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Direct Resolution of Enantiomers in High-Performance Immunoaffinity Chromatography under Isocratic Conditions

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This paper describes the application of stereoselective antibodies as tailor-made chiral selectors for the separation of enantiomers in HPLC under isocratic conditions. Stereoselective monoclonal antibodies to D- and L-α-amino acids, raised against protein conjugates of *p*-amino-D- and L-phenylalanine, were immobilized on a synthetic high-flow-through support material and used for rapid enantiomer separation of a number of amino acids at flow rates between 0.1 and 10 mL/min. Since separations could be performed in a mild buffer, column lifetime considerably exceeded that of classical immunoaffinity systems. Using an anti-D-amino acid antibody as chiral selector, the L-enantiomers eluted with the void volume, while the D-enantiomers eluted second. Inverted elution orders were obtained on chiral stationary phases prepared from an anti-L-amino acid antibody. These results demonstrate, for the first time, that antibody-based chiral stationary phases are useful for routine enantiomer separation under true high-performance chromatographic conditions.

High-performance liquid chromatography (HPLC) is one of the most popular techniques for the direct resolution of enantiomers, not only for the determination of enantiomeric purity at analytical scale but also for large-scale preparative purification.[1] Enantiomer separation is based on noncovalent differential interactions with a chiral selector that is either added to the mobile phase or immobilized on a solid support material, e.g., silica and synthetic polymers, to produce a chiral stationary phase (CSP). The use of CSPs is generally advantageous since consumption of chiral selector is minimized and interference with analyte detection, often observed if the selector is added to the mobile phase, is prevented. Chiral selectors commonly used for the production of CSPs include oligo- and polysaccharides,[2−5] macrocyclic antibiotics,[6−9] synthetic polymers,[10,11] π-donor/π-acceptor systems,[12−14] crown ethers,[15] ligand exchange selectors,[16,17] and various proteins.[18,19] With the exception of imprinted polymers,[11,20,21] these chiral selectors are not tailor-made for a specific separation problem, and reliable prediction of selectivity and suitable separation conditions is generally limited.[22−24] The rational design of chiral selectors therefore represents a challenging aim in order to facilitate enantiomer discrimination. As early as 1928, Karl Landsteiner demonstrated that suitably raised antibodies can differentiate between the enantiomers of chiral compounds.[25] Yet the stereoselective potential of antibodies has found only a few applications, mainly for the detection of drugs and metabolites in immunoassays.[26] The use of stereoselective antibodies for chromatographic enantiomer separation has been limited to low-pressure immunoaffinity systems in which mixtures of enantiomers, dissolved in a neutral buffer, were passed through a column containing antibodies covalently linked to agarose beads; after the unbound enantiomer was washed clear, elution of the bound enantiomer was achieved by altering to a mobile phase that disrupted its interaction with the antibody.[27−30] As is typical

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(1) Francotte, E. R. *J. Chromatogr., A* **2001**, *906*, 379−397.
(2) Ichida, A.; Shibata, T.; Okamoto, I.; Yuki, Y.; Namikoshi, N.; Toga, Y. *Chromatographia* **1984**, *19*, 280−284.
(3) Okamoto, Y.; Kawashima, M.; Hatada, K. *J. Am. Chem. Soc.* **1984**, *106*, 5357−5359.
(4) Armstrong, D. W.; DeMond, W. *J. Chromatogr. Sci.* **1984**, *22*, 411−415.
(5) Hinze, W. L.; Riehl, T. E.; Armstrong, D. W.; DeMond, W.; Alak, A.; Ward, T. *Anal. Chem.* **1985**, *57*, 237−242.
(6) Armstrong, D. W.; Tang, Y.; Chen, S.; Zhou, Y.; Bagwill, C.; Chen, J. R. *Anal. Chem.* **1994**, *66*, 1473−1484.
(7) Ward, T. J.; Farris, A. B. *J. Chromatogr., A* **2001**, *906*, 73−89.
(8) Berthod, A.; Chen, X.; Kullman, J. P.; Armstrong, D. W.; Gasparrini, F.; D'Acquarica, I.; Villani, C. *Anal. Chem.* **2000**, *72*, 1767−1780.
(9) Berthod, A.; Yu, T.; Kullman, J. P.; Armstrong, D. W.; Gasparrini, F.; D'Acquarica, I.; Misiti, D.; Carotti, A. *J. Chromatogr., A* **2000**, *897*, 113−129.
(10) Nakano, T. *J. Chromatogr., A* **2001**, *906*, 205−225.
(11) Sellergren, B. *J. Chromatogr., A* **2001**, *906*, 227−252.
(12) Mikeš, F.; Boshart, G.; Gil-Av, E. *J. Chromatogr.* **1976**, *122*, 205−221.
(13) Pirkle, W. H.; House, D. W. *J. Org. Chem.* **1979**, *44*, 1957−1960.
(14) Welch, C. J. *J. Chromatogr., A* **1994**, *66*, 3−26.
(15) Dotsevi, G.; Sogaah, Y.; Cram, D. J. *J. Am. Chem. Soc.* **1975**, *97*, 1259−1261.
(16) Rogozhin, S. V.; Davankov, V. A. *Chem. Commun.* **1971**, 490.
(17) Kurganov, A. *J. Chromatogr., A* **2001**, *906*, 51−71.
(18) Allenmark, S. G.; Andersson, S. *J. Chromatogr., A* **1994**, *666*, 167−179.
(19) Haginaka, J. *J. Chromatogr., A* **2001**, *906*, 253−273.
(20) Wulff, G.; Minarik, M. *HRC CC, J. High Resolut. Chromatogr. Chromatogr. Commun.* **1986**, *9*, 607−608.
(21) Sellergren, B.; Lepistö, M.; Mosbach, K. *J. Am. Chem. Soc.* **1988**, *110*, 583−560.
(22) Maier, N. M.; Franco, P.; Lindner, W. *J. Chromatogr., A* **2001**, *906*, 3−33.
(23) Booth, T. D.; Azzaoui, K.; Wainer, I. W. *Anal. Chem.* **1997**, *69*, 3879−3883.
(24) Lipkowitz, K. B. *J. Chromatogr., A* **2001**, *906*, 417−442.
(25) Landsteiner, K.; van der Scheer, J. *J. Exp. Med.* **1928**, *48*, 315−320.
(26) Cook, C. E. In *Drug Stereochemistry: Analytical Methods and Pharmacology*; Wainer, I. W., Drayer, D. E., Eds.; Marcel Dekker: New York, 1988; Chapter 3.
(27) Mertens, R.; Stüning, M.; Weiler, E. W. *Naturwissenschaften* **1982**, *69*, 595−597.
(28) Knox, J. P.; Galfre, G. *Anal. Biochem.* **1986**, *155*, 92−94.
(29) Takasaki, W.; Tanaka, Y. *Chirality* **1992**, *4*, 308−315.

