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Enantiomer separation of α-hydroxy acids in high-performance immunoaffinity chromatography

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Abstract

In this study, a monoclonal anti-D-hydroxy acid antibody was immobilized onto a synthetic high-flow-through chromatographic support material to produce a chiral stationary phase suitable for enantiomer separation of free α-hydroxy acids. Chiral separation of several aliphatic and aromatic members of this class of compounds was achieved in HPLC under mild isocratic buffer conditions using phosphate buffered saline, pH 7.4, as mobile phase. Due to the high degree of stereoselectivity exhibited by the immobilized antibody, in all cases the L-enantiomer eluted with the void volume, while the D-enantiomer was retained and eluted second. The effect of the mobile phase parameters flow rate, temperature, pH, and ionic strength on the enantiomer separation of the model analyte mandelic acid was investigated. While it was found that variations in the flow rate did not change the retention factor k_2, dramatic effects on the interaction between the immobilized antibody and D-mandelic acid were observed when any of the other mobile phase parameters were modulated.

Keywords: Antibody; Enantiomer separation; High-performance liquid chromatography; Hydroxy acid; Protein chiral stationary phase

1. Introduction

α-Hydroxy acids represent one of the most important classes of chiral compounds in nature. Lactic acid, for example, is a key intermediate and end product, respectively, of both prokaryotic and eukaryotic metabolism. In humans, abnormal accumulation of α-hydroxy acids in serum and urine is usually the result of an inherited autosomal recessive disorder causing an enzyme deficiency [1]; however, lactic acidosis, the accumulation of lactic acid in the blood, can also be a potential side-effect of some anti-HIV drugs [2] or be caused by increased bacterial fermentation in the gastrointestinal tract [3]. Furthermore, α-hydroxy acids are of great significance in the pharmaceutical and chemical industries, e.g., as building blocks for the production of chiral drugs and biodegradable polymers [4,5]. Recently, α-hydroxy acids have achieved new prominence because of their importance to the cosmetic industry. It has been found that cosmetics that contain these compounds may make users more sensitive to UV-light and, thus, increase the risk for developing skin cancer [6–8].

In order to elucidate their exact biochemical roles and metabolization routes, it is necessary to develop methods for the detection of α-hydroxy acids and for the determination of their three-dimensional structure. A number of analytical techniques such as enzymatic assays, GC, CE, CEC, and HPLC have been used for enantiomer analysis of α-hydroxy acids; direct enantiomer separation of this class of compounds in LC was previously accomplished utilizing ligand exchange selectors, macrocyclic antibiotics, π-donor-π-acceptor systems, and serum albumin [9–19]. While these chiral selectors often show a higher specificity for other analytes (e.g., amino acids), and have been identified on a trial and error basis, we have lately reported the production of tailor-made stereoselective antibodies to α-hydroxy acids [20].

Despite the fact that as early as in the first half of the last century it was recognized that stereoselective antibodies can be raised against virtually any low-molecular weight chiral compound [21,22], this class of proteins has only recently gained more attention as chiral selectors for enantiomer analysis [23], and very few applications in high pressure chromatography have

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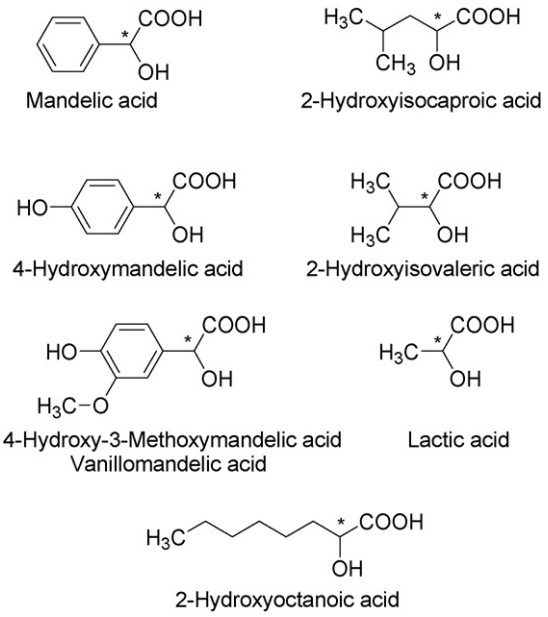

