Enantioseparation

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Strong Detrimental Effect of a Minute Enantiomeric Impurity of a Chiral Selector on the Enantioselectivity Factor**

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Separation of racemates into pure enantiomers by chiral chromatography is an essential process both in university laboratories and in the pharmaceutical industry. Up to 75% of small-molecule drugs approved by the US Food and Drug Administration (FDA) are single enantiomers, some of which undergo separation by chiral chromatography at the multi-ton scale during the manufacturing process.[1] The high enantioselectivity of chiral chromatography is important for producing pure enantiomers. The enantioselectivity of a chiral stationary phase (CSP), which is composed of a chiral selector covalently or adsorptively immobilized on the surface of a porous support material, is commonly represented by the enantioseparation factor α. It is anticipated intuitively that α depends on the enantiomeric excess (*ee*) of the chiral selector and is reduced upon decrease of the *ee* value. It was theoretically predicted,[2–5] however, that this reduction significantly depends on the enantioselectivity of the selector and becomes very substantial for chiral selectors with higher enantioselectivity. Herein, we report the first experimental confirmation that for highly enantioselective chiral selectors even trace amounts of the opposite enantiomer of the selector present in the stationary phase can lead to a drastic decrease of the observed enantioseparation factor. Our results provide an important additional criterion for the design of artificial, highly enantioselective, receptor-like chiral selectors and chromatographic systems.

Chiral anion-exchange-type stationary phases based on modified cinchona alkaloids developed by Lindner et al. show extremely high enantioselectivity towards different classes of chiral acids[6–8] and are suitable for our study. For the present work we chose the adamantyl, neopentyl derivative of quinine (AN-QN) and quinidine (AN-QD; Figure 1), which showed exceptionally high enantioselectivity for the enantioseparation of *N*-3,5-dinitrobenzoyl (DNB) α-amino acids (Figure 2).

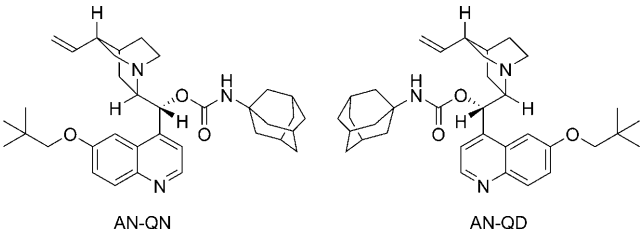

Figure 1. Structure of quinine- and quinidine-based chiral selectors (AN-QN and AN-QD, respectively) possessing exceptionally high and opposite enantioselectivity.

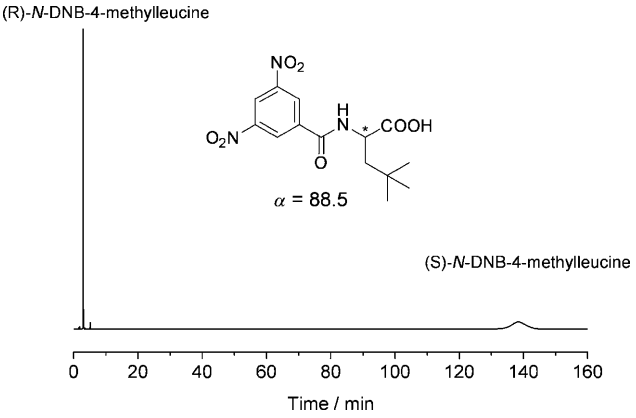

Figure 2. Chromatogram of the enantioseparation of (*R*,*S*)-*N*-3,5-dinitrobenzoyl-4-methylleucine on a stationary phase based on AN-QN chiral selector (CSP AN-QN100). The enantioseparation factor is 88.5.

It should be noted that natural quinine and quinidine are diastereomers having two (C8 and C9) out of five stereogenic centers of opposite configuration. Nevertheless, they represent a good model for our study because they show almost completely opposite enantioselectivity and thus behave quasi pseudo-enantiomerically.[6] Moreover, to our knowledge, another chiral selector possessing very high enantioselectivity and being easily accessible in both enantiomeric forms is unavailable.

