic mobilities $\Delta\mu = |\mu^{d}_{obs} - \mu^{l}_{obs}|_{max}$ is at

$$[CD]_{opt} = \frac{1}{\sqrt{K^{d}_{a}K^{l}_{a}}} \quad (10)$$

This implies that there exists an optimum CD concentration at which the separation of the electrophoretic peaks is largest [53–55]. Eq. (10) is also valid in our model RP-HPLC system due to the analogy with CE and shows that maximization of the separation not only requires a knowledge of the relative magnitudes of the association constants (Eq. (8)), but we must also know the pertinent absolute values in order to find the optimal CD concentration. It is noted that separation at $[CD]_{opt}$ does not necessarily give optimal resolution since its derivation does not involve the treatment of peak width and symmetry. Since Eqs. (8)–(10) are fundamental relations in the theory of dynamic separation with CDs as mobile phase additives, the exact knowledge of K^{d}_{a} and K^{l}_{a} from an independent method (NMR [30], UV [56]) determined at identical conditions (same solvent, pH, temperature, etc.) is a great help prior to chromatographic or electrophoretic work which aids the rational design of separation in either technique.

While the proposed mechanism of enantioseparation in HPLC and CE is manifested in the separation of two chromatographic or electrophoretic peaks

corresponding to the enantiomers, in NMR the situation is more complex. NMR spectroscopy in principle offers a multiple chance to observe enantiodiscrimination. Each NMR active nucleus carries the possibility to differ by their intrinsic chemical shifts in their diastereomeric complexes ($\delta^i_{d\mathrm{CD}} \neq \delta^i_{l\mathrm{CD}}$). This entails the possibility to discriminate between enantiomers even if the association constants for the binding of the two enantiomers to CD are the same ($K^d_\mathrm{a} = K^l_\mathrm{a}$) (Fig. 3A). On the other hand the opposite case, showing a similarity to the mechanism of enantioseparation in RP-HPLC and CE, is also possible: when the chemical shifts for the diastereomeric complexes are equal within experimental error ($\delta^i_{d\mathrm{CD}} \approx \delta^i_{l\mathrm{CD}}$) at a given spectral resolution, enantiodiscrimination is still possible if the association constants differ for the two complexes ($K^d_\mathrm{a} \neq K^l_\mathrm{a}$) (Fig. 3B).

A widely used measure of enantiomeric discrimination in NMR is the absolute value of the difference between the chemical shifts for the *i*th resonance of the enantiomers $\Delta\Delta\delta^i = |\delta^{d,i}_\mathrm{obs} - \delta^{l,i}_\mathrm{obs}|$—called the chemical shift non-equivalence [1,7,12]—at a given host–guest ratio and spectral resolution. We refer to the difference in the intrinsic chemical shifts of the complexes $\Delta\Delta\delta^i_\infty = |\delta^i_{d\mathrm{CD}} - \delta^i_{l\mathrm{CD}}|$ as *the limiting shift non-equivalence*. The basic difference between shift non-equivalence ($\Delta\Delta\delta^i$) and its limiting value ($\Delta\Delta\delta^i_\infty$) is that the former is experimentally measurable from a single spectrum of the racemate at a well-defined c_CD concentration while the latter is a theoretical value determined by fitting Eq. (7) to a series of chemical shift data points $\Delta\delta^{\mathrm{G},i}$ obtained from experiments with varied host concentration c_CD. Depending on the magnitude of K^G_a however, $\Delta\Delta\delta^i_\infty$ can be fairly well approximated by $\Delta\Delta\delta^i$ being measured at large excesses of the CD. The conceptual difference between Fig. 3A and 3B is that while $\Delta\Delta\delta^i < \Delta\Delta\delta^i_\infty$ is valid for Fig. 3A for any value of $\Delta\Delta\delta^i$, the reverse relationship $\Delta\Delta\delta^i > \Delta\Delta\delta^i_\infty$ holds for Fig. 3B for each value of $\Delta\Delta\delta^i$.

Setting up a correlation between shift non-equivalence ($\Delta\Delta\delta^i$) values and separation indices [12] found in CE was the method proposed by Owens et al. for the characterization of CDs with respect to the enantioselectivity in CE. Their screening method of measuring $\Delta\Delta\delta^i$ values for various CD complexes of the enantiomers is undoubtedly fast, but may be

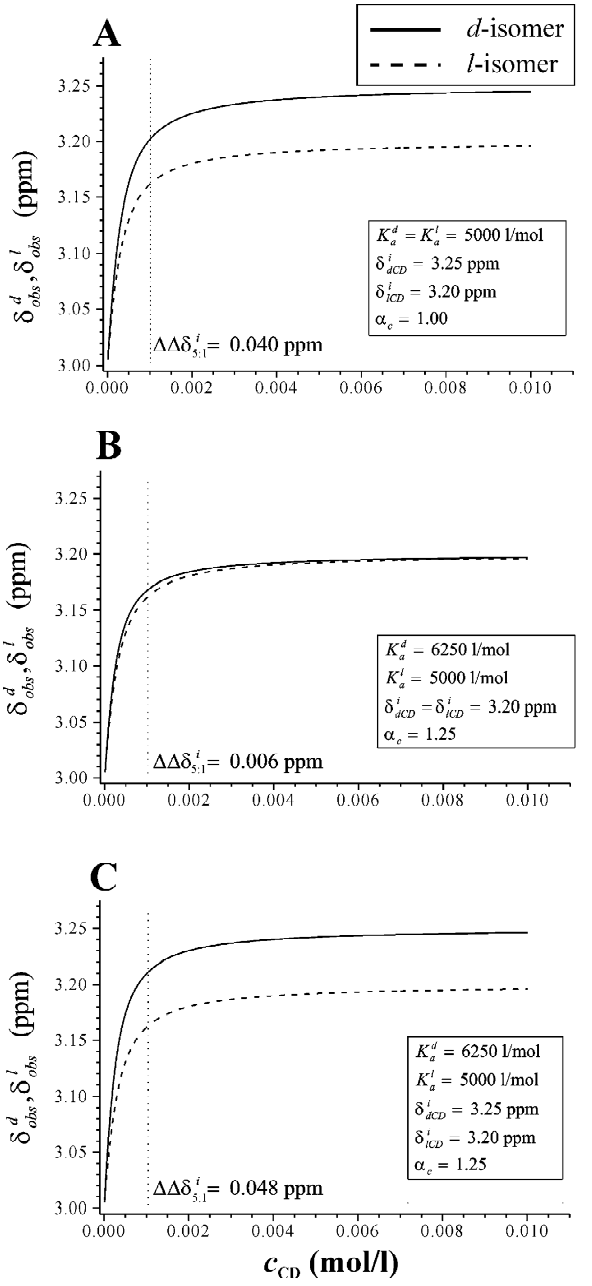

Fig. 3. Simulation of cases of enantiomeric discrimination in NMR. The titration curves were calculated using Eq. (7) with $c_\mathrm{G} = 2\mathrm{E}-4$ mol/l and $\delta^i_\mathrm{free} = 3.00$ ppm (other parameters are indicated in the parameter boxes). The $\Delta\Delta\delta^i_{5:1}$ values correspond to the 5:1 host/guest ratio (marked with a vertical dashed line).

unreliable in general, which can best be understood by the comparison of the two extreme cases shown in Fig. 3A,B. In Fig. 3A, the massive shift non-equivalence ($\Delta\Delta\delta^i$) observed in NMR (at, say, a host–guest = 5:1 molar ratio) does not imply a successful separation in chromatography, while in Fig. 3B, the unequal association constants may provide a successful separation in RP-HPLC or CE, even if NMR exhibits no or negligible enantioseparation. The result is a disagreement in both cases between NMR and RP-HPLC or CE.

