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DIRECT LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS ON IMMOBILIZED PROTEIN STATIONARY PHASES

III. OPTICAL RESOLUTION OF A SERIES OF N-AROYL D,L-AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON BOVINE SERUM ALBUMIN COVALENTLY BOUND TO SILICA

STIG ALLENMARK* and BJÖRN BOMGREN
Clinical Research Centre, Linköping University, S-58185 Linköping (Sweden)
and
HANS BORÉN
Department of Organic Chemistry, IFM, Linköping University, S-58183 Linköping (Sweden)
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SUMMARY

A series of N-aroyl D,L-amino acids was investigated with respect to direct optical resolution by high-performance liquid affinity chromatography on a stationary phase consisting of bovine serum albumin covalently bound to a 10-μm silica support. In all instances the k' values increased with decreasing pH of the mobile phase. Many of the compounds, such as the N-benzoyl and N-naphthoyl derivatives of D,L-alanine and D,L-phenylalanine, could be completely resolved into the optical antipodes. The 2-substituent of the amino acid was found to exert a great influence not only on the degree of resolution but also on the elution order of the enantiomers. A change in the N-aroyl substituent from a benzoyl to a 2-naphthoyl group caused a large increase in the retention of both enantiomers of a D,L-alanine derivative.

INTRODUCTION

In previous papers in this series we reported on the ability of an affinity sorbent, albumin–agarose, to act as a chiral discriminator in liquid chromatography and thereby resolve the optical antipodes of widely different compounds[1,2].

We found that the capacity ratios (k') of the enantiomers and the corresponding α values were greatly influenced by the composition of the mobile phase, such as buffer strength and pH[1,2], and also by the content of 1-propanol[3]. These parameters can therefore be used for the regulation of retention and thereby also for optimization of enantiomeric resolution.

In this paper the use of silica (10 μm), chemically modified to contain a surface layer of bovine serum albumin (BSA), for the optical resolution of a series of N-aroyl

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D,L-amino acids is described. In order to gain more insight into the complex problem area of the mechanism of retention and chiral recognition, a systematic investigation of substituent effects and the influence of the mobile phase composition was undertaken.

To the best of our knowledge, this is the first example of the use of a protein as a stationary phase in affinity chromatography of low-molecular-weight compounds by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chromatography

The chromatographic material used was a BSA-silica of 10 μm particle size for HPLC, developed in our laboratory and now available as Resolvosil from Macherey, Nagel & Co. (Düren, G.F.R.). This material was slurry-packed into a stainless-steel column (150 × 4.6 mm I.D.) with the use of a phosphate buffer (pH 7) as the slurry medium and at a pressure of *ca.* 400 bar. The column was inserted into an HPLC system composed of an Altex Model 110A solvent pump, a Rheodyne Model 7120 injection valve equipped with a 10-μl loop, an LDC Spectromonitor III variable-wavelength UV detector and a Hitachi Model 561 potentiometric recorder. All runs were performed isocratically with aqueous buffers as mobile phases.

Synthesis of the substituted amino acids

The amino acid (2 mmol) was dissolved in 5 ml of 1.0 *M* sodium carbonate (pH 9.1). To this solution was then added an equivalent amount (2 mmol) or less of the appropriate acid chloride by means of a microlitre syringe. The solid 2-naphthoyl chloride was first dissolved in a small amount of acetone. After the addition, the 100-ml erlenmeyer flask used for the reaction was shaken vigorously until an almost clear solution indicated that the acid chloride had been nearly completely reacted. While the reaction with benzoyl and 2-thenoyl chloride was sufficiently fast at room temperature, naphthoyl chloride required a temperature of *ca.* 50°C in order to react within 20 min.

The solution was then filtered and the filtrate acidified with concentrated hydrochloric acid until the precipitation of the aroylated amino acid was complete. The solid material was isolated on a glass filter by vacuum filtration, dried and finally recrystallized from 50% ethanol, chloroform or ethyl acetate.

N-Benzoyl-D,L-alanine, -D-alanine and -L-alanine, N-benzoyl-D,L-alanine methyl ester, N-benzoyl-D,L-leucine, N-benzoyl-D,L-phenylalanine and N-benzoyl-D,L-valine were obtained from Sigma (St. Louis, MO, U.S.A.).