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for classical affinity and immunoaffinity chromatography systems,[31−33] elution of bound material required harsh conditions, i.e., a drastic change in pH or addition of organic solvents or chaotropic salts. Such severe conditions generally cause protein denaturation and considerably shorten column lifetime. This, as well as lengthy cycles of binding, elution, and column regeneration, makes traditional immunoaffinity approaches impractical for routine enantiomer separation and confines their use to special applications. To be attractive for widespread use in academic and industrial laboratories, a CSP should combine selectivity with ease of use, short analysis times, and long-term column stability. Here, we demonstrate the application of antibody-based CSPs that possess these qualities.

In a series of recent publications, we reported the production of highly stereoselective antibodies to D- and L-α-amino acids, respectively.[34,35] These antibodies were successfully used in immunoassay and sensor approaches for the sensitive detection of enantiomeric impurities.[36−38] Lately, we demonstrated that monoclonal antibodies to D-amino acids, immobilized on agarose beads, can be used for enantiomer separation under isocratic conditions; that is, both binding and elution occur under mild buffer conditions.[35] The α-amino acids represent one of the most important classes of substances in nature that possess a stereogenic center and, therefore, exemplify an excellent model system to demonstrate the applicability of antibodies as chiral selectors. Hundreds of α-amino acids have been isolated from biological and other sources,[39] and amino acids are of utmost importance in the food, chemical, and pharmaceutical industries, e.g., as chiral building blocks.[40] Various chiral selectors have been used for enantiomer separation of amino acids;[41] notably, macrocyclic antibiotics have proven to be valuable for this class of substances.[8,9,42] However, these selectors were not specifically designed for α-amino acids and were found by trial and error. In contrast, our anti-amino acid antibodies were generated using rationally designed immunogens and possess predicted selectivity. Here, we describe the application of these antibodies as tailor-made chiral selectors for the direct resolution of amino acid enantiomers under isocratic conditions in a high-performance liquid chromatographic mode. Monoclonal antibodies both to D-amino acids and to L-amino acids, respectively, were used as chiral selectors covalently bound to a synthetic support material. Packed into standard analytical stainless steel columns, the CSPs enabled rapid enantiomer separations in a mild buffer at flow rates of up to 10 mL/min. Separations were routinely carried out at room temperature, and columns were stored under an azide-containing buffer at 4 °C only when not used for an extended period of time. However, after more than eleven months and 1000 injections, no significant change in column performance was observed. Thus, such antibody-based CSPs combine the advantages of tailor-made chiral selectors with the practical convenience of other routinely used phases.

EXPERIMENTAL SECTION

Instruments. The HPLC system consisted of a Hitachi L-7100 pump with a degasser, an L-7400 UV detector equipped with an analytical flow cell, and a D-7000 interface with System Manager V 4.0 software. Injections were performed using a Rheodyne 7725i injection valve with a 20-μL loop (Hitachi, Naperville, IL). Columns were packed using an Alltech slurry packer model 1666 (Alltech, Deerfield, IL).

