Journal of Chromatography, 316 (1984) 617–624
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 480

DIRECT LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS ON IMMOBILIZED PROTEIN STATIONARY PHASES

IV*. MOLECULAR INTERACTION FORCES AND RETENTION BEHAVIOUR IN CHROMATOGRAPHY ON BOVINE SERUM ALBUMIN AS A STATIONARY PHASE

STIG ALLENMARK*,**, BJÖRN BOMGREN and HANS BORÉN
IFM/Department of Chemistry, Linköping University, S-58183 Linköping (Sweden)

SUMMARY

Chromatography with the use of immobilized bovine serum albumin as a stationary phase and aqueous buffer systems as eluents has proved to be a highly selective method, capable of separating structurally very closely related compounds. Retention can be effectively regulated by changes in at least three independent parameters of the mobile phase, which may be used for an optimization of separation factors. Particularly, the enantioselective properties of the chiral stationary phase have been demonstrated to be useful for the analytical resolution of a variety of racemates into enantiomers. From the variation of the retention behaviour with substituent effects, as well as the mobile phase composition, some indications regarding the molecular interaction forces regulating the substrate–protein equilibria have been obtained.

INTRODUCTION

Studies of the stereodifferentiating properties of proteins with respect to low-molecular-weight compounds have been performed by a variety of techniques for the determination of protein–ligand equilibria[1,2]. A logical extension of the method of gel filtration according to Hummel and Dreyer[3] is the use of protein immobilized on a suitable support, such as agarose, a technique introduced in 1973 by Stewart and Doherty[4] and later used by Lagercrantz *et al.* for studies of drug binding to albumins[5,6]. In a series of papers from our group, investigations on the general use of this reversed-affinity chromatographic principle for the direct separation of enantiomers have been reported[7–11]. Successful binding of albumin to a microparticulate silica support produced an affinity-chromatographic sorbent fulfilling the require-

* For Part III, see *J. Chromatogr.*, 264 (1983) 63.

** Present address: Laboratory of Microbiological Chemistry, Institute of Medical Microbiology, University of Gothenburg, S-41346 Gothenburg, Sweden.

0021-9673/84/$03.00

ments of fast chiral high-performance liquid chromatographic (HPLC) separations[10–12]. This paper describes an investigation of the behaviour of different solutes on immobilized bovine serum albumin (BSA) under various mobile phase conditions, and its implications with respect to the possible molecular interaction forces responsible for chiral recognition.

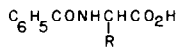
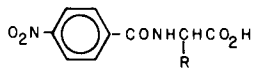
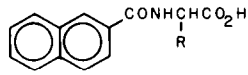
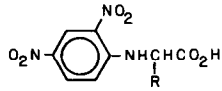
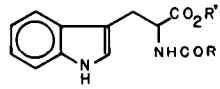
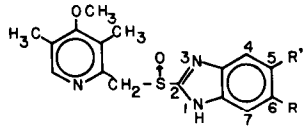
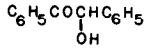
EXPERIMENTAL

The chromatographic set-up was the same as described previously[10]. An electronic integrator (Hewlett-Packard Model 3390A), interfaced with the UV detector, was used for the determination of peak areas and exact elution times. All chromatographic experiments were performed under isocratic conditions.

The compounds investigated (Table I) either were obtained commercially or had been synthesized in our laboratory[10] or by AB Hässle Pharmaceutical Company,

TABLE I

RACEMIC COMPOUNDS STUDIED WITH RESPECT TO OPTICAL RESOLUTION ON BSA-SILICA (RESOLVOSIL) COLUMNS

No.	*Compound*	*Substituent*	
I		R = CH_2OH	(Ia)
		CH_3	(Ib)
		$CH_2C_6H_5$	(Ic)
II		R = CH_2OH	(IIa)
		CH_3	(IIb)
III		R = CH_3	(IIIb)
		$CH_2C_6H_5$	(IIIc)
IV		R = CH_2CO_2H	(IVa)
		$CH_2CH_2CO_2H$	(IVb)
V		R = R′ = H	(Va)
		R = CH_3 R′ = H	(Vb)
		R = CH_3 R′ = C_2H_5	(Vc)
VI		R = H, R′ = Cl	(VIa)
		R = H, R′ = Br	(VIb)
		R = H, R′ = CH_3	(VIc)
		R = H, R′ = $(CH_3)_2CH$	(VId)
		R = H, R′ = $(CH_3)_3C$	(VIe)
		R = CH_3OCO, R′ = CH_3	(VIf)
VII			

Mölndal, Sweden[11]. They were all of a purity sufficient for the purposes of the investigation.