Fig. 1. Chemical structures of α-hydroxy acids investigated in this study.

been reported so far [23–26]. As antibodies typically exhibit considerable affinity toward their binding partners, analyte elution often requires harsh mobile phase conditions, i.e., a drastic change in pH or addition of organic solvents or chaotropic salts. In most cases, such conditions compromise the structural integrity of the immobilized chiral selector, which may significantly reduce column lifetime. However, using antibodies directed against the stereogenic center of α-amino acids, we have previously demonstrated that the lifetime of antibody-based CSPs can be increased significantly if they are operated under mild, isocratic buffer conditions [27,28].

In this study, a monoclonal anti-D-hydroxy acid antibody was immobilized onto a polystyrene-based support material in order to produce a CSP suitable for chiral discrimination of α-hydroxy acids. Enantiomer separation of a total of seven analytes comprising both aromatic and aliphatic compounds (Fig. 1) was achieved in phosphate buffer under isocratic conditions, and the effect of the mobile phase parameters flow rate, temperature, pH, and ionic strength on the enantiomer separation of the model analyte D,L-mandelic acid was investigated.

2. Experimental

2.1. Chemicals

POROS-OH was purchased from PerSeptive Biosystems (Cambridge, MA). *N,N′*-disuccinimidyl carbonate (DSC) and dimethylaminopyridine (DMAP) were from NovaBiochem (La Jolla, CA), HPLC-grade acetonitrile, ethanol, methanol, 1-propanol, and 2-propanol were obtained from Sigma (St. Louis, MO). Inorganic salts were from ACROS/Fisher (Fair Lawn, NJ); all other chemicals were from Sigma (St. Louis, MO).

Water was purified using a MilliQ water system (Millipore, Bedford, MA). Phosphate buffered saline (PBS) was prepared according to reference [29] and adjusted to pH 7.4 with 0.1 N HCl. All α-hydroxy acid analytes were of the highest purity available. D-Mandelic acid, D,L-4-hydroxy-3-methoxymandelic acid (vanillomandelic acid), and 2-hydroxyoctanoic acid were purchased from Sigma (St. Louis, MO). 4-hydroxymandelic acid monohydrate and 2-hydroxyisocaproic acid were from Aldrich (Milwaukee, WI). L-Mandelic acid, D-2-hydroxyisovaleric acid, L-2-hydroxyisovaleric acid, and L-lactic acid were from Fluka (Allentown, PA). D-Lactic acid was obtained from ACROS/Fisher (Fair Lawn, NJ).

2.2. Monoclonal antibody production

Antibody production was carried out in accordance with institutional animal care and safety guidelines. The monoclonal anti-D-hydroxy acid antibody 8E10.9 was produced as previously described [20,30]. In brief, 8-week old BALB/c mice were immunized with conjugates of keyhole limpet hemocyanin and *p*-amino-D-phenyllactic acid, prepared by diazotization, following a standard immunization protocol [31]. For the production of monoclonal antibodies, splenocytes were fused with P3X63-AG8.653 myeloma cells using polyethylene glycol, and hybridomas were selected in hypoxanthine/aminopterin/thymidine medium. Large quantities of the anti-D-hydroxy acid antibody secreted by clone 8E10.9 were obtained by the preparation of ascites fluid, which was carried out by OEM Concepts (Boca Raton, FL). The antibody was isolated from ascites fluid by ammonium sulfate precipitation followed by purification using the commercially available Melon Gel Monoclonal IgG Purification Kit (Pierce Biotechnology, Rockford, IL). The antibody did not contain any impurities as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Coomassie staining.