Reagents. Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) were from Sigma (Rehovot, Israel). DEAE-Sephacel was purchased from Pharmacia (Uppsala, Sweden) and POROS-OH was from PerSeptive Biosystems (Cambridge, MA). *N*,*N*′-Disuccinimidyl carbonate (DSC) and (dimethylamino)pyridine (DMAP) were from NovaBiochem (La Jolla, CA).

Water was purified using a MilliQ water system (Millipore, Bedford, MA). Phosphate-buffered saline (PBS) was prepared according to ref 43 and adjusted to pH 7.4 with 0.1 N HCl. All amino acids were of the highest purity available. D-Tryptophan, D- and L-kynurenine, D- and L-phosphotyrosine, D- and L-*p*-aminophenylalanine, D- and L-phenylalanine, and D-histidine were purchased from Sigma (Deisenhofen, Germany). L-Tryptophan and D-tyrosine were from Aldrich (Munich, Germany), D- and L-DOPA were from Fluka (Neu-Ulm, Germany). L-Tyrosine, L-histidine, and D- and L-cyclohexylalanine were kindly provided by Degussa (Frankfurt, Germany).

Production of Monoclonal Antibodies. Monoclonal antibodies were produced as previously described.[35] In brief, eight-week-old BALB/c mice were immunized with *p*-azo-D-phenylalanine−KLH or *p*-azo-L-phenylalanine−KLH conjugates in complete Freund's adjuvant. Booster injections of immunogen in incomplete Freund's adjuvant were administered twice at intervals of two weeks. Four and three days prior to fusion, final boosts were given intraperitoneally. Splenocytes of two mice showing strong immune responses were fused with NS0 myeloma cells using poly(ethylene glycol).[44] Hybridomas were selected in hypoxanthine/aminopterin/thymidine medium, and supernatants were screened by a noncompetitive enzyme-linked immunosorbent assay using three different solid-phase coatings: BSA, *p*-azo-D-phenylalanine−BSA and *p*-azo-L-phenylalanine−BSA. Hybridomas producing stereoselective antibodies were cloned at least twice by limiting dilution. Large quantities of the anti-D-amino acid antibody secreted by the clone 67.36 (anti-D-AA 67.36) and the anti-L-amino acid antibody produced by clone 29.2 (anti-L-AA 29.2) were obtained by the

(30) Nevanen, T. K.; Söderholm, L.; Kukkonen, K.; Suortti, T.; Teerinen, T.; Linder, M.; Söderlund, H.; Teeri, T. T. *J. Chromatogr., A* **2001**, *925*, 89−97.
(31) Wilchek, M.; Miron, T.; Kohn, J. *Methods Enzymol.* **1984**, *104*, 3−55.
(32) Phillips, T. M. *Adv. Chromatog.* **1989**, *29*, 133−173.
(33) Cutler, P. In *High Resolution Chromatography: A Practical Approach*; Millner, P., Ed.; Oxford University Press: Oxford, 1999; Chapter 9.
(34) Hofstetter, O.; Hofstetter, H.; Schurig, V.; Wilchek, M.; Green, B. S. *J. Am. Chem. Soc.* **1998**, *120*, 3251−3252.
(35) Hofstetter, O.; Hofstetter, H.; Wilchek, M.; Schurig, V.; Green, B. S. *Int. J. Bio-Chromatogr.* **2000**, *5*, 165−174.
(36) Hofstetter, O.; Hofstetter, H.; Wilchek, M.; Schurig, V.; Green, B. S. *Chem. Commun.* **2000**, 1581−1582.
(37) Silvaieh, H.; Schmid, M. G.; Hofstetter, O.; Schurig, V.; Gübitz, G. *J. Biochem. Biophys. Methods*, **2001**, accepted.
(38) Hofstetter, O.; Hofstetter, H.; Wilchek, M.; Schurig, V.; Green, B. S. *Nature Biotechnol.* **1999**, *17*, 371−374.
(39) Barrett, G. C. *Chemistry and Biochemistry of the Amino Acids*; Chapman and Hall: London, 1985.
(40) Coppola, G. M., Schuster, H. F. *Asymmetric Synthesis: Construction of Chiral Molecules Using Amino Acids*; Wiley: New York, 1987.
(41) Bhushan, R.; Joshi, S. *Biomed. Chromatogr.* **1993**, *7*, 235−250.
(42) Berthod, A.; Liu, Y.; Bagwill, C.; Armstrong, D. W. *J. Chromatogr., A* **1996**, *731*, 123−137.
(43) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1988; p 684.
(44) Eshhar, Z. In *Hybridoma Technology in the Biosciences and Medicine*; Springer, T. A., Ed.; Plenum Publishing Corp.: New York, 1985; Chapter 1.

preparation of ascites fluid. The antibodies were purified by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE-Sephacel using a gradient of 0−400 mM NaCl in 10 mM tris(hydroxymethyl)aminomethane (TRIS), pH 8.5, for elution.