RESULTS

Retention behaviour as a function of molecular structure

Studies of substituent effects on molecular retention show that BSA as a stationary phase acts on a series of structurally similar compounds, partly by an effect of hydrophobic interaction in much the same way as a reversed-phase chromatographic material. This phenomenon is illustrated by Fig. 1, which shows, apart from the resolution of the racemic compounds Ia–c, an order of retention that parallels their hydrophobicity. The same type of effect is found on increasing the hydrophobicity of the aroyl substituent, *i.e.* on changing a benzoyl- to a 2-naphthoyl group. Thus, Compounds III are much more retained than I[10] and will require the presence of 1-propanol in the mobile phase for desorption.

However, optical resolution necessitates the operation of yet another type of bonding interaction between the stationary phase and the solute, according to the "three-point mode" of interaction proposed by Dalgliesh[13]. We find that polar groups, such as ester and free carboxyl groups, may interact strongly with BSA, thus enhancing the overall affinity. Fig. 2 shows the different behaviour of the sulphoxides VIc and VIf, from which the large effect of the methoxycarbonyl group (R, Table I) in the 6-position of VIf on the chromatographic retention is evident.

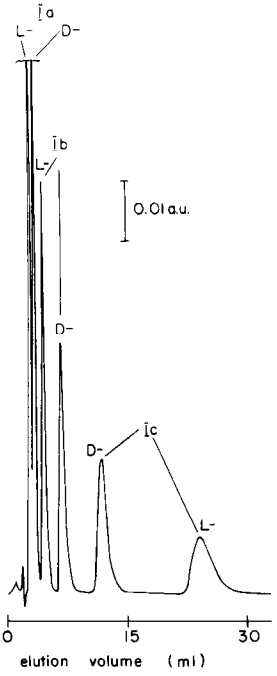


Fig. 1. Optical resolution of the N-benzoyl-D,L-amino acids Ia–c (all 0.3 m*M*, 10 μl of each injected); 50 m*M* phosphate buffer (pH 7.1) +5% 1-propanol; flow-rate, 1.5 ml/min. UV detection (225 nm, 0.1 a.u.f.s.).

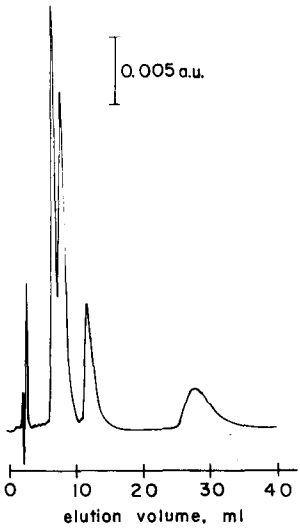


Fig. 2. Optical resolution of the sulphoxides (*cf.* the formula given in Table I) VIc (R = H, R′ = CH_3) and VIf (R = CH_3OCO, R′ = CH_3) (all 0.1 m*M*, 20 μl of each injected); 40 m*M* phosphate buffer (pH 6.6) + 3% 1-propanol; flow-rate, 1.0 ml/min. UV detection (292 nm, 0.05 a.u.f.s.). VIc eluted prior to VIf; elution order within each enantiomeric pair unknown.

Similarly, steric effects will greatly affect the difference in affinity of the two enantiomers for the protein, which will influence the α-value. Thus, α has been found to increase progressively in the series VIc–e, as well as from R = Cl (VIa) to R = Br (VIb). Generally, however, it is not possible to make any detailed predictions regarding the retention behaviour of a racemic solute owing to the great complexity of the interactions involved.

Fig. 3 shows the enantiomeric pairs of N-formyl- (Va) and N-acetyl-D,L-tryptophan (Vb) in a mixture, where the remarkable retention of L-Va is obvious. A similar phenomenon is obtained on the introduction of a *p*-nitrosubstituent in Ib. The two enantiomers of IIb show very different affinities for the immobilized BSA under the conditions used, giving an α-value of 17 in 50 m*M* phosphate buffer (pH

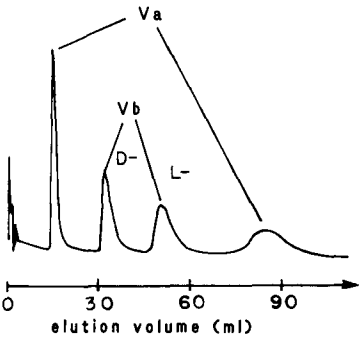


Fig. 3. Chromatogram showing the large difference in α value between N-formyl-(Va) and N-acetyl-D,L-tryptophan (Vb) (all 0.1 m*M*, 20 μl of each injected); 50 m*M* phosphate buffer (pH 6.0) + 2% 1-propanol; flow-rate, 1.0 ml/min. UV detection (225 nm, 0.1 a.u.f.s.).