2.3. Chiral stationary phase

3.5 g POROS-OH (20 μm particles) were reacted with 350 mg DSC and 287 mg DMAP in 17.5 ml dry acetone for 1.5 h at 4 °C. The activated support material contained 6.67 μmol active sites/g of support material as determined spectrophotometrically following the procedure by Wilchek and Miron [32]. A fraction of 1.25 g was reacted for 2 days with 38 mg of the anti-D-hydroxy acid antibody 8E10.9 in PBS at 4 °C under salting-out conditions, i.e., in the presence of 0.125 M sodium sulfate. Remaining active groups on the support were quenched for 1 h by treatment with 0.1 M Tris(hydroxymethyl)aminomethane (TRIS), adjusted to pH 7.4 with 0.1 N HCl. After extensive washing with PBS, the amount of immobilized antibody was determined spectrophotometrically as the difference of the absorbance (at 280 nm) of the antibody solution before and after the immobilization step, and was found to be 29 mg/g of support material.

2.4. Chromatography and instrumentation

The HPLC system consisted of a Jasco PU-1586 preparative scale HPLC pump (Jasco Inc., Easton, MD), a Hitachi 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).

A stainless steel column (4.6 mm × 150 mm) was slurry packed with the antibody-derivatized support in PBS at 160 bar using an Alltech Slurry Packer Model 1666 (Alltech, Deerfield, IL). No leakage of antibody was detected during column equilibration. Unless stated otherwise, all chromatographic separations were performed at room temperature under isocratic conditions using PBS, pH 7.4, as mobile phase. The column was stored under azide-containing PBS at 4 °C only when not used for an extended period of time. Flow rates used in this study ranged from 0.5 to 8 ml/min. Analytes, dissolved in PBS, were injected either as mixtures of the pure enantiomers (when available) or racemates. All analytes were detected utilizing their UV-absorbance at 205 nm. The elution order was determined by injection of the pure enantiomers as well as by spiking; this only applies to compounds that were available as pure enantiomers. The void volume for the determination of chromatographic data was measured using buffer [33]. For the short retention times obtained in this study (especially for the first eluting enantiomer), errors in the determination of the void volume affect the calculation of chromatographic parameters. Slight variations may be explained by this fact. To ensure reproducibility of results, all measurements were carried out at least in triplicate. Standard deviation of chromatographic parameters was typically less than 10%.

2.4.1. Influence of the flow rate

The effect of the flow rate on antibody-based chiral separations was investigated using mandelic acid as model analyte and flow rates between 0.5 ml/min and 8 ml/min.

2.4.2. Effect of temperature

The temperature dependence of the enantiomer separation of mandelic acid was studied between 10 °C and 40 °C at 4.5 ml/min. The antibody column and buffer reservoir were immersed in a water bath (Haake, Berlin, Germany), and the temperature was increased in increments of 5° and 2.5°, respectively. The antibody column was equilibrated for at least 30 min at a flow rate of 4.5 ml/min before the corresponding series of injections were performed at the respective temperature.

2.4.3. Effect of pH

The effect of the pH on enantiomer separations of mandelic acid was studied using 10 mM phosphate buffer in the range between pH 5.5 and 9. The buffers were prepared by mixing 10 mM solutions of Na_2HPO_4 or KH_2PO_4 in the proportions necessary to reach the desired pH. The column was equilibrated for at least 30 min before the corresponding series of injections were performed. Separations of mandelic acid were carried out at a flow rate of 6 ml/min.

2.4.4. Effect of ionic strength

To study the influence of the ionic strength on the separation of mandelic acid, the molarity of NaCl in a 10 mM phosphate buffer was changed by adding varying amounts of the salt to a solution containing 10 mM Na_2HPO_4 and KH_2PO_4, which was adjusted to pH 7.4. The concentration of NaCl ranged from 0 to 900 mM. The antibody column was equilibrated for at least 30 min at a flow rate of 6 ml/min before the corresponding series of injections were performed.