Preparation of the Chiral Stationary Phase. A 3.5-g sample of POROS-OH (20-μm particles) was reacted with 350 mg of DSC and 287 mg of DMAP in 17.5 mL of dry acetone for 1.5 h at 4 °C. The support material was then washed successively with absolute acetone, 5% acetic acid in dioxane, methanol, and 2-propanol. The material contained 3.4 μmol of active groups/g of stationary phase as determined spectrophotometrically.[45] The material was washed with PBS and stored under 2-propanol until further use.

Column D1. One fraction of the support material (1 g) was reacted overnight with 30 mg of anti-D-AA 67.36 in PBS at 4 °C.

Column D2. Another fraction of the support material (1 g) was reacted overnight at 4 °C with 30 mg of anti-D-AA 67.36 under salting-out conditions; i.e., sodium sulfate was added to the antibody solution to a final concentration of 0.125 M.

Column L1. The remaining activated support material (1.5 g) was reacted with 50 mg of anti-L-AA 29.2 overnight at 4 °C under salting-out conditions.

All batches were washed with PBS and remaining active groups on the support were quenched by treatment with 0.2 M TRIS for 1 h, followed by extensive washing with PBS. The concentration of immobilized antibody was determined using the dye-binding method of Bonde et al.[46] and found to be as follows: D1, 12 mg/g of support material (wet); D2, 16 mg/g; and L1, 15.5 mg/g.

Chiral Immunoaffinity Chromatography. Stainless steel columns (D1, D2: 2.3 × 200 mm. L1: 4.6 × 250 mm) were slurry packed at a pressure of 160 bar. The slurry and packing medium was PBS. No leakage of antibody was detected during column equilibration. All chromatographic separations were performed at room temperature (22 °C) under isocratic conditions using PBS as mobile phase. Flow rates used in this study varied from 0.1 to 10 mL/min. Ten-microliter samples of mixtures of the pure enantiomers (concentration 0.3 mM) in PBS were injected. Tryptophan was detected at 280 nm, *p*-aminophenylalanine at 240 nm, phosphotyrosine at 254 nm, tyrosine and DOPA at 220 nm, kynurenine at 215 nm, and histidine, cyclohexylalanine, and phenylalanine at 205 nm. The elution order was determined by injection of the pure enantiomers as well as by spiking.

The void volume for the determination of chromatographic data was measured using water or buffer.[47] For the short retention times obtained in this study, error in the determination of the void volume affects the calculation of the retention factor *k* and the separation factor α. Slight variations may be explained by this fact. To ensure the reproducibility of the results, all measurements were carried out at least in triplicate. Standard deviation of chromatographic parameters was typically less than 10%.

RESULTS AND DISCUSSION

In this study, monoclonal antibodies to both D- and L-α-amino acids were immobilized on a highly porous synthetic support material based on poly(styrene−divinylbenzene). This material,

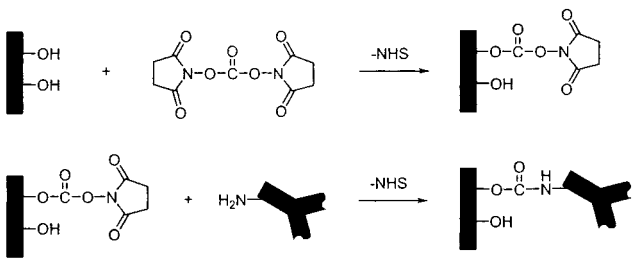

Figure 1. Activation of the stationary phase with DSC and binding of the antibody.

which possesses excellent chemical and mechanical stability, was originally developed for the rapid separation of high molecular weight biomolecules in perfusion chromatography.[48,49] In previous publications, we demonstrated that this support is also well-suited for the preparation of chiral stationary phases. Using BSA as chiral selector, fast enantiomer separations of a variety of drugs, amino acids, and derivatives thereof were achieved at flow rates up to 10 mL/min.[50−52] Numerous strategies for the immobilization of proteins have been described in the literature.[53] Here, we covalently linked the antibodies to the support material via reaction of primary amines on the protein with DSC-activated hydroxyl groups on the solid phase (Figure 1).[45]

The main advantages of this method are that the coupling step can be carried out in a neutral buffer and that the resulting uncharged carbamate bond is very stable;[45] thus, leakage of bound protein is minimized. The coupling yield, i.e., the amount of immobilized protein per gram of support material, is dependent not only on the number of activated groups on the carrier but also on the ratio of protein to carrier and the exact coupling conditions. Here, two columns with anti-D-AA 67.36 as chiral selector (columns D1 and D2) and one column with anti-L-AA 29.2 as chiral selector (column L1) were prepared and used for enantiomer separations. D1 and D2 differ only with regard to the coupling conditions used for antibody immobilization. In the preparation of the D1 CSP, DSC-activated support was incubated with a solution of 20 mg/mL anti-D-AA 67.36 in PBS. The D2 CSP was prepared under the same conditions, except that sodium sulfate was added to the antibody solution to a concentration of 0.125 M. Although the solid phase is relatively hydrophilic, the addition of salt is believed to induce a partial precipitation of the protein on the solid phase, which results in an increased local surface concentration.[54] Thus, the amount of immobilized antibody could be increased from 12 (D1) to 16 mg of antibody/g of support material (D2). Salting-out conditions were also used for the preparation of the L1 CSP, resulting in the immobilization of 15.5 mg of anti-L-AA 29.2/g of support.

