Journal of Chromatography, 447 (1988) 297–303
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 589

MICRO-LIQUID CHROMATOGRAPHY AND THE CHIRAL RECOGNITION MECHANISM ON ALBUMIN-COATED SILICA GEL

LARGE SELECTIVITY CHANGES WITH SAMPLE SIZE

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(First received March 31st, 1988; revised manuscript received April 25th, 1988)

SUMMARY

The suitability of micro-liquid chromatography (micro-LC) (liquid chromatography on packed fused-silica capillary columns) for enantiomer separations was demonstrated for bovine serum albumin (BSA)-coated silica gel. Micro-LC has the great advantage of needing only very small amounts of possibly very expensive chiral stationary phases. The elution order for a series of N-nitroaroylamino acids on BSA-coated silica gel was determined. Column overload effects on BSA-coated silica gel occur at much lower concentrations than on other stationary phases. These overload effects result in strange retention time shifts whereby the later eluting peak is mostly affected. The resulting large selectivity change with sample size is unusual in LC. An explanation is attempted.

INTRODUCTION

One of the disadvantages of direct enantiomer separation by liquid chromatography (LC) on chiral stationary phases (CSPs) is that, with the current "state-of-the-art", it is difficult to predict which one of the several available phases should be used to solve a particular problem. Although with some phases, such as the Pirkle-type[1] or cyclodextrin phases[2], an *a posteriori* rationalization is possible, this is certainly not yet so for protein-based phases as introduced by Hermansson[3] and Allenmark *et al.*[4]. The enantioselectivity observed for a series of N-nitroaroylamino acids suggests that both electronic and steric conditions determine the elution order.

For such studies the miniaturization of LC, resulting in a need for smaller amounts of stationary and mobile phases, is particularly attractive. In this study micro-LC was examined[5–7].

EXPERIMENTAL

Chromatography was performed on a Varian (Walnut Creek, CA, U.S.A.) Model 5000 chromatograph equipped with a split-flow system and a 60-nl Valco

0021-9673/88/$03.50

(Houston, TX, U.S.A.) sample loop injector. A Varian 2050 variable-wavelength detector was modified for micro-LC detection as described previously[8].

Columns were constructed of 320-μm I.D. fused-silica capillaries (RSL/Altech Europe, Eke, Belgium) and packed with wide-pore 5-μm RoSil (a spherical silica gel from RSL/Altech Europe) with a mean pore size of 25 nm and a specific surface area of 50 m^2/g (data from the supplier). After slurry packing with carbon tetrachloride, followed by *n*-hexane at 500 bar, the columns were tested [typically 40 000 plates/m with dimethyl phthalate; *n*-hexane–isopropanol (95:5) at 3 μl/min], rinsed with methanol and water, equilibrated with 0.05 *M* phosphate buffer (pH 5.0) and coated with bovine serum albumin (BSA)[9] using a conventional Valco sample loop injector with a 1-ml loop. Before coating, the columns were painted black to avoid photodecomposition of the adsorbed protein.

All analyses were performed with 0.05 *M* phosphate buffer (pH 7.0) with 2% isopropanol as the eluent at flow-rates of 2.5–3 μl/min.

Analytes (N-benzoyl-, N-*p*-nitrobenzoyl-, N-*m*-nitrobenzoyl- and N-dinitrobenzoylamino acids) were prepared by esterification of commercial amino acids (DL- and L-leucine, DL- and L-alanine, DL- and L-aminobutyric acid) with methanol–thionyl chloride. The methyl esters were derivatized with various nitrobenzoyl chlorides (in acetonitrile at 65–70°C for 2 h in the presence of dimethylaminopyridine). The benzoylated esters were hydrolysed with 0.1 *M* hydrochloric acid and recrystallized from hot water.

RESULTS AND DISCUSSION

In situ stationary phase modification of micro-LC columns

One of the advantages of micro-LC[5–7] is the reduced amount of packing material needed for the column and, consequently, the reduced amount of reagent needed to modify the stationary phase by coating or derivatization. Coating a 15-cm micro-column with BSA required only a few milligrams of protein, as opposed to the several tens of milligrams needed to coat a shorter, conventional column. This approach could be especially interesting for use with expensive proteins or when refined fragments of proteins[10] are used for retention mechanism studies.

Micro-LC as optimized in this laboratory uses inner wall-coated fused-silica capillaries. The inner wall elastic polymer coatings holds the stationary phase in position, which is a very important point for column efficiency and prolonged column stability[11]. In this work with an *in situ* BSA coating, an adverse effect of the presence of an inner wall coating was observed. The results were much better without the inner wall polymer coating. Our interpretation is that, during switching from the apolar solvent used for packing and testing the columns to the polar buffer used for coating and analysis, too large differences in swelling of the inner wall coating results in rupture of the packing and, consequently, in a poor BSA coating and poor chromatography.