The origin of the above disagreement stems from the fact that enantioseparation in NMR cannot be modelled by ignoring the difference between the intrinsic properties of the complexes ($|\xi_{d\mathrm{CD}} - \xi_{l\mathrm{CD}}| \neq 0$), while those differences can be neglected (as discussed above) when describing the retention and migration behaviour in RP-HPLC and CE. Although in most cases the primary resolving factor in NMR is the large difference between the chemical shift of the nuclei of the enantiomers in their diastereomeric complexes ($\delta^i_{d\mathrm{CD}} \neq \delta^i_{l\mathrm{CD}}$) (Fig. 3A), in general neither the stability constants $K^d_a \neq K^l_a$ nor the specific chemical shifts of the complexes $\delta^i_{d\mathrm{CD}} \neq \delta^i_{l\mathrm{CD}}$ are equal and both factors contribute to enantiodiscrimination in liquid-phase NMR (Fig. 3C).

The need to estimate δ^i_{GCD} from preliminary $\Delta\delta^{\mathrm{G},i}$ measurements in the excess of CD is vital for two reasons. (1) Firstly, by the titration of each nucleus (aiming to find K^{G}_a) after adding a certain amount of CD to the solution of the analyte, some resonances (those having rather different δ^i_{GCD} values relative to δ^i_{free}) tend to show considerable shift displacement while others (where $\delta^i_{\mathrm{GCD}} \approx \delta^i_{\mathrm{free}}$) may not shift at all. This influences the accuracy and precision of the association constant determined from NMR chemical shift data, since larger shift displacements ($\Delta\delta^{\mathrm{G},i}$) give smoother titration curves at a given spectral resolution. (2) Secondly, from the point of view of the optimization of enantioseparation in NMR, two extreme cases (represented by Fig. 3A,B) may occur: (i) when the largest shift non-equivalence is found at an optimal value of the cyclodextrin concentration (optimization is necessary for best enantioseparation), or (ii) when $\Delta\Delta\delta^i$ is largest at a large excess of the CD (no optimization is required). The efficiency of NMR as an analytical tool in enantioseparation is largely due to the simultaneous occurrence of both types of nuclei in the analyte molecules [57].

2.6. Factors influencing the value of the apparent association constant in NMR

The association constant (K^{G}_a) which appears in Eqs. (5) and (7) has been derived on the basis of the simplest complexation model described by Eq. (1), since it is regarded as a theoretical value for the quantification of binding equilibria. In a real-life situation, however, deviations from the ideal case are frequent since both species (host and guest) may take part in additional competitive complexation processes in solution [58,59]. Depending on their nature, the competitive complexation processes may act by slightly changing the analytical concentration of the species which influences the measured chemical shift data points, i.e. the appearance of the titration curve. If Eqs. (5) and (7) are fitted to these data points, the association constant obtained will be some value different from the theoretical value and must be regarded as an *apparent association constant* denoted as K'^{G}_a.

In NMR one may estimate the value of the apparent association constants of the enantiomers (K'^d_a, K'^l_a) in two ways: (a) by carrying out two independent titrations using single enantiomers, or (b) via performing a single titration measurement with the racemic drug. The latter is more practical since the effort it requires is just half of the former method. In the case of molecules where one of the enantiomers is not available in enantiomerically pure form—as in the case of norgestrel—titration of the racemic sample is the only way to estimate the apparent association constant of the missing enantiomer (say K'^l_a).

The coexistence of the equations

$$d + \mathrm{CD} \overset{K^d_a}{\rightleftharpoons} d\mathrm{CD}; \quad l + \mathrm{CD} \overset{K^l_a}{\rightleftharpoons} l\mathrm{CD}$$

which will be referred to as *enantiomeric competition* in the following—is the most probable competing mechanism that influences the results of the racemate titration in dilute solutions. Since, in principle, $K^d_a \neq K^l_a$, the apparent association constants are expected to be different ($K'^d_a \neq K^d_a$ and $K'^l_a \neq K^l_a$) whether determined by method (a) or (b). It seems

that only the results of method (a) can be safely transferred to RP-HPLC or CE where enantiomeric competition is absent because of the spatial separation of the enantiomers.

In the case of norgestrel, determination of K_a^l required titration of the racemate due to the non-availability of the *l*-isomer. However we found that the $K_a'^d$ values of method (b) and (a) do not differ significantly if the effect of enantiomeric competition is compensated by taking the total concentration of both enantiomers into account during non-linear fitting. By analysis of the experimental results (see discussion of Fig. 9 later), this was exploited to estimate K_a^l found in RP-HPLC with $K_a'^l$ determined by NMR using method (b). Behind the compensation for the enantiomeric competition there are two basic ideas. On the one hand, the mutual dependence of the parameters K_a^G and c_G in Eq. (7) can be exploited to influence the value of $K_a'^G$ (during the course the non-linear analysis of the racemate data) by calculating with an apparent analyte concentration c_G' instead of c_G. On the other hand, the enantiomers are mathematically indistinguishable if $K_a^d = K_a^l$, which to some degree can be extended to cases where the two association constants are not equal but of similar orders of magnitude $K_a^d \approx K_a^l$.

In this section, the effect of enantiomeric competition on $\delta_{obs}^{d,i}$ is analysed by numerical simulations. We aimed to show that Eq. (7)—although derived for the competition free model—is also useful for the determination of $K_a'^d$ and $K_a'^l$ values from the racemate titration data. Later (in connection to Fig. 9), this gains importance by comparison of the *dl*-norgestrel results with those of the competition-free cases: titration of *d*-norgestrel and HPLC results [18,19]. The equations describing the titration curve of the *d*-enantiomer in the racemate were derived by combining Eqs. (1), (2) and (6) (written for both enantiomers) with the following Eqs. (11) and (12):

$$c_{CD} = [CD] + [dCD] + [lCD] \tag{11}$$

$$c_d = c_l \tag{12}$$

where, c_{CD}, c_d, c_l are the total concentrations of the CD and the enantiomers (*d* and *l*). The mathematical derivation led to a third-order polynomial describing the variation of $[d]$ with the total concentration of the cyclodextrin c_{CD}:

$$A[d]^3 + B[d]^2 + C[d] + D = 0 \tag{13}$$

where:

$$A = K_d K_l - K_d^2 \tag{14}$$

$$B = (K_d K_l - K_d^2)c_{CD} + (K_d^2 - 3K_d K_l)c_d - K_d + K_l \tag{15}$$

$$C = (K_d - 2K_l - K_d K_l c_{CD})c_d - 2K_d K_l c_d^2 \tag{16}$$

$$D = K_l c_d^2 \tag{17}$$

By analyzing the roots of Eqs. (13)–(17) for positive concentrations, Eq. (13) can be solved numerically for $[d]$ and thus the observed chemical shift δ_{obs}^d can be calculated (simulated) using Eqs. (18) and (19)

$$[dCD] = c_d - [d] \tag{18}$$

$$\delta_{obs}^d = \frac{[d]\delta_{free} + [dCD]\delta_{dCD}}{c_d} \tag{19}$$

The effect of enantiomeric competition is first demonstrated through the comparison of the three titration curves in Fig. 4. While Curves 1 and 2 were simulated by Eq. (7) until Curve 3 was calculated by

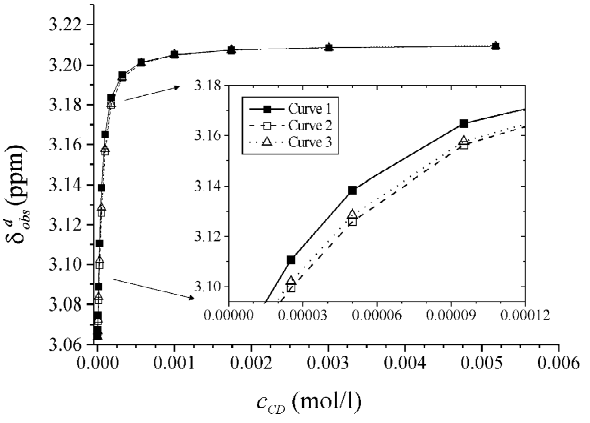

Fig. 4. Simulation of the effect of enantiomeric competition. The curves were calculated for the observed *d*-isomer ($K_a^d = 3E+4$ l/mol in each case). Curve 1 and Curve 2 were simulated by Eq. (7) with $c_G = 3E-5$ and $6E-5$ mol/l, respectively, whereas Curve 3 represents a titration curve of the *d*-isomer in the racemate calculated by Eqs. (13)–(19) with $c_d = c_l = 3E-5$, $K_a^l = 2E+4$ l/mol.