As is known from previous studies,[34−38] the antibodies only bind to α-amino acids that have the correct configuration; i.e., anti-

(45) Wilchek, M.; Miron, T. *Appl. Biochem. Biotechnol.* **1985**, *11*, 191−193.
(46) Bonde, M.; Pontoppidan, H.; Pepper, D. S. *Anal. Biochem.* **1992**, *200*, 195−198.
(47) Kennedy, G. J.; Knox, J. H. *J. Chromatogr. Sci.* **1972**, *10*, 549−556.
(48) Afeyan, N. B.; Gordon, N. F.; Mazsaroff, I.; Varady, L.; Fulton, S. P.; Yang, Y. B.; Regnier, F. E. *J. Chromatogr.* **1990**, *519*, 1−29.
(49) Regnier, F. E. *Nature* **1991**, *350*, 634−635.
(50) Hofstetter, H.; Hofstetter, O.; Schurig, V. *J. Chromatogr., A* **1997**, *764*, 35−41.
(51) Hofstetter, H.; Hofstetter, O.; Schurig, V. *Biochemica* **1997**, *101*, 29.
(52) Hofstetter, H.; Hofstetter, O.; Schurig, V. *GIT Spec. Ed.* **1997**, 46−47.
(53) Hermanson, G. T.; Mallia, A. K.; Smith, P. K. *Immobilized Affinity Ligand Techniques*; Academic Press: San Diego, 1992; Chapter 3.
(54) *Operating Instructions: POROS 20 AL, EP, OH, NH, and HY*; PerSeptive Biosystems: Cambridge, MA. http://docs.appliedbiosystems.com/pebiodocs/00102017.pdf (accessed Dec 2001).

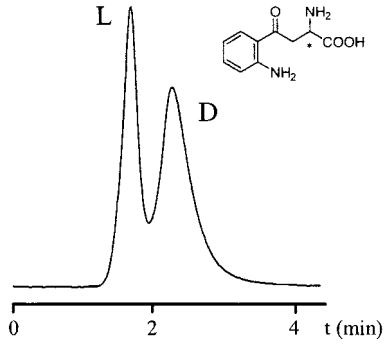

Figure 2. Separation of D,L-kynurenine on column D1 at a flow rate of 0.8 mL/min.

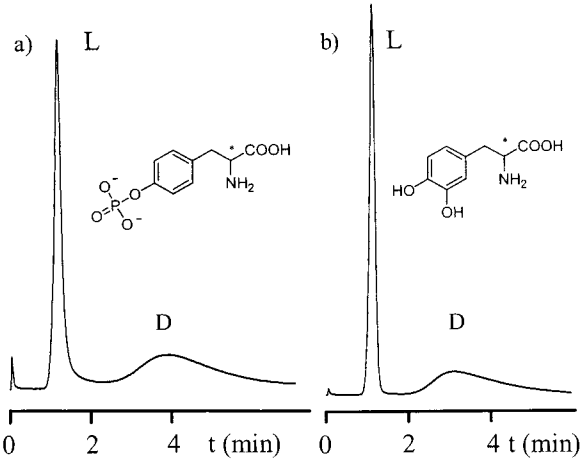

Figure 3. Separations of D,L-phosphotyrosine (a) and D,L-DOPA (b) on column D1 at a flow rate of 1 mL/min.

D-amino acid antibodies bind to D-amino acids and anti-L-amino acid antibodies bind to L-amino acids, while their interaction with the opposite enantiomer is negligible. Since these antibodies were elicited against protein conjugates of D- and L-*p*-aminophenylalanine, respectively, their affinity is highest toward structurally similar compounds. However, the antibodies also bind stereoselectively to α-amino acids with different, e.g., aliphatic, side chains, though with lower affinity. The dissociation constants for the amino acids investigated in this study are in the millimolar to micromolar range; the antibody−analyte interactions are therefore of weak to medium affinity. It has long been known that the weak interaction between a protein and a ligand can be used for affinity purification under isocratic conditions. In 1982, Allenmark et al. demonstrated that chiral separations can be achieved under isocratic conditions using BSA as chiral selector.[55] Enantiomer separations of three amino acids were based on the protein's preferred interaction with the naturally occurring L-enantiomers. Although the observed interactions were relatively weak, the difference in the retention between the L- and D-enantiomers was sufficient to enable baseline separations. Since then this principle has found numerous applications, and other protein CSPs, which all utilize weak stereoselective interactions, have been developed.[18,19] In 1988, Ohlson et al. showed that weak affinity antibodies can be used for achiral separations under isocratic conditions[56] and introduced the term "weak affinity chromatography".[57] In contrast to classical affinity systems, which are basically adsorption−desorption systems, weak affinity systems allow true chromatographic procedures.[58] In addition, separations can generally be performed under mild buffer conditions, which are favorable for the structural integrity and activity of the protein. Due to the resulting improved column stability, column lifetimes of several months to years, enabling hundreds of injections, are not unusual. Consequently, our first experiments were aimed at the chiral separation of an amino acid that was known to have a relatively weak affinity to the immobilized stereoselective antibodies. It was found that, using column D1, partial enantiomer separation of kynurenine was possible within 3 min under true isocratic conditions in PBS at a flow rate of 0.8 mL/min (Figure 2).