Determination of enantiomeric elution order

The elution order was established from consecutive injections of samples containing the racemic form unspiked and spiked with one enantiomer. The absence of peak overlap, caused by possible contaminants in the sample, was demonstrated by the constant peak-height ratio obtained on detection at different wavelengths.

Optical rotations were measured at 546 nm with a Perkin-Elmer Model 243 B photoelectric polarimeter using a 1-ml quartz microcell of 1 dm path length.

^{1}H NMR spectra were recorded with a JEOL Model FX 100 Fourier transform spectrometer. Acetone-d_6 and methanol-d_4 were used as solvents with TMS as the internal standard for the δ values given.

TABLE I

CAPACITY RATIOS (k') AND SEPARATION FACTORS (α) FOR THE D- AND L-FORMS OF A SERIES OF N-AROYL-D,L-AMINO ACID DERIVATIVES, Ar–CONH–CHR–COOR'

Buffer: (A) 0.05 *M* phosphate (pH 7.0); (B) 0.05 *M* phosphate + 5% 1-propanol (pH 8.1). All compounds were studied at the same concentration level, *viz.*, as *ca.* $5 \cdot 10^{-5}$ *M* solutions.

No.	*Compound*			k'		α	*Buffer*
	Ar	*R*	*R'*	*L-*	*D-*		
I	Ph	CH_3	H	0.5	1.8	3.6	A
II	Ph	$PhCH_2$	H	3.05	2.3	1.3	A
III	Ph	$HOCH_2$	H	0.36	0.56	1.56	A
IV	Ph	$(CH_3)_2CH$	H	2.5	2.5	1.0	A
V	Ph	$(CH_3)_2CHCH_2$	H	1.7	1.5	1.13	A
VI	2-C_4H_3S	CH_3	H	0.56	0.78	1.4	A
VII	2-$C_{10}H_7$	CH_3	H	7.1	3.5	2.0	B
VIII	2-$C_{10}H_7$	$PhCH_2$	H	2.95	9.32	3.17	B
IX	Ph	CH_3	CH_3	0.3	0.3	1.0	A

RESULTS

Resolution experiments

The series of compounds investigated with respect to optical resolution is shown in Table I. The structural variation in the N-aroyl substituent as well as the 2-substituent of the amino acid obviously has a great influence on both capacity ratios and resolution. In Table II are given some physical data used for the characterization of the more investigated compounds

The large variation in k' with the nature of the substituents implies that the BSA-silica can be used for the separation not only of enantiomeric forms but also of differently substituted amino acid derivatives. This is well illustrated by Fig. 1, which shows, in one chromatogram, the separation of a mixture of N-benzoyl-D,L-alanine and N-benzoyl-D,L-phenylalanine into the four possible optically active species.

Effects of changing the pH and buffer strength of the mobile phase

Fig. 2 shows the influence of the mobile phase composition on the k' values for two of the compounds investigated. Although the k' values are much larger for II than I the same general trend is evident, *viz.*, an increase in k' of both enantiomers with decreasing pH and with decreasing buffer strength. This general behaviour was used in order to decrease the retention of the naphthoyl derivatives which could not be eluted from the column at pH 5.7 within a reasonable time. In these instances the pH of the mobile phase was changed to 8.12 and 5% of 1-propanol was added[3] in order to decrease the retention further.

The α values, however, did not follow any general trend, *i.e.*, no regular change with increasing k' values was observed. For benzoyl-D,L-alanine, however, the α value increased with decreasing k' and a change from 2.0 to 3.7 was found on changing the pH of the mobile phase from 5.0 to 8.0.

Observations indicating a stereoselective biodegradation of N-benzoyl-D,L-alanine

It was found that the dilute solutions of N-benzoyl-D,L-alanine used in this investigation were not stable. If samples from a solution were analysed over a 24-h

TABLE II

MELTING POINTS, SPECIFIC ROTATIONS AND ^1H NUCLEAR MAGNETIC RESONANCE DATA OF SOME OF THE N-AROYLAMINO ACIDS INVESTIGATED

M.p.s given within parentheses are those given in Beilstein's Handbook on organic compounds.