3. Results and discussion

We have recently reported the production of both polyclonal and monoclonal antibodies exhibiting exquisite stereoselectivity and class-specificity toward free α-hydroxy acids [20]. The antibodies were raised against a protein-conjugate of the hapten *p*-amino-phenyllactic acid and, therefore, display highest affinity for structurally similar compounds, i.e., aromatic α-hydroxy acids. However, the antibodies also bind stereoselectively to aliphatic members of this class of compounds, e.g., lactic acid. Using a polyclonal anti-D-hydroxy acid antibody immobilized onto silica, we were able to demonstrate in a preliminary study the general utility of such antibodies to perform chiral separations of phenyllactic acid, which is bound rather strongly [20]; however, we were not able to separate other analytes.

Therefore, a large amount of the monoclonal anti-D-hydroxy acid antibody 8E10.9 was produced from ascites fluid and, following a simple two-step purification, was immobilized onto the synthetic support material POROS [34,35]. This support, which is based on poly(styrene-divinylbenzene), is extremely porous and can be operated at high flow rates without a significant increase in back pressure. We have previously demonstrated that POROS is well suited for the preparation of protein-based CSPs [36], and that the enormous flexibility with regard to the applied flow rate is particularly useful to adjust the chromatographic separation to the strength of interaction between antibody-based CSPs and appropriate analytes [27,28]. While numerous chemistries for the immobilization of proteins onto solid phases are available [37], here, the antibody was covalently linked to the solid phase via reaction of primary amines on the protein surface with DSC-activated hydroxyl groups on the POROS material. This coupling chemistry, which can be performed under mild buffer conditions, leads to the formation of a stable, uncharged carbamate bond. Yields are typically very high and leakage of antibody is negligible. In this study, approximately 95% of the total amount of antibody invested was linked to the support.

In order to investigate the utility of the prepared antibody-based CSP for enantiomer separation of α-hydroxy acids, the derivatized support was packed into a standard size 4.6 mm × 150 mm stainless steel column, and mixtures of D- and L-mandelic acid were injected at various flow rates using PBS as mobile phase. It was found that baseline separations of this analyte were achieved between 0.5 ml/min and 8 ml/min (Fig. 2). Since the antibody stereoselectively binds to the D-enantiomers of α-hydroxy acids, D-mandelic acid was retained on the column, while the L-enantiomer eluted with the void volume. As seen in Fig. 2, the interaction between the immobilized antibody and D-mandelic acid caused significant peak broadening, especially at lower flow rates; rapid enantiomer separation with significantly improved peak shapes was achieved at higher flow rates. The

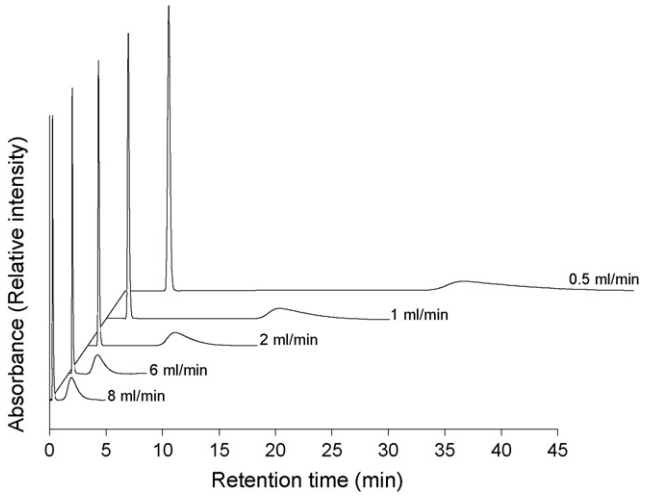

Fig. 2. Enantiomer separation of D,L-mandelic acid at various flow rates. All separations were performed using PBS, pH 7.4, as mobile phase. The first peak corresponds to the L-enantiomer, the second to the D-enantiomer.