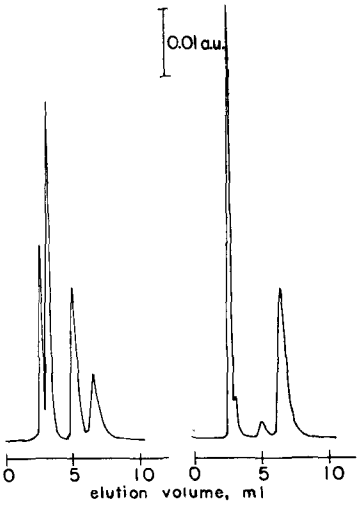


Fig. 4. Chromatogram showing the effect of a *p*-nitro substiuent in N-benzoyl-D,L-alanine. A mixture of N-benzoyl-D,L-alanine (Ib) and N-(*p*-nitrobenzoyl)-D,L-alanine (IIb) recorded (all 0.1 m*M*, 20 µl of each injected); 40 m*M* phosphate buffer (pH 6.6) + 3% 1-propanol; flow-rate, 1.0 ml/min. UV detection [225 nm (left) and 269 nm (right), 0.05 a.u.f.s.]. Elution order (left to right): L-IIb, L-Ib, D-Ib, D-IIb.

8.5). In Fig. 4, the optical resolutions of Ib and IIb are shown. Possibly, some degree of charge-transfer interaction between the nitrophenyl and the tryptophan ring systems in the solute and protein, respectively, may account for the effect of the nitro-substitution.

Table II gives a summary of representative retention data (k' and α) in the optical resolution of Compounds I–VII.

Retention behaviour as a function of mobile phase parameters

The effect of three independent mobile phase parameters, *viz.* pH, ionic strength, and 1-propanol content, upon the k' and α-values of compounds Ia–c was investigated, and the results are shown in Fig. 5. The pH study demonstrates that all k' values increase rapidly with decreasing pH and that the elution order is maintained throughout the pH range investigated. Upon variation of the 1-propanol content, all k' values increase with decreasing amount of co-solvent with maintainence of the elution order. However, the effect of the buffer concentration appears to be more complex. Here, a minimum in k' is reached, as clearly shown for both enantiomers of compound Ic.

DISCUSSION

With no exceptions found so far with respect to solute retention behavior, the effect of adding 1-propanol to the mobile phase is a decrease in all k' values, indicating the importance of hydrophobic interaction in this type of chromatography. This phenomenon agrees well with other data found for BSA by different methods, such as aqueous two-phase partition[14] or hydrophobic-interaction chromatography[15,16], all of which support the overall hydrophobic character of this protein[17].

TABLE II

ENANTIOSELECTIVITY OBTAINED ON HPLC OF COMPOUNDS I–VII

Compound no.	*k' (enantiomer)*		*α*	*Mobile phase composition [conc.(M), pH, % 1-propanol]*
Ia	1.3 (L)	2.4 (D)	1.9	Phosphate (0.05, 6.5, 1)
Ib	3.6 (L)	9.9 (D)	2.7	Phosphate (0.05, 6.5, 1)
Ic	15.1 (D)	28.3 (L)	1.9	Phosphate (0.05, 6.5, 1)
IIa	0.45 (L)	2.6 (D)	5.7	Phosphate (0.05, 5.7, 0)
IIb	0.5 (L)	4.9 (D)	9.8	Borate (0.05, 8.0, 0)
IIIb	3.5 (L)	7.1 (D)	2.0	Phosphate (0.05, 8.1, 5)
IIIc	2.95 (L)	9.3 (D)	3.2	Phosphate (0.05, 8.1, 5)
IVa	0.8	2.5	3.1	Phosphate (0.04, 8.1, 2.5)
IVb	0.75	2.2	2.9	Phosphate (0.04, 8.1, 2.5)
Va	1.7 (D)	8.45 (L)	4.9	Phosphate (0.05, 8.15, 3)
Vb	2.95 (D)	6.20 (L)	2.10	Phosphate (0.05, 8.15, 3)
Vc	2.5 (D)	12.7 (L)	5.15	Phosphate (0.05, 6.0, 3)
VIa	12.0	27.0	2.2	Phosphate (0.05, 6.1, 2)
VIb	21.7	58.1	2.7	Phosphate (0.05, 6.1, 2)
VIc	5.3	18.9	3.5	Phosphate (0.08, 5.8, 0)
VId	7.4	48.0	6.5	Phosphate (0.05, 6.1, 2)
VIe	10.6	79.0	7.5	Phosphate (0.05, 6.1, 2)
VIf	19.1	52.6	2.7	Phosphate (0.05, 7.5, 5)
VII	1.7	3.4	2.0	Phosphate (0.05, 8.15, 3)