As expected, the L-enantiomer did not show significant interaction with the CSP and eluted with the void volume, while the D-enantiomer was retained due to its interaction with the im-

Table 1. Chromatographic Data for the Separation of α-Amino Acids on Column D1

amino acid	flow rate,[a] mL/min	k_2	R_s	α
kynurenine	0.4	0.7	1.60	4
phosphotyrosine	1	2.1	1.28	25
DOPA	2	2.4	1.28	48
tryptophan	3	4.8	1.25	15
phenylalanine	4	4.5	1.24	54
p-aminophenylalanine	4	7.5	1.25	41
tyrosine	4	9.5	1.19	136

[a] If applicable, values are given for the fastest flow rate still leading to baseline separation.

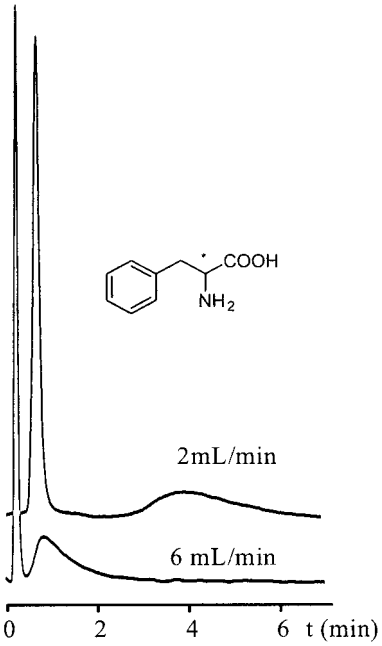

Figure 4. Separation of D,L-phenylalanine on column D1 at 2 and 6 mL/min. The first peak corresponds to the L-enantiomer and the second peak to the D-enantiomer.

(55) Allenmark, S.; Bomgren, B.; Borén, H. *J. Chromatogr.* **1982**, *237*, 473−477.

(56) Ohlson, S.; Lundblad, A.; Zopf, D. *Anal. Biochem.* **1988**, *169*, 204−208.

(57) Zopf, D.; Ohlson, S. *Nature* **1990**, *346*, 87−88.

(58) Ohlson, S.; Bergström, M.; Påhlsson, P.; Lundblad, A. *J. Chromatogr., A* **1997**, *758*, 199−208.

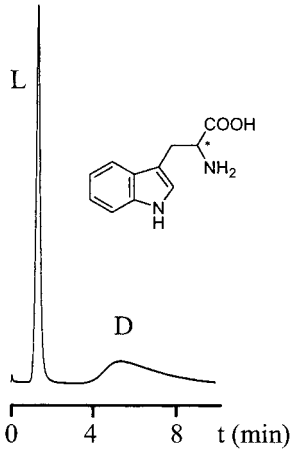

Figure 5. Separation of D,L-tryptophan on column D1 at a flow rate of 1 mL/min after 1009 injections.

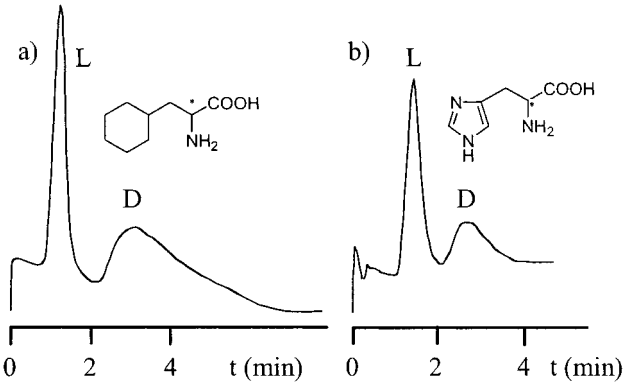

Figure 6. Separations of D,L-cyclohexylalanine (a) and D,L-histidine (b) on column D2 at flow rates of (a) 1.5 and (b) 1 mL/min.