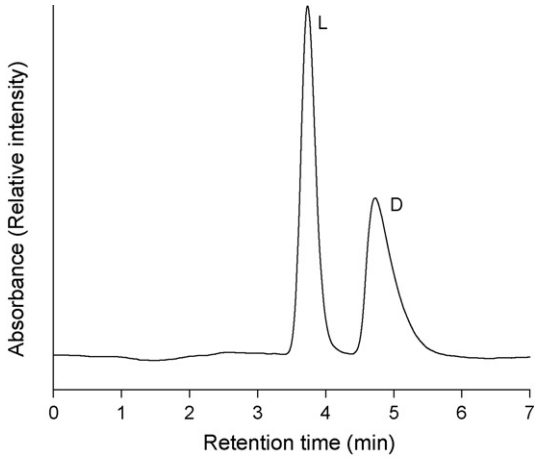

Fig. 4. Enantiomer separation of D,L-lactic acid at 0.5 ml/min in PBS.

resolution R_s, however, dropped from 5.6 at 0.5 ml/min to 2.1 at a flow rate of 8 ml/min.

We have previously demonstrated that when POROS is used as support material, the loss in resolution at higher flow rates is lower than predicted for conventional solid phases [28,36]; evaluation of the chromatographic data for enantiomer separation of mandelic acid at different flow rates confirmed that this is also the case here (data not shown). In addition, it was found that the retention factor k_2 remained basically constant over the investigated range of flow rates. Using anti-amino acid antibodies as chiral selectors, we have previously found that k_2 depends on the affinity between the analyte and the immobilized antibody, and is not independent of the flow rate [28]. However, the affinity between the anti-D-hydroxy acid antibody 8E10.9 and D-mandelic acid is not high enough to lead to a discernible change in k_2.

In addition to mandelic acid, enantiomer separation of its derivatives 4-hydroxymandelic acid and vanillomandelic acid was achieved (Fig. 3). Representative chromatographic data for these and all other analytes are given in Table 1. While in our previous studies using anti-amino acid antibodies as chiral selectors, it was particularly challenging to achieve enantiomer separation of the more weakly bound aliphatic amino acid analytes, we found that the CSP investigated in this study was very well suited for enantiomer separation of the analogous aliphatic α-hydroxy acids. Besides 2-hydroxyoctanoic acid, 2-hydroxyisocaproic acid, and 2-hydroxyisovaleric acid (Table 1), even the smallest naturally occurring chiral member of this class of compounds, lactic acid, could be separated (Fig. 4). Since, based on their different side chain structures, the immobilized antibody displays different affinities to the D-enantiomers of the compounds investigated, changes in flow rate were utilized to optimize enantiomer separation of each analyte. Thus, relatively slow flow rates were required to separate lactic acid.

For further characterization of the antibody-based CSP, mandelic acid was chosen as a model analyte, and the influence of the mobile phase parameters temperature, ionic strength, and pH on enantiomer separation of this compound was investigated. All of these parameters are known to potentially have a significant effect on protein-based chiral discrimination and are often used to optimize enantiomer separations [38,39]. Although the effect of organic mobile phase additives on protein-based enantiomer separations is also commonly investigated, our previous studies