Eqs. (13)–(19). For Curves 2 and 3, the total analyte concentration is equal (since $2c_d = c_d + c_l$), whereas for Curves 1 and 3, the total *d*-enantiomer concentration is the same. Although the appearance of Curve 3 is slightly different from the two others, it clearly shows more resemblance to Curve 2 than to Curve 1. This is rationalized as follows. The conditions described for Curve 2 are equivalent to the situation when the association constants of the enantiomers are equal in the racemate ($K_a^d = K_a^l$). This means that Curve 2 can be simulated in two ways: (1) either by using Eq. (7) with a total analyte concentration $c_G = 2c_d$ or, (2) by using Eqs. (13)–(19) with the conditions $c_l = c_d$ and $K_a^l = K_a^d$. Accordingly, if the association constants of the enantiomers do not differ significantly (see the interpretation of the results in Fig. 5 below), the titration curve of the observed enantiomer in the racemate will be similar within experimental error to Curve 2. Eqs. (13)–(19) have reproduced this property for a wide range of parameters.

Because of the overall similarity of the three curves in Fig. 4, the equation of the competition free model (Eq. (7)) also fits very well to the data set of the *d*-enantiomer in the racemate, but it yields different $K_a'^d$ values depending on the choice of the analyte concentration [actual (c_d) or total ($2c_d$)] used for the non-linear fitting. This difference between the $K_a'^d$ values in the racemate is demonstrated in Fig. 5 as a function of the association constant of the competing enantiomer (K_a^l). Data points in Fig. 5 were obtained by a two-step process, (i) first titration data points (not shown) were calculated using equations (Eqs. (13)–(19)), (ii) then equations of the competition free model (Eqs. (5) and (7)) were fitted to the simulated data points to obtain the apparent association constant $K_a'^d$ for various K_a^l values of the competing partner. We were interested in the deviation of $K_a'^d$ from its theoretical value K_a^d at constant c_d.

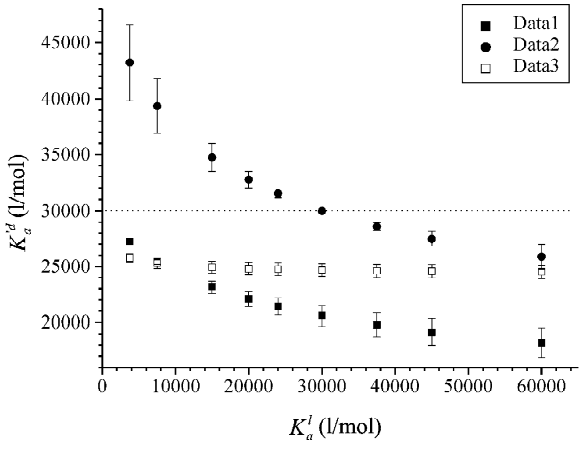

Fig. 5. Simulation of the dependence of $K_a'^d$ on the choice of the fitting method as a function of the association constant of the competing *l*-isomer (K_a^l). All data points were created with $K_a^d = 3E+4$ l/mol. Data 1 was obtained by fitting Method 1 ($c_G = 3E-5$ mol/l), whereas Data 2 was obtained by Method 2 ($c'_G = c_d + c_l = 6E-5$ mol/l). Data 3 represents results of the linear fitting to data points with the condition $10 \leq c_{CD}/c_d$.

When, during the fitting, the concentration of the analyte in Eq. (7) was set equal to the actual concentration of the *d*-isomer ($c_G = c_d$, Method 1), an increasing affinity of the competing partner (*l*-enantiomer) yielded a decreasing apparent association constant (represented by Data 1 in Fig. 5)—with increasing error—for the *d*-enantiomer ($K_a'^d$). The apparent association constant $K_a'^d$ reaches the value of the theoretical association constant K_a^d near $K_a^l = 0$. The situation is different for Data 2 where the $K_a'^d$ values were calculated by the substitution of the total concentration of the enantiomers into Eq. (7) ($c_G = c_d + c_l = 2c_d$, Method 2), since $K_a'^d$ reaches the value of K_a^d at $K_a^l = K_a^d$. For $K_a^l < K_a^d$ values $K_a'^d$ is overestimated, whereas for $K_a^l > K_a^d$ values, $K_a'^d$ is underestimated. We may conclude that for complexes of cyclodextrins (where often $1 \leq \alpha_c < 1.25$ is expected), K_a^d can be fairly well approximated by the apparent association constant $K_a'^d$, determined by Method 2 rather than by Method 1.

The apparent association constants obtained from the Benesi–Hildebrand method (for data points $10 < c_{CD}/c_d$) (Data 3)—similarly to Data 1—are also underestimated ($K_a'^d < K_a^d$), but their error is less affected by the magnitude of K_a^l than for Data 1. The major difference between the non-linear and the linear fitting methods is that in the former case, the mutual dependence of the parameters K_a^G and c_G in Eq. (7) allows us to compensate for enantiomeric competition using an apparent analyte concentration ($c'_G = c_d + c_l$), which is not possible to do for Eq. (5).