The chiral selectors AN-QN and AN-QD (Figure 1) were synthesized from natural quinine and quinidine, respectively (for experimental details see the Supporting Information). The enantiomeric excess of each selector was at least 99.7% according to HPLC analysis.[9] The diastereomerically pure selectors were immobilized onto γ-mercaptopropyl-modified silica (5 μm particle size) using 2,2′-azobisisobutyronitrile as an initiator, thus generating the AN-QN100- and AN-QD100-type CSPs. Selector loadings measured by elemental analysis were found to be (145 ± 0.5) and (158 ± 0.5) μmol g^{-1} for silica modified with AN-QN and AN-QD, respectively. Portions of

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the silica gel modified with the two selectors were then mixed physically to yield seven batches of CSPs with different molar fractions, x, of AN-QN in the mixture of AN-QN and AN-QD: $x=1$ (AN-QN100), 0.989 (AN-QN99), 0.946 (AN-QN95), 0.867 (AN-QN87), 0.736 (AN-QN74), 0.483 (AN-QN48), and 0 (AN-QD100). Thus, the whole range of compositions corresponding, in our model system, to enantiomeric excess of the chiral selector from $ee \approx 0$ ($x=0.483$) to $ee=100\,\%$ ($x=1$) was covered. This protocol simulates the same situation as that of changing the stereochemical excess of the chiral selector per se. Seven HPLC columns (15 cm × 4.0 mm i.d.) were packed with these silica batches by using a slurry method. The eighth column (SH) was packed with γ-mercaptopropyl silica particles and was used as a reference column to access the extent of nonstereoselective binding of the target analytes.

Six racemic α-amino acid derivatives were selected to cover a large range of enantioseparation factors α obtained on the enantiomerically pure CSP AN-QN100: *N*-Fmoc-phenylalanine ($\alpha=2.46$; Fmoc = 9-fluorenylmethoxycarbonyl), *N*-benzoylmethionine ($\alpha=4.29$), *N*-DNB-alanine ($\alpha=18.4$), *N*-DNB-2-allylglycine ($\alpha=30.9$), *N*-DNB-leucine ($\alpha=51.8$), and *N*-DNB-4-methylleucine ($\alpha=88.5$). Chromatographic separation of the racemic analytes on the eight HPLC columns was carried out in a polar organic mode by using methanol modified with acetic acid (2 % v/v) and ammonium acetate (0.5 % w/w) as the mobile phase. The values of the enantioseparation factors (Table S1 in the Supporting Information) were plotted against the molar fraction of the AN-QN selector (see Figure 3 and Figure S1 in the Supporting Information for all analytes).

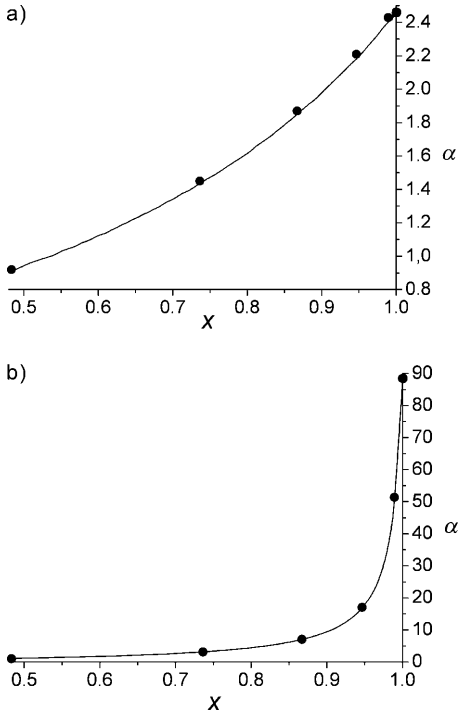

Figure 3. Experimental (●) and calculated (——) enantioseparation factors α versus molar fraction x of AN-QN selector in a mixture of AN-QN and AN-QD. Analytes: a) (*R*,*S*)-*N*-Fmoc-phenylalanine, b) (*R*,*S*)-*N*-3,5-dinitrobenzoyl-4-methylleucine.

Figure 3a shows the behavior of the enantioseparation factor α of *N*-Fmoc-phenylalanine as a function of x. Its value on the enantiomerically pure CSP AN-QN100 is relatively low ($\alpha^*=2.46$) and, as a result, the plot of α versus x is close to linear. Decrease of x from 1 to 0.99 (corresponding to 1.1 % of the AN-QD selector) results in a minute decrease of α from 2.46 to 2.43. A further decrease of x to 0.95 (corresponding to 5 % of the AN-QD selector) leads to a decrease of α to 2.21 (Table S1 in the Supporting Information). As the *ee* value is proportional to the molar fraction x, the appearance of the curves does not change if the *ee* value is used instead of x.