The effect of pH is also largely understandable. With decreasing pH, the negative net charge of BSA will also decrease numerically. This means that the Coulomb interaction between amino acids and the stationary phase will decrease and result in lower k' values. However, carboxylic acids having no positive charge carrier in the molecule, such as N-acyl- or -aroyl-amino acids and other uncharged carboxylic acids, will experience the opposite effect, giving higher k' values. The effect of pH on non-protolytic solutes such as Vc or VII (Table I) has not yet been fully investigated.

The results from a study of the effect of ionic strength on the retention of compounds Ia–c imply that, at a certain buffer strength, there exists a minimal affinity to BSA. We interprete these data as a net effect of the buffer strength on the two main types of binding forces. The rapid decrease in k' with the buffer strength up to *ca.* 100 m*M* should mainly reflect the decrease in Coulomb attraction, whereas the increase above *ca.* 200 m*M* should be due to the increasing role of binding by means of hydrophobic interaction[18]. This probably represents an oversimplified picture; it is, however, consistent with all our available data. It is also supported by the current theory of affinant–sorbent bonding in affinity chromatography[19], and by some experimental results concerning the influence of the ionic strength on affinity-chromatographic desorption processes[20].

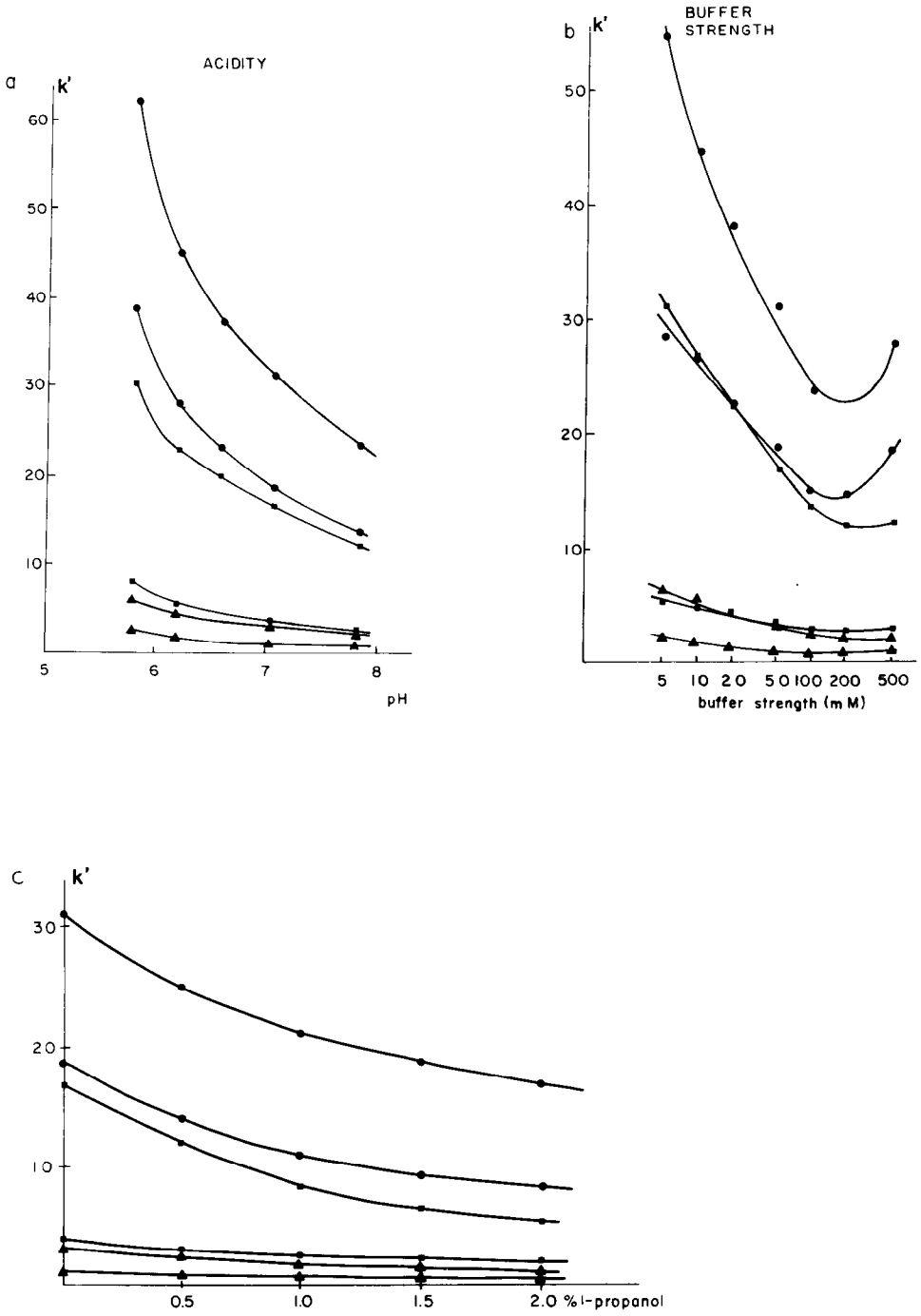


Fig. 5. The effect of (a) pH, (b) buffer strength and (c) organic solvent modifier (1-propanol) on capacity ratios (k') and enantiomeric separation factors (α) of the N-benzoyl-D,L-amino acids Ia–c. The two enantiomers of each compound are represented by the same symbol: Ia (▲), Ib (■), Ic (●).