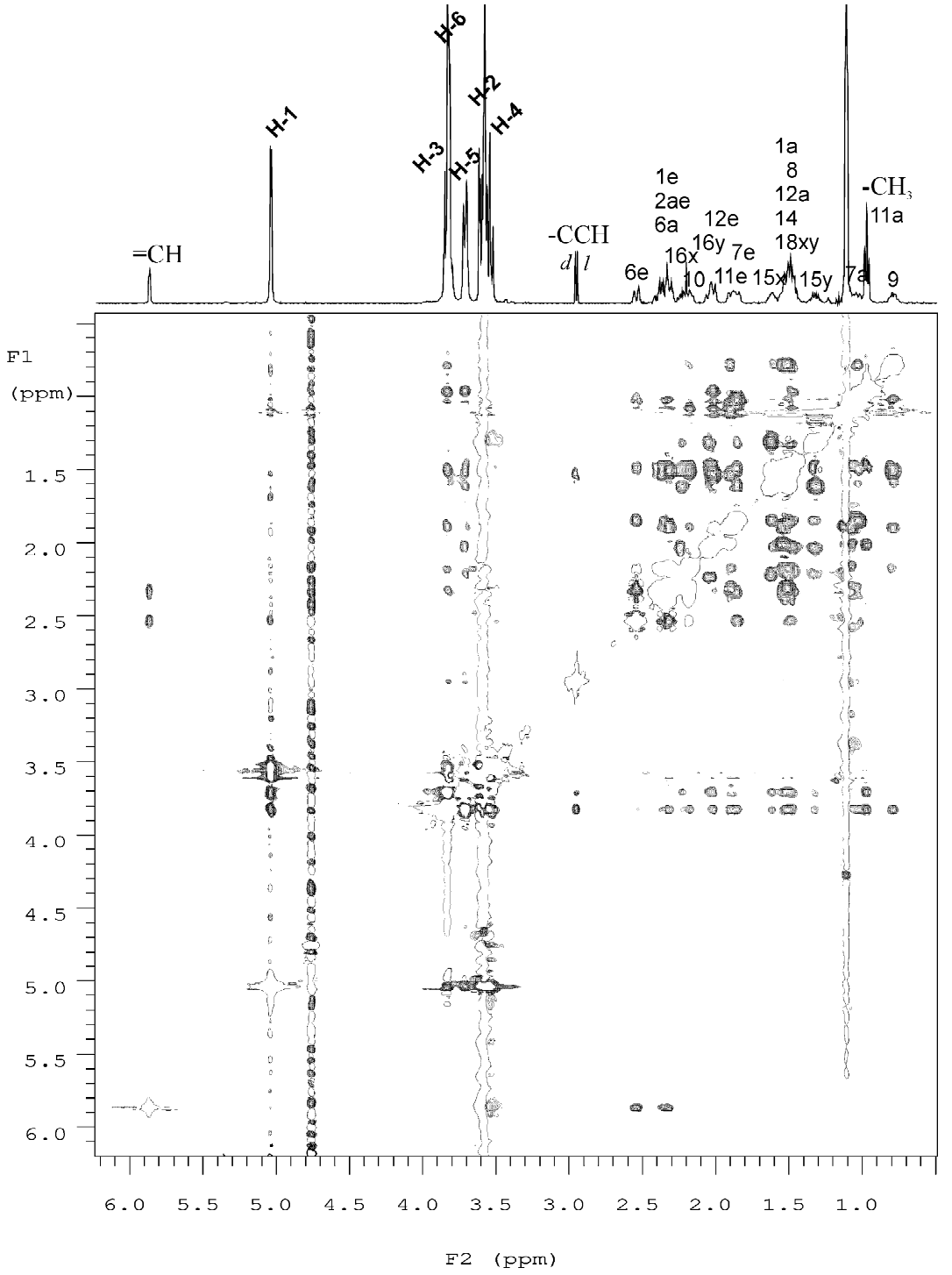

Fig. 6. ROESY-2D spectrum of *dl*-norgestrel (2.9 m*M*) in the presence of γ-CD (2.9 m*M*).

3. Experimental

Spectra were recorded on a Varian Inova NMR spectrometer (500 MHz for ^{1}H) at 30 °C using a triple resonance $^{1}H\{^{13}C,^{15}N\}$ 5-mm probe equipped with a waveform generator and Z-gradient shimming. Chemical shifts in D_2O are referenced to the solvent shifts $\delta_{MeOD} = 3.41$ ppm, $\delta_{EtOD} = 1.11$ ppm. Norgestrel samples were supplied by Synthetic Laboratory I of Gedeon Richter Ltd. Analytical-grade cyclodextrins were purchased from Cyclolab (Hungary), whereas 99.5% deuterated NMR solvents were purchased from Merck. Titration experiments were carried out in a standard 5-mm NMR sample tube by adding volumes of cyclodextrin solution with a Gilson® 100-μl analytical pipette (±0.1 μl) keeping the total analyte concentration constant. Extreme dilute solutions (0.02–0.50 m*M*) of the steroid were prepared by dilution of standard solutions (16 m*M*) at room temperature. Job's plots were determined at 3.2 m*M* total concentration of the species. Composition of the water–methanol mixtures are given as v/v ratios. For practical reasons, we used deuterium lock internal referencing following the guidelines described by Matsui et al. [60–62] for methanol containing solutions of CDs. The ^{1}H NMR spectra were recorded by collecting 64–128 scans using water presaturation technique and line-broadening prior Fourier-transformation of the FID. Digital resolution was 0.061 Hz. For simulation and data processing commercially available software Mathcad® 2000 Professional and Microcal Origin® 6.0 (licensed to Gedeon Richter Ltd.) were used. The 13 data points used for simulation of the titration curves were evenly spaced on the logarithmic concentration scale.

The phase-sensitive ROESY-2D spectrum was measured at 22 °C in EtOD/D_2O = 1:1 with a 0.32 s mixing time, 1.2 kHz spin-lock amplitude, 3.6 s water presaturation delay. We used 0.114 s acquisition time and States-Haberkorn phase cycling to acquire 1024×128 data points in F2 and F1 dimensions; 32 scans were collected at each increment. For data processing, Gaussian line broadening was used in both dimensions and the data points in the F1 dimension were linear predicted to 384 and zero filled to 1024 points.

For the resonance assignments, we followed the conventional assignment strategies based on the measurement of homonuclear $^{1}H–^{1}H$ (gMQFCOPS) and heteronuclear $^{1}H–^{13}C$ scalar connectivities (gHSQC, gHMQC).

Preliminary molecular simulations involved in vacuo docking studies using the CVFF force-field engine of the Docking module of InsightII® software from Molecular Simulations Inc.

4. Results and discussion

4.1. Verification of the model

The 1:1 stoichiometry of complexes was verified both by using the method of continuous variations (Job's plot method) [13,30,63] and by the analysis of the titration data. The linearity of the plots in Fig. 7D–F [33] reinforced the 1:1 stoichiometry. To explore the nature of complexation between norgestrel and γ-CD, ROESY experiments were carried out (Fig. 6). Intermolecular ROESY cross-peaks were detected between the protons (H-3, H-5) located at the internal face of the cyclodextrin (Fig. 2) and protons of the steroid, proving that inclusion complex formation took place between norgestrel and the CD. According to preliminary molecular simulations [64], the manyfold of ROESY cross-peaks between the steroidal protons and H-3, H-5 may stem from the superposition of different inclusion modes. As no evidence of interaction was observed between the outer surface (checked on H-2 and H-4 protons) of the CD and the analyte, this validates the chromatographic model described in Section 2.3. Detailed characterisation of the geometry of the α-, β-, and γ-CD complexes of norgestrel will be published elsewhere [64].

4.2. Determination of the association constants for the α-, β, and γ-CD complexes of d-norgestrel by NMR

Since both the precision and accuracy of the determination of K_a^G are affected by $|\Delta\delta_{\infty}^{G,i}|$, determination of the association constants should preferably begin with the analysis of the shift displacement values found in different norgestrel-CD systems. In general, the larger the magnitude of the