Compound No.	*Enantiomers*	*M.p. (°C)*	$[\alpha]_{546}^{20} \cdot (C_2H_5OH)$	*δ ppm (J, Hz)*
I	D,L-	159–160 (166)		7.90–7.80, m, 2H; 7.50–7.42, m, 3H; 4.60, q (*J* 7.3), 1H; 1.51, d (*J* 7.3), 3H
	L-	141–142 (150–151)	−11.9, *c* 1.3	
II	D,L-	179–181 (187–188)		7.88–7.78, m, 3H; 7.48–7.23, m, 7H; 4.93, m (J_1 9.0, J_2 5.2), 1H;
	L-	183–185	−15.1, *c* 1.0	3.27, m (J_1 9.0, J_2 5.2, J_3 −13.9), 2H
III	D,L-	161–162 (167–169)		7.93–7.83, m, 2H; 7.57–7.40, m, 3H; 4.72, t (*J* 4.6), 1H; 4.00, d (*J* 4.6), 2H
	L-	146.5-147	+48.5, *c* 0.27	
VI	D,L-	182.5–183		7.69, m, 2H; 7.12, m, 1H; 4.56, q (*J* 7.6),
	L-	156–157	+15.7, *c* 0.38	1H; 1.50, d (*J* 7.6), 3H
VII	D,L-	209.5–210.5		8.35, s, 1H; 7.86, m, 4H; 7.61–7.44, m,
	D-	212.5–213	−44.0, *c* 0.30	2H; 4.64, q (*J* 7.6), 1H; 1.56, d (*J* 7.6), 3H
VIII	D,L-	179–181		8.44, s, 1H; 7.93, m, 4H; 7.70–7.53, m,
	D-	152.5–154	−62.9, *c* 0.23	2H; 7.53–7.21, m, 5H; 5.04, m (J_1 9.2, J_2 5.4), 1H; 3.31, m (J_1 9.2, J_2 5.4, J_3 −13.7), 2H

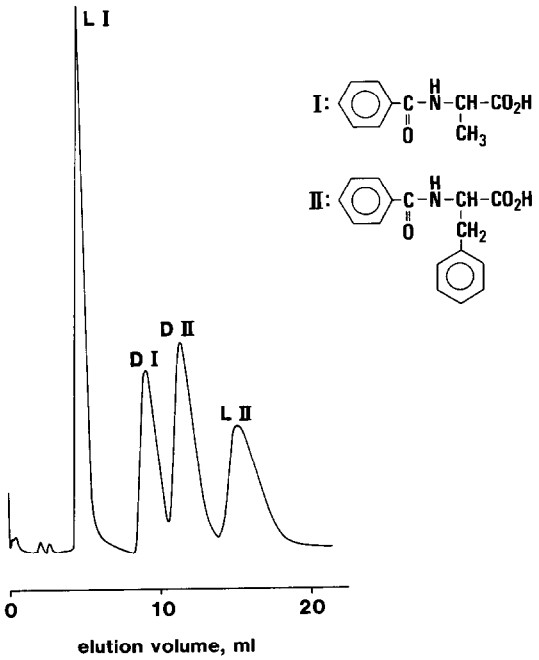

Fig. 1. Chromatograms resulting from an injection of 10 μl of a mixture of N-benzoyl-D,L-alanine ($8.3 \cdot 10^{-5}$ *M*) and N-benzoyl-D,L-phenylalanine ($9.8 \cdot 10^{-5}$ *M*). A 0.05 *M* phosphate buffer (pH 5.70) was used as the mobile phase at 1.0 ml/min. UV detection (235 nm, 0.02 a.u.f.s.).

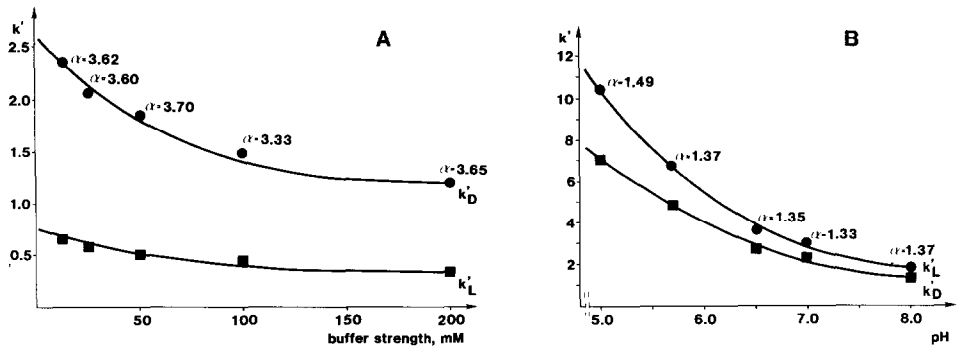

Fig. 2. Illustration of the variation of capacity ratios, k', and enantiomeric separation factors, α, with mobile phase composition. (A) N-Benzoyl-D,L-alanine, effect of buffer strength; (B) N-benzoyl-D,L-phenylalanine, effect of pH.

period, the two peaks from the enantiomers decreased significantly with time and, more interestingly, the first, corresponding to the L-enantiomer, decreased much more rapidly, as shown in Fig. 3. This phenomenon might be interpreted as an enzymatic degradation with a preference for the L-form and caused by microorganisms under aerobic conditions. Although these results are preliminary, they suggest an interesting area for the application of this type of enantioselective liquid chromatography.