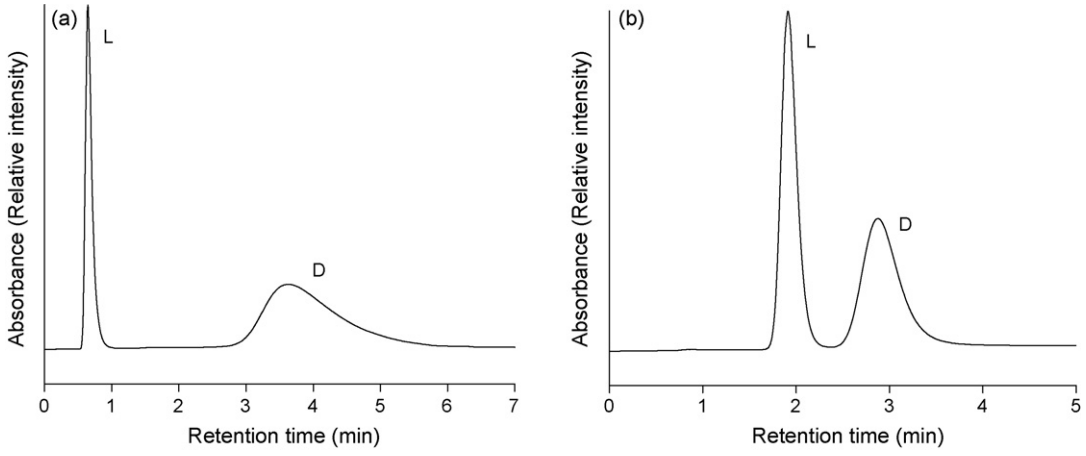

Fig. 3. Enantiomer separation of (a) D,L-4-hydroxymandelic acid at 3 ml/min, and (b) D,L-vanillomandelic acid at 1 ml/min using PBS as mobile phase.

Table 1
Chromatographic data for the enantiomer separation of α-hydroxy acids

Analyte	Flow rate (ml/min)	k_2	α	R_s
Mandelic acid	6	6.66 ± 0.02	660 ± 2	2.31 ± 0.01
4-Hydroxymandelic acid	3	4.63 ± 0.02	4622 ± 22	2.72 ± 0.03
Vanillomandelic acid	1	0.508 ± 0.001	508 ± 1	1.83 ± 0.03
2-Hydroxyoctanoic acid	7	4.49 ± 0.04	4489 ± 41	1.12 ± 0.09
2-Hydroxyisocaproic acid	6	4.60 ± 0.22	455 ± 22	1.60 ± 0.11
2-Hydroxyisovaleric acid	6	3.07 ± 0.15	304 ± 15	1.17 ± 0.07
Lactic acid	0.5	0.278 ± 0.003	27.6 ± 0.3	1.72 ± 0.02

showed that organic solvents in the mobile phase are likely to permanently compromise the structural integrity of antibody-based CSPs [28]; therefore, the effect of organic mobile phase additives was not examined here.

Depending on whether the interaction between a protein and a binding partner is enthalpically or entropically favored, a decrease in temperature may result in a stronger or weaker apparent affinity. In chromatography, changes in the overall free energy of binding manifest themselves as increased or decreased retention times, typically expressed as retention factors. In order to study the effect of temperature on the antibody-based enantiomer separation of mandelic acid, the temperature was varied between 10 °C and 40 °C. As seen in Fig. 5, a decrease in temperature led to a significantly more retained second peak, while an increase in temperature resulted in less interaction between the immobilized antibody and D-mandelic acid. The van't Hoff plot of $\ln k_2$ as a function of $1/T$ (Fig. 6) has a positive slope, which indicates that the interaction between the antibody and D-mandelic acid is enthalpically favored. Although a linear fit of the data obtained at temperatures between 10 °C and 40 °C seems reasonable ($y = 4.74x - 14.32$; $R^2 = 0.995$), closer analysis suggests a more complex temperature dependence. It appears that the van't Hoff plot can be divided into three linear regions, one between 10 °C and 20 °C ($y = 4.08x - 12.06$; $R^2 = 0.999$), a second one between 20 °C and 30 °C ($y = 4.56x - 13.\ 86$; $R^2 = 0.999$), and a third one between 35 °C and 40 °C ($y = 5.85x - 17.93$; $R^2 = 0.999$). Non-linear van't Hoff plots have been reported for protein-based chiral separations as well as for antibody–antigen interactions and are typically attributed to temperature-dependent conformational changes in the protein structure [28,40–42]. It is noteworthy that temperature changes did not affect the retention factor of the first eluting L-enantiomer, so that a higher selectivity for the enantiomer separation of α-hydroxy acids can be achieved by lowering the temperature.