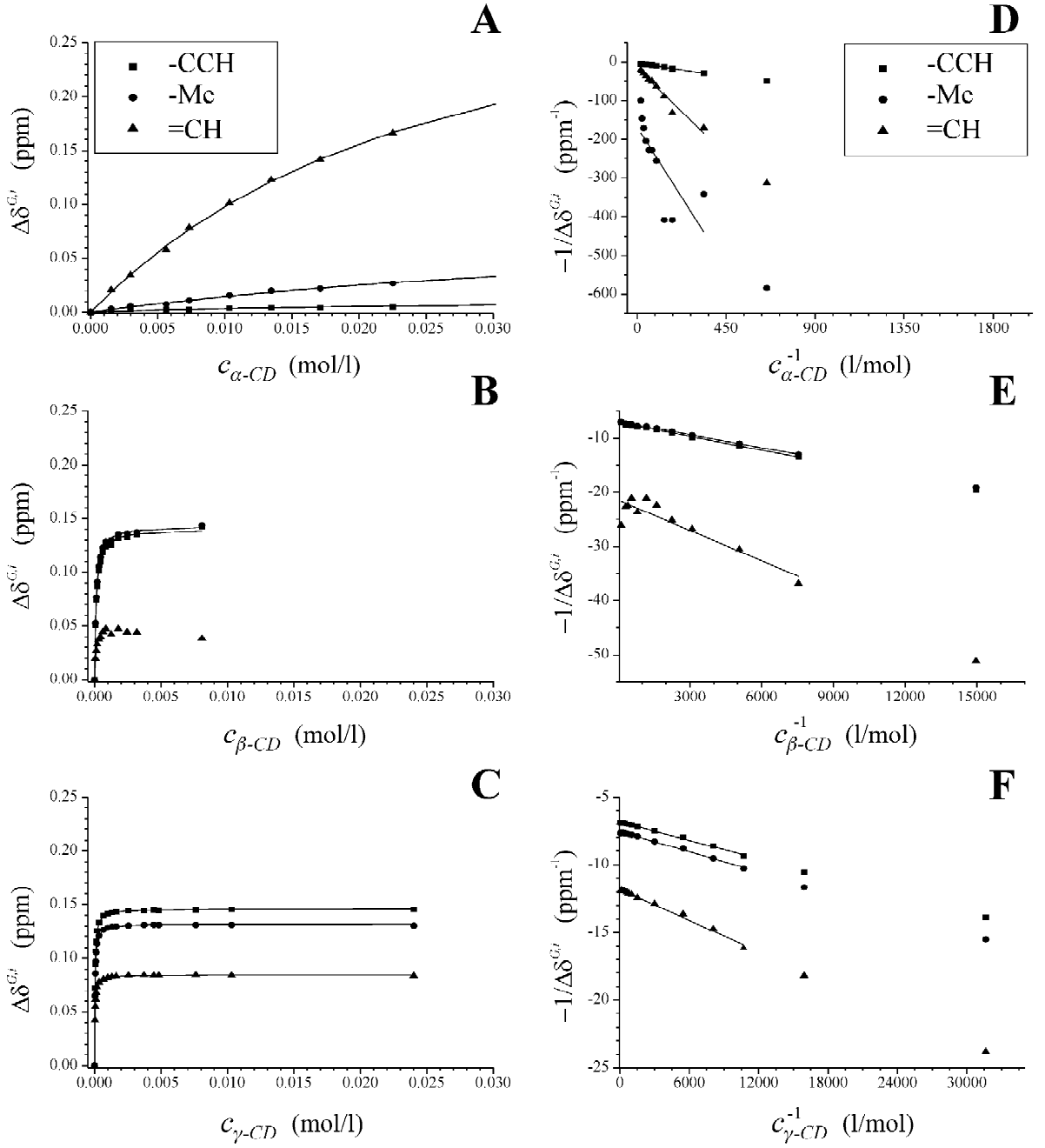

Fig. 7. Experimental titration curves for *d*-norgestrel/α-, β-, γ-CD systems (MeOD/D_2O=1:100). (A)–(C) show the non-linear, whereas (D)–(F) show the linear representation of the titration curves. Note the different vertical and horizontal scales for (D)–(F).

limiting shift displacement $|\Delta\delta_{\infty}^{G,i}|$ of the given nucleus, the smaller the error of K_a^G at a given spectral resolution. Three intensive resonances with simple scalar coupling patterns were selected for the binding analysis: the singlet of the ethynyl proton (–CCH), the doublet–doublet of the olefinic proton (=CH) and the triplet of the methyl group (–Me) of 17-Et. In the case of α-CD, significant shift displace-

Table 1
Association constants and limiting shift displacements for the –CCH, –Me and =CH protons of *d*-norgestrel/α-, β-, γ-CD systems as calculated by non-linear fitting using Eq. (7)

	Association constants K_a^d (l/mol)			Limiting shift displacements (Hz)		
	CCH	Me	=CH	$\Delta\delta_\infty^{d,CCH}$	$\Delta\delta_\infty^{d,Me}$	$\Delta\delta_\infty^{d,=CH}$
α-CD	34±14	19±2	37±1	6.5±1.4	46.2±2.5	183±3
β-CD	8400±300	8800±200	8200±700	70.2±0.4	71.9±0.3	26.4±0.6
γ-CD	31 900±400	31 800±400	32 500±600	72.9±0.2	66.0±0.1	42.2±0.1

$MeOD/D_2O$=1:100 v/v.

ment was found only for the =CH moiety whereas for β-CD and γ-CD systems, all three candidates showed measurably large displacements. Fig. 7 shows the experimental titration curves for the α-, β-, and γ-CDs complexes of *d*-norgestrel whereas Table 1 summarizes the results of the non-linear fitting. Fig. 7A,C are typical examples of titration curves characteristic of host–guest systems having a relatively small- and a large association constant, respectively. For β-CD, a two orders of magnitude larger association constant was found than in the case of α-CD, and γ-CD showed a further one order of magnitude larger K_a^d than β-CD. This is in excellent agreement with the behaviour of the retention factors in RP-HPLC found in the literature [18], where the change in the eluent CD composition from α-CD to β-CD and from β-CD to γ-CD (in the same concentration) manifested in a significant decrease in the retention factor of *d*-norgestrel (k'^d) in each case. It was also reported [18] that by using γ-CD as a mobile phase additive, the retention factors did not change on the addition of β-CD and α-CD (equimolar with γ-CD), which further indicates the order of magnitude difference between the association constants of the different CDs. The results of linear fitting shown in Fig. 7D–F are collected in Table 2.

The comparison of the K_a^d data of Table 1 with the corresponding values of Table 2 confirms the compatibility of the two (non-linear and linear) methods in titration of the enantiomerically pure drug. As expected, for a given type of CD the same association constants were found for all three moieties (CH_3, =CH, ≡CH) although they exhibited different limiting shift displacements ($\Delta\delta_\infty^{d,i}$). The advantage of the Benesi method over non-linear regression is the reduced number of data points needed for a rough estimate of K_a^d which speeds up the screening process. For small limiting shift displacements however, the Benesi-method seems to be less precise for K_a^d than non-linear regression. The error in K_a^d seen in Tables 1 and 2 confirms that the larger the limiting shift displacement, the better the precision of the determination of K_a^d at a given spectral resolution. The most reliable data are those with the large $\Delta\delta_\infty^{d,i}$ values: =CH for α-CD, –Me or –CCH for β-CD and –CCH for γ-CD.

4.3. Investigation of the effect of solvent composition

Screening of CDs from the point of view of their complexing power in various eluents is essential for the optimisation of separation in RP-HPLC (refer to Eq. (10)). The effect of solvent composition on the appearance of the titration curves of the –CCH^d

Table 2
Association constants and limiting shift displacements for the –CCH, –Me and =CH protons of *d*-norgestrel/α-, β-, γ-CD systems as calculated by linear fitting using Eq. (5)

	Association constants K_a^d (l/mol)			Limiting shift displacements (Hz)		
	CCH	Me	=CH	$\Delta\delta_\infty^{d,CCH}$	$\Delta\delta_\infty^{d,Me}$	$\Delta\delta_\infty^{d,=CH}$
α-CD	209±103	33±12	32±2	3.0±0.6	30.2±9.5	199±13
β-CD	8400±180	8600±160	11 000±2000	70.1±0.4	72.2±0.4	23.3±0.9
γ-CD	30 400±600	30 600±600	31 000±900	73.0±0.2	66.1±0.1	27.3±0.1

$MeOD/D_2O$=1:100 v/v.

moiety in *d*-norgestrel/γ-CD systems is illustrated in Fig. 8A while the results of the non-linear fitting are given in Table 3. A monotonic decrease in K_a^d with increasing amount of methanol in the solvent was observed. This is expected since methanol, being more hydrophobic than water, occupies the cavity of the cyclodextrin more and solvates norgestrel better than water molecules do. As a result of the competing equilibria involving methanol molecules, the relative hydrophobicity of the CD's cavity—compared to that of the bulk solvent—is reduced, which results in a smaller association constant with increasing methanol concentration.