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Natural Science Research Council and the Swedish Board for Technical Development.

REFERENCES

1 C. F. Chignell, in G. D. Fasman (Editor), *Handbook of Biochemistry and Molecular Biology, Proteins*, Vol. II, CRC Press, Cleveland 1976, 3rd ed., p. 554.
2 T. Alebic-Kolbah, S. Rendic, Z. Fuchs, V. Sunjic and F. Kajfez, *Acta Pharm. Jugoslav.*, 29 (1979) 53.
3 J. P. Hummel and W. J. Dreyer, *Biochem. Biophys. Acta*, 63 (1962) 530.
4 K. K. Stewart and R. F. Doherty, *Proc. Nat. Acad. Sci. U.S.*, 70 (1973) 2850.
5 C. Lagercrantz, T. Larsson and H. Karlsson, *Anal. Biochem.*, 99 (1979) 352.
6 C. Lagercrantz, T. Larsson and I. Denfors, *Comp. Biochem. Physiol.*, 69C (1981) 375.
7 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 237 (1982) 473.
8 S. Allenmark, *Chem. Ser.*, 2 (1982) 5.
9 S. Allenmark and B. Bomgren, *J. Chromatogr.*, 252 (1982) 297.
10 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 264 (1983) 63.
11 S. Allenmark, B. Bomgren, H. Borén and P.-O. Lagerström, *Anal. Biochem.*, 136 (1984) 293.
12 S. Allenmark, B. Bomgren and S. Andersson, *Prep. Biochem.*, 14 (1984) 139.
13 C. Dalgliesh, *J. Chem. Soc.*, (1952) 137.
14 E. Keshavarz and S. Nakai, *Biochim. Biophys. Acta*, 576 (1979) 269.
15 B. H. J. Hofstee, *Anal. Biochem.*, 52 (1973) 430.
16 A. H. Nishikawa and P. Bailon, *Anal. Biochem.*, 68 (1975) 274.
17 R. H. McMenamy, in V. M. Rosenoer, M. Oratz and M. A. Rothschild, *Albumin Structure, Function and Uses*, Pergamon Press, Oxford, 1977, p. 143.
18 S. Hjertén, *Methods Biochem. Anal.*, 27 (1981) 89.
19 J. Turkova, *Affinity Chromatography*, Elsevier, Amsterdam, 1978, p. 51.
20 R. M. Morrow, R. G. Carbonell and B. J. McCoy, *Biotechnol. Bioeng.*, 17 (1975) 895.