DISCUSSION

There are two parameters obtained from the chromatographic data that are important to analyse in detail for an insight into the retention process. The first is k', which is directly proportional to the equilibrium constant for the solute–stationary phase interaction in a given chromatographic system[4]. Thus, the greater is k' the stronger are the forces involved in the binding of the solute to the stationary phase, in this instance to the protein. These forces are certainly of different types and accordingly influenced in different ways by changes in the chromatographic conditions, such as the properties of the mobile phase. The findings that an increase in the number of

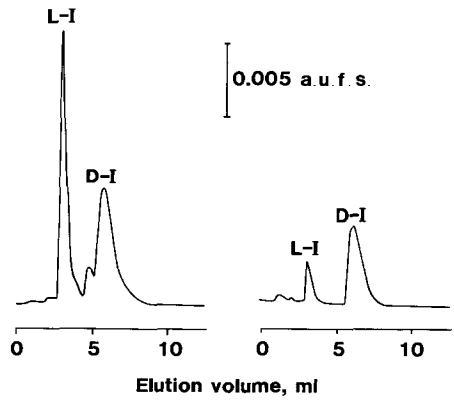

Fig. 3. Chromatograms of a $9.8 \cdot 10^{-5}$ M solution of N-benzoyl-D,L-alanine in 0.05 M phosphate buffer (pH 7.0); a freshly prepared solution (left) and the same solution after standing for 19 h at room temperature (right). A 0.05 M phosphate buffer (pH 7.0) was also used as the mobile phase at 1.0 ml/min. UV detection (235 nm, 0.02 a.u.f.s.). The small middle peak in the left chromatogram originates from an impurity present in the substrate.

aromatic rings in the molecular structure of the solute will increase k' rather drastically and that a reduction in k' occurs with a decrease in the surface tension of the mobile phase are both indications of one type of binding process that is based on hydrophobic interaction. On the other hand, the effect of the buffer strength seems to be the opposite of that which would be expected and also found experimentally in "pure" hydrophobic interaction chromatography[5]. This indicates a second type of binding process based on Coulombic interactions, *i.e.*, an ion-exchange process. One should also expect hydrogen bonding, which may be of great importance in non-polar, organic mobile phases[6], to contribute to only a minor extent in chromatography where aqueous buffers are used.

The second parameter, the enantiomeric separation factor, α, is directly related to the degree of enantioselectivity exerted by the stationary phase[3]. Because α is defined as k'_2/k'_1, where k'_1 and k'_2 denote the capacity ratios of the first and last eluted enantiomer, respectively, and k' by definition is directly proportional to the equilibrium constant, it follows that α is related to the difference in free energy change associated with the chiral recognition phenomenon by the expression

$$\Delta\Delta G = RT \ln \alpha$$

Thus, the enantioselectivity in the interaction between the immobilized BSA and N-benzoyl-D,L-alanine under the mobile phase conditions A in Table I can therefore be expressed in energy units and is calculated to be $\Delta\Delta G = 3.16$ kJ/mol.

Because the efficiency of the BSA-silica column is relatively high, *i.e.*, of the order HETP = 0.7 mm as calculated for the least retained enantiomer, a small α value will be sufficient for baseline separation. For the column used in this study, this limit may be calculated to be $\alpha = 1.4$, which corresponds to a $\Delta\Delta G$ value of only 0.83 kJ/mol.

The data in Table I are fully consistent with the assumption that the carboxyl group is involved in one of the bonding interactions with the stationary phase necessary within the framework of a "three-point interaction" view of chiral recognition[7]. This is perhaps not surprising because it is well known that fatty acids are bound to and transported by serum albumins[8]. It is also evident, however, that one strongly bonding interaction, giving high retention, is generally not favourable for an effective chiral discrimination.

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