Table 3 indicates that the limiting shift displacement values ($\Delta\delta_\infty^{d,i}$) also decrease with increasing methanol content of the solvent. We interpret this as follows: similarly to the behaviour of the association constant, the relative hydrophobicity of the chemical environment may also be reflected in the chemical shifts of the resonances in the complex. Moreover the presence of methanol may alter the relative population of the individual inclusion modes of the analyte by influencing the internal hydrogen bond network (and thus the conformation) of the CD. This may influence K_a^d and $\Delta\delta_\infty^{d,i}$ differently.

4.4. Titration of the racemate

Fig. 8B,C shows titration data for the CCH protons of *dl*-norgestrel enantiomers measured for γ-CD in various water–methanol mixtures. All three fitting strategies were applied to each set of data to assess (and allow the comparison of) the apparent association constants in the racemate: Method 1, 2 (with reference to the theoretical part) and the Benesi-method (Eq. (5)). The apparent association constants of *dl*-norgestrel determined by Method 1 are listed in Table 4 whereas the corresponding limiting shift displacements are collected in Table 5. Due to their large $\Delta\delta_\infty^{d,i}$ values, the data sets for the CCH moiety were selected to compare the $K_a'^d$, $K_a'^l$ values of the three fitting methods with K_a^d found for *d*-norgestrel (Fig. 9). Method 1 (BAR≠3) and the Benesi–Hildebrand approach (BAR≠5) yielded underestimated $K_a'^d$ values of the corresponding association constants K_a^d (BAR1) determined for *d*-norgestrel. This was attributed to the existence of enantiomeric competition in the racemate. The ex-

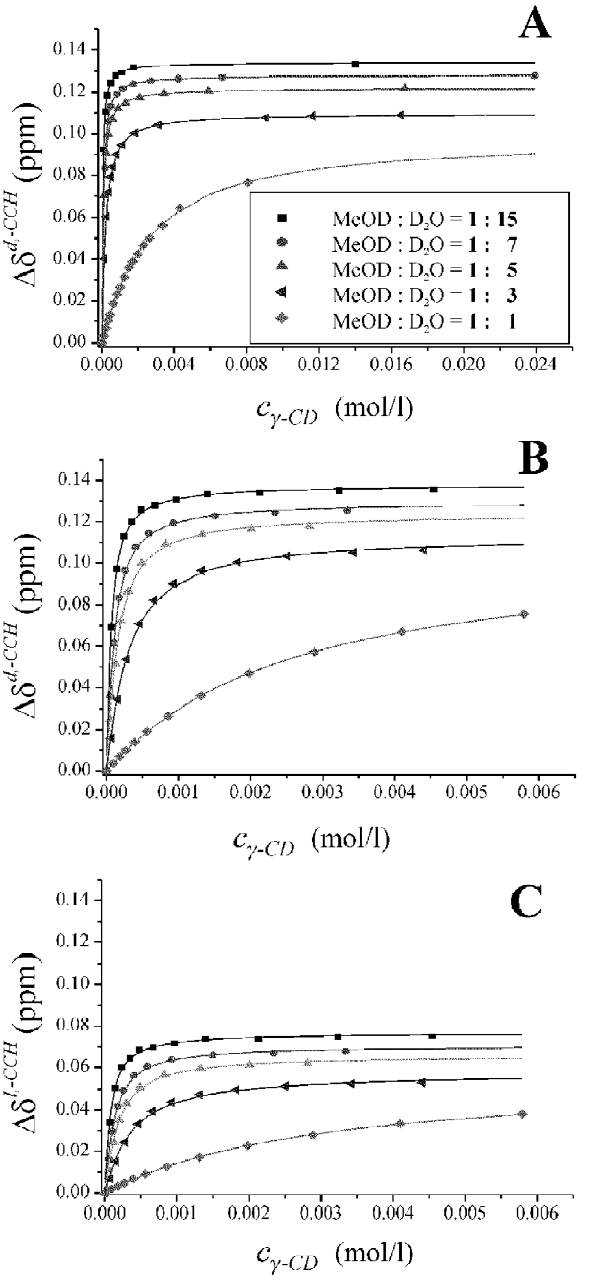

Fig. 8. Titrations for the optically pure *d*-norgestrel/γ-CD (A) and racemic *dl*-norgestrel/γ-CD systems (B,C) at varied solvent composition. Data points represent experimental values whereas the curves are results of non-linear fitting (Eq. (7)).

Table 3
Association constants and limiting shift displacements for the –CCH, –Me and =CH protons of *d*-norgestrel/γ-CD systems as calculated by non-linear fitting using Eq. (7) in various MeOD/D_2O systems

V_{MeOD}/V_{D_2O}	Association constants (K_a^d) (l/mol)			Limiting shift displacements (Hz)		
	CCH	Me	=CH	$\Delta\delta_\infty^{d,CCH}$	$\Delta\delta_\infty^{d,Me}$	$\Delta\delta_\infty^{d,=CH}$
1:15	28 500±400	30 600±900	30 000±1700	66.8±0.1	61.5±0.2	41.8±0.2
1:7	19 400±100	19 800±400	19 400±600	63.9±0.1	60.8±0.1	42.8±0.1
1:5	13 400±100	13 400±100	13 400±200	60.9±0.1	58.9±0.1	42.8±0.1
1:3	6030±30	6210±40	6110±40	54.9±0.1	55.1±0.1	43.0±0.1
1:1	480±10	450±8	470±10	49.3±0.4	43.9±0.3	38.5±0.4

Table 4
Apparent association constants and chiral selectivities for the –CCH, –Me and =CH protons of *dl*-norgestrel/γ-CD systems as calculated by Method 1 in various MeOD/D_2O systems

V_{MeOD}/V_{D_2O}	Apparent association constants $K_a'^d$ (l/mol)					
	CCH^d	CCH^l	Me^d	Me^l	$=CH^d$	$=CH^l$
1:15	24 200±300	18 700±300	21 300±100	20 400±800	24 200±500	18 200±500
1:7	15 100±300	12 000±300	13 500±200	13 600±500	15 700±400	12 500±500
1:5	11 500±300	9200±300	10 200±100	10 800±500	11 600±300	10 000±400
1:3	4130±200	3390±180	3690±150	3837±250	4160±240	3530±170
1:1	410±4	362±7	372±2	372±2	386±10	374±13

perimental titration curves were predicted successfully by theoretical calculations using Eqs. (13)–(19), indicating that they describe the system well in the absence of additional homo- and heterochiral self-association processes. As an example, Fig. 5, Data 1 shows a particular situation with parameters similar to those found in MeOD/D_2O=1:15 for *dl*-norgestrel/γ-CD system.

To compensate for the effect of enantiomeric competition, some of the $K_a'^d$ values (relevant for the comparison with the chromatographic results) were re-calculated by Method 2 using the apparent analyte concentration $c'_G = c_d + c_l = 2c_d$ (see data in Table 7). According to the simulations represented by Data 2 in Fig. 5, these recalculated apparent association constants are closer to the K_a^d values of the competition-free model, which is very well confirmed by the similarity of the experimental values (see BAR1 and BAR7) in Fig. 9. Moreover, both K_a^d and K_a^l values found in RP-HPLC systems could be reproduced very well from titrations of the racemate (Table 7), which makes this method very useful in the estimation of K_a^d and K_a^l for drugs not available as single enantiomers.