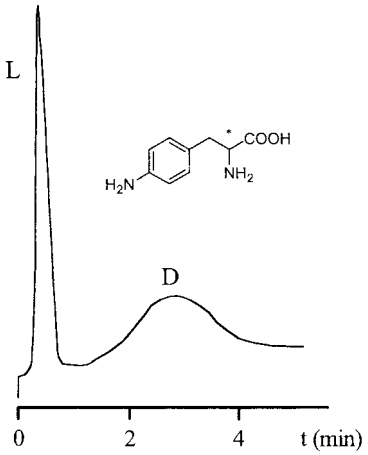

Figure 7. Separation of D,L-*p*-aminophenylalanine on column D2 at a flow rate of 10 mL/min.

mobilized anti-D-amino acid antibody. In subsequent experiments, a clear relation between the retention time of the more retained enantiomer and its affinity toward the immobilized antibody was observed. For example, while D-kynurenine eluted after 2.5 min at a flow rate of 1 mL/min, D-phosphotyrosine eluted after 6 min (Figure 3a).

Similarly, baseline separation of DOPA was possible within 6 min with a resolution R_s of 1.34 at a flow rate of 1 mL/min (Figure 3b). Baseline separations were also achieved for the enantiomers of tryptophan, phenylalanine, *p*-aminophenylalanine, and tyrosine (Table 1).

Table 2. Chromatographic Data for the Separation of α-amino Acids on Column D2

amino acid	flow rate,[a] mL/min	k_2	R_s	α
histidine	1	0.9	1.07	70
cyclohexylalanine	1.5	0.5	1.10	25
kynurenine	1.5	1.0	1.22	59
phosphotyrosine	4	4.1	1.29	34
DOPA	4	4.1	1.30	27
tryptophan	4	7.5	1.99	61
phenylalanine	10	4.8	1.22	91
p-aminophenylalanine	10	6.4	1.47	20

[a] If applicable, values are given for the fastest flow rate still leading to baseline separation.

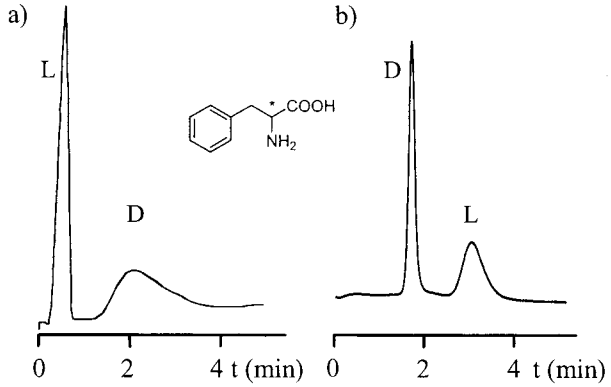

Figure 8. Inversion of the elution order for separations of D,L-phenylalanine on D2 at 10 mL/min (a) and on L1 at 2 mL/min (b).

The increased interaction of the D-enantiomers of these amino acids with the CSP, which is also reflected in higher *k* values, resulted in significant broadening of the second peak at lower flow rates. However, rapid enantiomer separations with improved peak shapes were possible by simply increasing the flow rate. Due to the properties of the support material, the flow rate could be increased without the limitations of high back pressure. Figure 4 shows the relation between retention time, peak shape, and flow rate for the enantiomer separation of phenylalanine.

It is well known that the chromatographic performance of protein CSPs can be influenced by varying, for example, the pH, the ionic strength, or the constituents of the mobile phase or by adding organic modifiers.[59] Changing these parameters mainly affects hydrophobic and electrostatic interactions between selector and selectand. The same type of noncovalent binding forces is disrupted in classical affinity chromatography in order to elute bound material. This is achieved by either a stepwise or a continuous change of the buffer composition. It is likely that the chromatographic performance of the antibody-based CSPs used here can also be further improved by varying the composition of the mobile phase. However, none of the above-mentioned parameters was varied in order to minimize protein denaturation. Since

(59) Allenmark, S. In *A Practical Approach to Chiral Separations by Liquid Chromatography*; Subramanian, G., Ed.; VCH: Weinheim, 1994; Chapter 7.

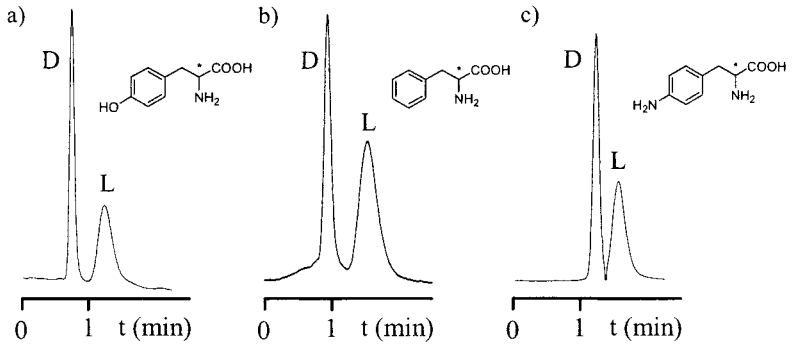

Figure 9. Separations of D,L-tyrosine (a), D,L-phenylalanine (b), and D,L-*p*-aminophenylalanine on column L1 at flow rates of 4 (a, b) and 2 mL/min (c).

separations were performed under isocratic conditions using a mild buffer as mobile phase, the same column could be used for an extended period of time. After more than eleven months, no significant change in column performance was observed, and high-quality separations were still obtained after more than 1000 injections (Figure 5).