The effect of the pH of the mobile phase on the enantiomer separation of mandelic acid was studied at pH values between 5.5 and 9. Fig. 7 shows a plot of the retention factor k_2 as a function of pH. Interestingly, no traditional optimum curve was obtained in the investigated pH range, but a significant decrease of the interaction between the antibody and D-mandelic acid was observed with increasing pH. In contrast, our previous studies using anti-amino acid antibodies as chiral selectors for the separation of amino acid enantiomers showed maximum interaction between the investigated antibodies and their corresponding binding partners at around neutral pH [28]. However, considering the fact that α-hydroxy acids are negatively charged at physiological pH (at which antibodies are produced by the immune system), it is reasonable to assume that a positively charged amino acid residue in the antibody's binding site is involved in the interaction with the carboxylate of mandelic acid ($pK_a = 3.36$), and that at low pH values a larger portion of this residue is protonated and, hence, positively charged; thus, it is not surprising

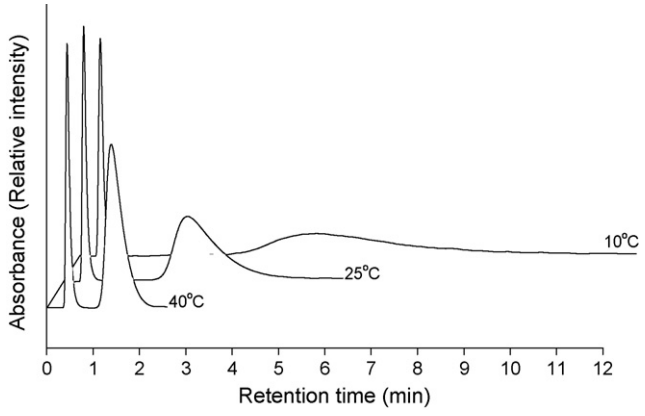

Fig. 5. Separation of D,L-mandelic acid at 10 °C, 25 °C, and 40 °C at 4.5 ml/min in PBS. The first peak corresponds to the L-enantiomer, the second to the D-enantiomer.

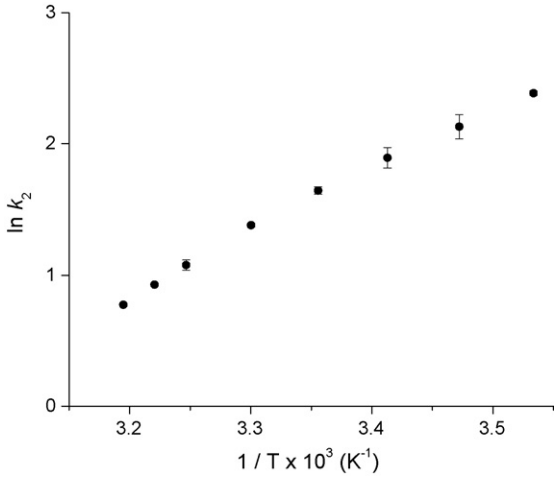

Fig. 6. Van't Hoff plot for the enantiomer separation of D,L-mandelic acid at 4.5 ml/min. Separations were carried out using PBS as mobile phase. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

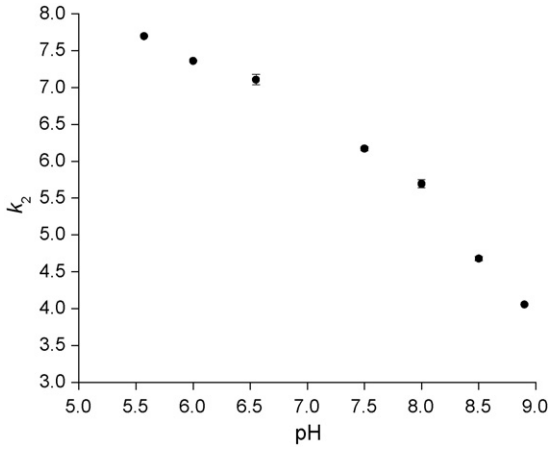

Fig. 7. Relationship between the retention factor k_2 and the pH. Enantiomer separation of mandelic acid was performed at 6 ml/min in 10 mM phosphate buffer containing NaCl at a concentration of 138 mM, which corresponds to the concentration in PBS. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