The limiting shift displacement values ($\Delta\delta_\infty^{G,i}$) showed an average variation of ±0.6 Hz depending on whether determined by Method 1 or 2. The comparison of the limiting shift non-equivalence $\Delta\Delta\delta_\infty^i$ values of the –CCH, –Me, =CH moieties (collected in Table 6), reinforced the theoretical

Table 5
Limiting shift displacements for the –CCH, –Me and =CH protons of *dl*-norgestrel/γ-CD systems as calculated by Method 1 in various MeOD/D_2O mixtures

V_{MeOD}/V_{D_2O}	c_G (mmol/l)	Limiting shift displacements (Hz)					
		$\Delta\delta_\infty^{d,CCH}$	$\Delta\delta_\infty^{l,CCH}$	$\Delta\delta_\infty^{d,Me}$	$\Delta\delta_\infty^{l,Me}$	$\Delta\delta_\infty^{d,=CH}$	$\Delta\delta_\infty^{l,=CH}$
1:15	0.02	68.9±0.3	38.3±0.2	65.1±0.2	65.5±0.4	43.2±0.2	29.7±0.1
1:7	0.04	64.7±0.4	35.3±0.3	63.3±0.3	63.1±0.7	43.7±0.3	30.8±0.3
1:5	0.09	61.9±0.5	32.9±0.4	62.0±0.6	62.2±0.7	44.5±0.6	31.4±0.4
1:3	0.14	56.7±0.5	28.7±0.3	59.2±0.4	57.9±0.7	44.9±0.5	32.2±0.3
1:1	0.28	54.2±0.2	28.3±0.2	50.1±0.1	50.0±0.1	43.7±0.5	31.6±0.5

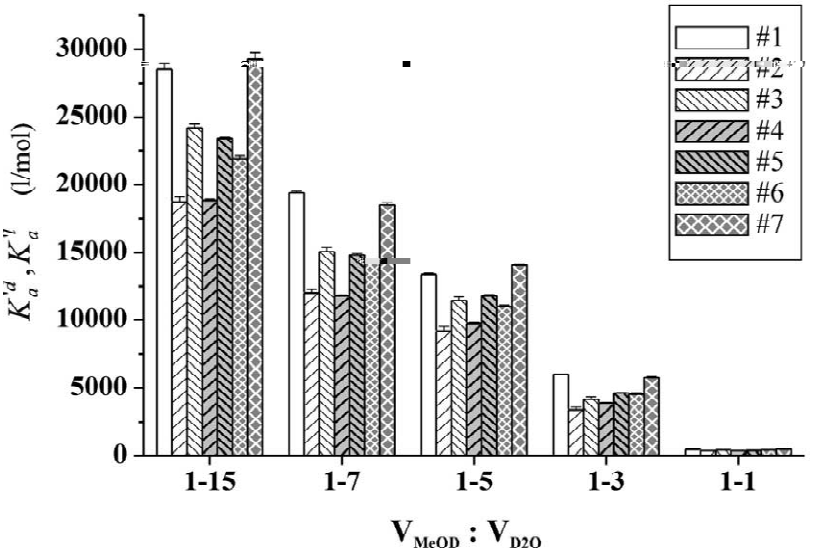

Fig. 9. Comparison of the association constants determined for *d*-norgestrel (BAR≠1) and racemic *dl*-norgestrel (BARs ≠2–≠7) in various MeOD/D_2O solvents. BARs ≠2–≠7 represent results from the same data sets but according to the following fitting strategies: BARs ≠2(*l*)–≠3(*d*): Method 1; BARs ≠4(*l*)–≠5(*d*): Benesi–Hildebrand-method; BARs ≠6(*l*)–≠7(*d*): Method 2.

considerations of the previous sections concerning the ''dual'' mechanism of enantiomeric discrimination in NMR: the experimental titration curves (for the *d* and *l* enantiomers) of the CCH and =CH moieties were analogous to the situation represented by Fig. 3C, whereas those of the –Me moiety showed resemblance to Fig. 3B.

4.5. Evaluation of the chiral selectivities

Chiral selectivities determined by NMR titration of the racemate were approximated by substituting the apparent association constants into Eq. (8). As the absolute values of the apparent association constants differed in Fig. 9, the different fitting methods also resulted in slightly different α_c values (Fig. 10). The decrease in α_c was found with increasing methanol content of the solvent for MeOD/D_2O v/v ratios between 1:15 and 1:3. For larger methanol content of the solvent (1:1 ratio) α_c dropped more significantly, indicating the loss of selectivity of native cyclodextrins with increasing organic content of the solvent. Fig. 11 shows the

Table 6
Limiting shift non-equivalence for the –CCH, –Me and =CH protons of *dl*-norgestrel/γ-CD systems as calculated by Method 1 in various MeOD/D_2O systems

$\frac{V_{MeOD}}{V_{D_2O}}$	c_G (mmol/l)	Limiting shift non-equivalences (Hz)		
		$\Delta\Delta\delta_{\infty}^{CCH}$	$\Delta\Delta\delta_{\infty}^{Me}$	$\Delta\Delta\delta_{\infty}^{=CH}$
1:15	0.03	30.6±0.3	0.4±0.4	13.5±0.2
1:7	0.07	29.4±0.4	0.2±0.7	12.9±0.3
1:5	0.09	29.0±0.5	0.2±0.7	13.1±0.6
1:3	0.14	28.0±0.5	1.3±0.7	12.7±0.5
1:1	0.28	25.9±0.2	0.1±0.1	12.1±0.5

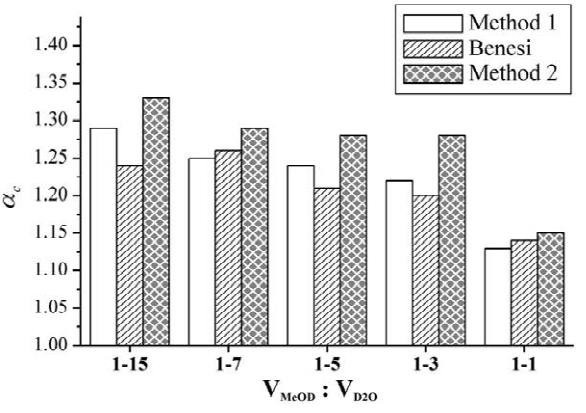

Fig. 10. Variation of the chiral selectivities as determined by the different fitting methods from titration data of the CCH moiety for *dl*-norgestrel/γ-CD systems at various solvent compositions.

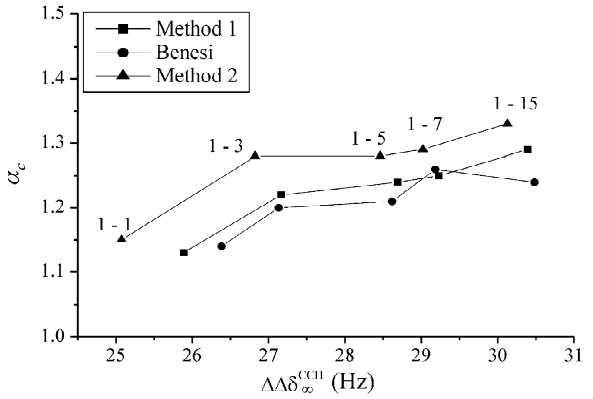

Fig. 11. Correlation between the chiral selectivities and the limiting shift displacement values of *dl*-norgestrel/γ-CD systems estimated from the –CCH^d and –CCH^l chemical shift data of the enantiomers at varied solvent compositions.

correlation between chiral selectivities and limiting shift non-equivalence values ($\Delta\Delta\delta^i_\infty$) measured (for the same nucleus and same CD) in different water–methanol systems. The roughly linear relationship between α_c and $\Delta\Delta\delta^i_\infty$ suggests that as methanol partially occupies the cavity of the CD, not only the differential binding of the enantiomers is diminished, but also the difference in the diastereomeric chemical environment (of protons CCH^d and CCH^l) is ''blurred''.

The chiral selectivities calculated from NMR titrations in a *dl*-norgestrel-γ-CD system were compared with those estimated by Eq. (4) from retention factors found in a few chromatographic systems in the literature [18–20] (Table 7). For *dl*-norgestrel/β-CD systems in MeOD/D_2O=1:3 and AcCN/D_2O=1:3, we found that α_c=1.00±0.04 which corresponded to the none [18–20] or to the relatively poor separation of the enantiomers in β-CD containing RP-HPLC systems [36] compared to those having γ-CD in the mobile phase [18–22].