Enantiomeric resolution of amino acids that possess a lower affinity toward anti-D-amino acid antibody 67.36 than kynurenine, e.g., histidine and cyclohexylalanine, was not achieved on D1. Due to closely eluting peaks, a precise assessment of the chromatographic parameters of the partial separation was not possible. Enantiomer separation of these two amino acids was however possible on D2 (Figure 6), which contained ~33% more antibody/g of support.

Although it is known that the antibodies used in this study also bind stereoselectively to amino acids with smaller side chains (e.g., alanine), chromatographic enantiomer separation of such amino acids was not possible. It is believed that this may be achieved by further increasing column capacities through, for example, the immobilization of smaller antigen-binding fragments, the use of different immobilization procedures, or the use of larger columns. The higher capacity of D2 compared to D1 resulted in increased retention times of the second peak. Therefore, separations could be performed at higher flow rates; e.g., baseline separation of *p*-aminophenylalanine was still possible at flow rates as high as 10 mL/min (Figure 7). The practical limit was given by the maximum delivery of the pump.

Table 2 summarizes the results of the enantiomer separations obtained on column D2.

Immobilization of anti-L-AA 29.2 resulted in a CSP with an inverted elution order. As a result of their stereoselective interaction with the immobilized antibody, the L-enantiomers of several amino acids were retained on the column, while the corresponding D-enantiomers eluted with the void volume. Figure 8 shows a comparison of the inverted elution profiles of separations of phenylalanine on D2 and L1.

The inverted elution order provides unambiguous evidence that the observed chiral separations are based on the specific stereoselective interaction between the analytes and the antibodies' antigen-binding sites. Although both the anti-L-amino acid antibody and the anti-D-amino acid antibody are themselves made from L-amino acid building blocks, the specific three-dimensional arrangement of L-amino acid residues in the complementarity-determining regions generates binding sites that can accommodate *either* D-amino acids *or* L-amino acids. The specific interaction between an antibody and an antigen is typically the result of a certain shape complementarity and noncovalent attractive and repulsive forces between the antigen and amino acid side chains in the antibody-binding site.[60] Thus, the observed chiral discrimination is not simply based on the proteins' "inherent chirality".[19] Anti-L-AA 29.2 has an overall lower affinity to L-amino acids than anti-D-AA 67.36 has toward the respective D-enantiomers. However, using L1, enantiomer separations of tryptophan, DOPA, *p*-aminophenylalanine, tyrosine, and phenylalanine were achieved under isocratic conditions in PBS at flow rates between 0.1 and 4 mL/min (Table 3).

Table 3. Chromatographic Data for the Separation of α-amino Acids on Column L1

amino acid	flow rate,[a] mL/min	k_2	R_s	α
tryptophan	0.1	0.65	1.14	2
DOPA	0.5	0.15	0.69	9
p-aminophenylalanine	2	0.8	1.34	18
tyrosine	4	0.8	1.78	29
phenylalanine	4	0.9	1.90	10

[a] If applicable, values are given for the fastest flow rate still leading to baseline separation.

Baseline separations of *p*-aminophenylalanine, phenylalanine, and tyrosine were possible in less than 2 min at flow rates of 2 and 4 mL/min, respectively (Figure 9).

CONCLUSIONS

This study demonstrates that antibodies are useful tailor-made chiral selectors for enantiomer separation in HPLC under true chromatographic conditions. Since separations can be performed in a mild buffer, columns can be used for an extended period of time. The application of a stationary phase that allows the use of high flow rates furthermore enables rapid separations within a few minutes. The increased flexibility with regard to the flow rate can be employed to adjust the chromatographic separation to the strength of interaction between selector and selectand.

(60) Van Oss, C. J. In *Immunochemistry*; Van Oss, C. J., Van Regenmortel, M. H. V., Eds.; Marcel Dekker: New York, 1994; Chapter 23.

Since antibodies can be raised against virtually any compound of interest,[61,62] this appears to be a general approach applicable to a variety of separation problems. The rational design of immunogens and screening procedures enables the generation of antibodies with desired binding properties and selectivities. Furthermore, the progression of molecular biological techniques for the generation and engineering of antibodies nowadays enables the inexpensive production of large amounts of antibodies and antibody-binding fragments.[63] Therefore, the use of antibody-based CSPs is not limited to analytical applications but may be extended to large-scale industrial preparations.

(61) Landsteiner, K. *The Specificity of Serological Reactions;* Dover Publications: New York, 1962.
(62) Pressman, D.; Grossberg, A. L. *The Structural Basis of Antibody Specificity;* W. A. Benjamin: New York, 1968; Chapter 3.
(63) *Antibody Engineering: A Practical Approach;* McCafferty, J., Hoogenboom, H. R., Chiswell, D. J., Eds.; IRL Press: Oxford, 1996.

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