that low pH values favor the interaction between the antibody and D-hydroxy acids. Although pH values lower than 5.5 were not investigated in this study, it can be expected that protonation of the carboxyl group of mandelic acid has a negative effect on its interaction with the CSP. Since, on the other hand, an increase in the pH of the mobile phase is likely to result in the deprotonation of amino acid residues in the binding site (while the anionic form of the α-hydroxy acid is favored), it is not surprising that k_2 drops significantly at higher pH values.

As mentioned above, it is reasonable to assume that electrostatic interactions play an important role in the interaction of the anti-hydroxy acid antibody 8E10.9 with D-α-hydroxy acids. Thus, it could be expected that, in the chromatographic separation process, changes in the ionic strength of the mobile phase would affect k_2 of the retained enantiomer. In order to test this hypothesis, enantiomer separation of mandelic acid was performed in a 10 mM phosphate buffer (pH 7.4) that contained varying concentrations of NaCl. As seen in Fig. 8, changes in the effective ionic strength of the mobile phase had a dramatic effect. While an increase in the salt concentration resulted in a weaker interaction between analyte and antibody, and, thus, a decrease in k_2, a decrease in the salt content of the mobile phase led to a significant increase of k_2. Apparently, a decrease in the ionic strength of the aqueous buffer favors electrostatic interactions between the antibody and D-mandelic acid, while these non-covalent forces are weakened at higher ionic strengths. As the retention of the first eluting enantiomer is not affected by changes in the ionic strength, variations in the salt content of the mobile phase can also be utilized to modulate column selectivity.

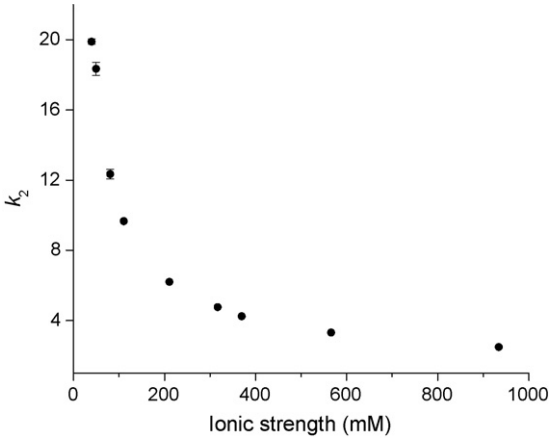

Fig. 8. Influence of the ionic strength on the retention factor k_2. Enantiomer separation of mandelic acid was performed at 6 ml/min in 10 mM phosphate buffer, pH 7.4, containing varying amounts of NaCl. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

4. Conclusions

Antibodies have only recently started to gain popularity as a new class of chiral selectors. Besides molecularly imprinted polymers and aptamers [43,44], they are the only chiral host molecules that can currently be produced in a rational, i.e., targeted way, and be tailor-made for specific analytes. While antibodies have proven their versatility for enantiomer detection and separation in a variety of analytical techniques such as immunoassays, sensors, and chromatography [23], they have not yet been fully adopted by the scientific community for routine enantiomer analysis. However, this study, as well as our previous work on anti-amino acid antibodies, illustrates that CSPs prepared with suitably raised stereoselective antibodies can compete with many more widely used CSPs with regard to column selectivity and ease of use. Moreover, immunoaffinity columns are surprisingly stable, especially if they are operated under mild, isocratic buffer conditions that do not cause denaturation of the immobilized protein; in this study, no significant changes in column performance were observed after almost 300 injections. Our results also show that a number of mobile phase parameters can be conveniently varied in order to modulate analyte retention, and to fine-tune chromatographic conditions to achieve enantiomer separation of structurally different compounds that may be bound by the antibody with varying affinities.

Acknowledgements

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