The correlation between α_c and $\Delta\Delta\delta^i_\infty$ seen in Fig. 11 does not (in principle) hold for the comparison of values measured for different CDs, due to the possible (and very probable) differences between the nature and the relative population of the inclusion geometries. This gives a serious limitation to the screening methods that are based on the comparison of non-limiting chemical shift differences (either $\Delta\delta^{G,i}$ or $\Delta\Delta\delta^i$) only [12,28]. We monitored this failure for *dl*-norgestrel in β- and γ-CD containing solvents (MeOD/D_2O=1:3 v/v). Fig. 12 shows that the –CCH and =CH protons showed visibly greater enantiomeric splitting for γ-CD than for β-CD (both at 5:1 and at 2:1 host–guest ratio). This is in accordance with the relative magnitudes of the chiral selectivities in the two systems [α_c(β-CD)<α_c(γ-CD)] and reinforces the qualitative observations of Owens et al. [12]. However the –Me group shows the opposite correlation: the larger shift non-equivalence values are now found for β-CD (Fig. 12) which contradicts our understanding of the apparent correlation between α_c and $\Delta\Delta\delta^i$. Since however δ^i_{GCD} and K^G_a are independent parameters, so are $\Delta\Delta\delta^i$ and α_c, and whether or not there exists a qualitative correlation between $\Delta\Delta\delta^i$ and α_c seems to be circumstancial depending on the choice of resonance selected for analysis. The structural details of the ''anomalous behaviour'' of $\Delta\Delta\delta^{-Me}$ will be discussed elsewhere [64].

Table 7
Comparison of the RP-HPLC association constants and chiral selectivities of *dl*-norgestrel/γ-CD systems calculated from the retention factors of Refs. [18,19] using Eq. (4) with those determined by NMR from titration data for –CCH protons using Method 2

$\frac{V_{MeOH}}{V_{D_2O}}$	CD	RP-HPLC (literature)			NMR (experimental)		
		K^d_a (l/mol)	K^l_a (l/mol)	α_c	K'^d_a (l/mol)	K'^l_a (l/mol)	α_c
1:3	γ-CD	5830[c]	4480[c]	1.30	5780±80	4510±120	1.28±0.05
1:1	γ-CD	541[b]	472[b]	1.15	469±8	410±9	1.14±0.02
		385[a]	332[a]	1.16			

[a] Retention factor data are from Table 1 of Ref. [18].
[b] Retention factor data are from Table 2 of Ref. [18].
[c] Retention factor data are from Table 1 of Ref. [19].

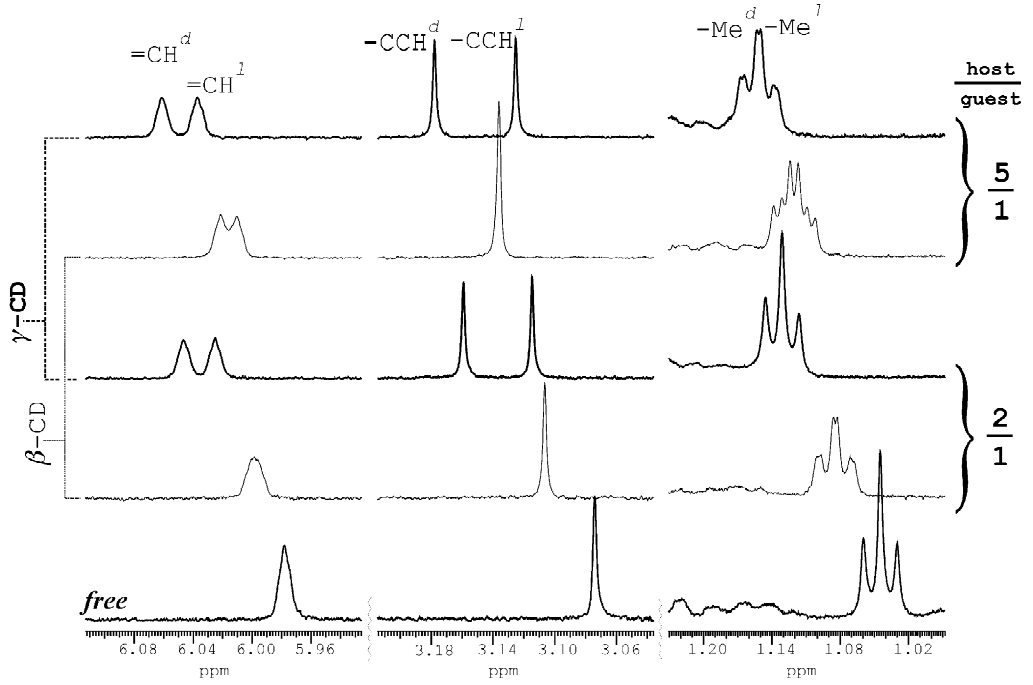

Fig. 12. Comparison of the observed shift non-equivalences ($\Delta\Delta\delta^{=CH}$, $\Delta\Delta\delta^{-CCH}$, $\Delta\Delta\delta^{-Me}$) for the =CH, –CCH and –Me resonances found in *dl*-norgestrel/β-CD and *dl*-norgestrel/γ-CD systems at two well-defined host/guest ratios 2:1 and 5:1 (MeOD/D_2O=1:3).

5. Conclusions

Our strategy proposed for the screening of cyclodextrins with respect to their success in the separation of enantiomers in RP-HPLC or CE is based on the determination of the association constants K_a^d, K_a^l from NMR chemical shift data of single enantiomers measured at identical experimental conditions. On the basis of the equations valid for the presented chromatographic model, two rather general ''selection rules'' can be established: (1) maximization of both the ratio (α_c) and (2) the absolute values of the association constants K_a^d, K_a^l. The larger the ratio of the association constants, the better separation of the enantiomeric peaks is expected, whereas the larger the magnitude of the association constants the smaller is the optimal concentration of the chiral selector necessary for the best peak-separation. Due to the analogy between the mechanism of enantioseparation in RP-HPLC and CE, NMR also aids the optimization of chiral separations in CE according to similar ''rules of thumb''. However maximization of the difference $|\xi_{GCD} - \xi_{free}|$ in Eq. (2), in order to achieve good separation in RP-HPLC and CE especially in case of relatively small K_a^G values, remains the task of the chromatographer. This cannot be modelled by NMR.

By comparing the mechanism of enantioseparation in NMR and RP-HPLC and CE it was shown that in NMR, in order to achieve good enantioseparation, α_c does not necessarily have to be a large value. Instead, it is the sufficiently large absolute value of the association constants (even if $K_a^d \approx K_a^l$) that serves as the dominant factor in invoking the inherently existing chemical shift non-equivalence ($\Delta\Delta\delta^i$) of the nuclei in their diastereomeric complexes (*d*CD and *l*CD). Since $\Delta\Delta\delta^i_\infty$ may range from almost zero to a few hundred Hertz, we found that measurement of $\Delta\Delta\delta^i$ at a single c_{CD} is not a reliable way for screening CDs to aid chromatographic or electrophoretic work. However, the association constant is independent of the limiting shift displacement $\Delta\delta^{G,i}_\infty$ and can be determined by either linear or non-linear fitting of the appropriate equations (Eqs. (5) and (7))

from NMR titration data. For compounds available only as racemates, α_c and K'^{d}_{a}, K'^{l}_{a} were successfully determined from titration data of the racemate via non-linear fitting compensated for enantiomeric competition in Eq. (7). The reliability of these estimated K'^{d}_{a}, K'^{l}_{a} values were verified by comparisons with the competition free results from NMR and RP-HPLC.

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