Figure 3b shows the behavior of α for *N*-3,5-DNB-4-methylleucine as a function of x. For this analyte the chiral selectors, AN-QN and AN-QD, are highly enantioselective and, as a result, we observe a very strong exponential-like decrease of α. Thus, the enantioseparation factor of *N*-3,5-DNB-4-methylleucine drops from 88.5 to 51.4 and to 17 upon very small decreases of x from 1 to 0.99 and to 0.95, respectively.

To corroborate these experimental results with theory, we calculated the enantioseparation factors α of the analytes for different values of x. The calculation was based on the experimental retention data obtained using stationary phases AN-QN100, AN-QD100, and SH (for details see the Supporting Information). Figure 3 and Figure S1 in the Supporting Information show that calculated values of the enantioseparation factors (solid lines) clearly comply with the experimental data.

As follows from our study, for highly enantioselective CSPs the use of the enantioseparation factor might become confusing. Therefore, we developed[5,10] a different term that can be used to record and compare enantioselectivities of chiral selectors. This term was called "retention excess", $re=(k_2-k_1)/(k_2+k_1)$, where k_1 and k_2 are the retention factors of the first and second eluted enantiomers, respectively. The retention excess re and the separation factor α are correlated as follows: $\alpha=(1+re)/(1-re)$ and $re=(\alpha-1)/(\alpha+1)$. A plot of re versus ee of a selector gives a straight line independently of the enantioselectivity of the selector (Figure S2 in the Supporting Information) and, therefore, seems to be more convenient than the enantioseparation factor α.

To conclude, we have shown experimentally that the enantiomeric purity of highly enantioselective chiral selectors critically determines their observed enantioselectivity. Even traces of the opposite enantiomer of a selector or an impurity possessing strong interactions with the analyte can cause a strong decrease of the observed enantioseparation factor. The reason for this is that to achieve very high enantioselectivity, it is equally important for a selector to have both very strong interactions with the analyte enantiomer eluted as the second peak and very weak interactions with the analyte enantiomer eluted as the first peak. However, since the reverse is true for the opposite enantiomer of the selector, its presence in a stationary phase becomes detrimental for achieving high enantioseparation factors. In other words, the higher the enantioselectivity of a selector, the stronger the interaction we expect for its opposite enantiomer with the analyte enantiomer eluted as the first peak. This leads to the observed very pronounced drop of the enantioseparation factor.

The practical conclusions of this study are that in order to achieve the highest possible enantioseparation factor, it is vitally important 1) to avoid any nonstereoselective interactions of the analyte with the selector and the support material representing together the CSP, and 2) to use 100% enantiomerically pure and nonracemizable chiral selectors. To realize the latter requirement, chiral selectors with several stereogenic centers, which are less prone to complete racemization, might be a better choice than selectors with single stereogenic centers—just as nature avoids complete racemization by the existence of numerous chiral centers in proteins (enzymes) and other biomolecules, for example, carbohydrates and alkaloids. Thus, we have established for the first time utmost enantiomeric purity as an essential condition for the development of chiral selectors with receptor-like enantioselectivity, and stress the need to devise effective strategies for the detection and removal of even trace amounts of enantiomeric impurities. We also want to point out that these findings will have equal implications for processes in which multiple stereoselective adsorption and binding phenomena are involved.

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[1] A. M. Thayer, *Chem. Eng. News* **2007**, *85*, 11 – 19.
[2] U. Beitler, B. Feibush, *J. Chromatogr. A* **1976**, *123*, 149 – 166.
[3] V. A. Davankov, *Chromatographia* **1989**, *27*, 475 – 482.
[4] W. H. Pirkle, C. J. Welch, *J. Chromatogr.* **1996**, *731*, 322 – 326.
[5] P. A. Levkin, V. Schurig, *J. Chromatogr. A* **2008**, *1184*, 309 – 322.
[6] M. Lämmerhofer, W. Lindner, *J. Chromatogr. A* **1996**, *741*, 33 – 48.
[7] M. Lammerhofer, W. Lindner, *Adv. Chromatogr.* **2008**, *46*, 1 – 107.
[8] N. M. Maier, L. Nicoletti, M. Lammerhofer, W. Lindner, *Chirality* **1999**, *11*, 522 – 528.
[9] C. V. Hoffmann, M. Lammerhofer, W. Lindner, *Anal. Bioanal. Chem.* **2009**, *393*, 1257 – 1265.
[10] V. Schurig, *J. Chromatogr. A* **2009**, *1216*, 1723 – 1736.