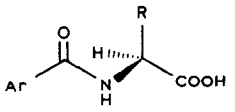
Elution order of some N-nitroaroylamino acids

Elution orders were obtained by comparing the chromatograms of the racemates with those of the corresponding pure L-enantiomers. As can be seen from Table I, the elution order is determined by both the nitroaroyl moiety and the size

TABLE I

ELUTION ORDERS OF N-NITROAROYL-SUBSTITUTED AMINO ACIDS

Eluent: 0.05 *M* phosphate buffer (pH 7.0) + 2% isopropanol.



Ar	*I* (*R* = CH_3)	*II* (*R* = $CH_2CH_2CH_3$)	*III* [*R* = $CH_2CH(CH_3)_2$]
(a) Benzoyl	L < D	D < L	D < L
(b) *m*-Nitrobenzoyl	L < D	D < L	D < L
(c) *p*-Nitrobenzoyl	L < D	L < D	D < L
(d) 3,5-Dinitrobenzoyl	L < D	L < D	L < D

of the alkyl side-chain in the amino acid. A low electron density in the aroyl substituent seems to favour retention of the D-enantiomer whereas a bulkier side-chain favours retention of the L-enantiomer.

It has been argued[12] that enantiomer separations on albumin-based phases are governed by hydrophobic interactions, although the role of polar interactions is recognized. Certainly the situation is complex, as is shown by the elution order reversals in Table I. The series that we investigated suggests that hydrophobic interactions may be less important than is generally acknowledged: resolution increases in the alanine series (compound I in Table II) by introducing a 3,5-dinitrobenzoyl group, the L-enantiomers eluting first. This is in agreement with the elution order

TABLE II

CAPACITY FACTORS AND SELECTIVITIES

Eluent and compound numbers as in Table I; 21 ng injected.

Compound	k'_1	k'_2	α
Ia	0.54	0.54	1.00*
Ib	0.68	0.68	1.00*
Ic	0.45	0.85	1.89
Id	0.68	2.86	4.22
IIa	0.66	1.26	0.53**
IIb	0.56	2.06	3.69**
IIc	0.69	2.17	3.13
IId	0.40	1.69	4.24
IIIa	0.87	2.23	2.58**
IIIb	0.95	1.46	1.54**
IIIc	1.55	1.89	1.22**
IIId	1.47	2.13	1.44

* The elution order could be established at lower concentration.
** Elution order reversal (D < L).

found by Allenmark and Andersson[13] for N-(2,4-dinitrophenyl)amino acids. On the other hand, they found that replacing the benzoyl group in Ia by the more electron-dense naphthoyl group caused an elution order reversal, the D-enantiomer eluting before the L-enantiomer[12].

Overload effects

The low capacity for preparative-scale applications of albumin-coated silica gel phases has been mentioned previously[9]. It is common to express sample loading as the amount of analyte injected onto a given amount of stationary phase. Although more fundamental expressions have been proposed[14], this is practical as a rule-of-thumb for daily chromatographic work. When comparing different stationary phases, some conditions are assumed: a fixed specific surface area, as determined by the silica gel, and a fixed adsorption site surface concentration, as determined by the nature of the adsorbing monolayer. With its molecular weight of 65 000, BSA as the active monolayer influences both parameters. The need for larger pores in the silica gel, to make the surface accessible[15], results in a lower specific surface area[16]. In proteins, as opposed to polymers such as cellulose, which are built from repetitive units, it can be assumed that the number of adsorption sites does not increase with increasing molecular weight, as these sites probably occur at a few specific, local geometric configurations. A single BSA molecule will cover a relatively large portion of the surface, but contributes only a single unit, or only very few units, to the number of adsorption sites. With these assumptions, the low capacity of protein phases is understandable.

Figs. 1 and 2 show overload effects, observed here as a decrease in the capacity factors of the enantiomers in a racemic mixture with increasing sample loading. On a conventional 15 × 0.46 cm I.D. column, holding 1–2 g of stationary phase, a 10-μl injection of a 0.1% solution would result in a column loading of 5–10 μg of compound per gram of silica gel. To obtain the same level of loading on a micro-LC column, holding 5–10 mg of stationary phase, only 25–50 ng of injected material are needed, which is arrived at by reducing the injection volume to 60 nl. On most high-capacity stationary phases, such as octadecylated silica gel, even a multiple of that loading level would still be regarded as analytical, *i.e.*, free from mass overload effects (linear chromatography). That this is not so with BSA-coated silica gel simply reflects the low capacity of this kind of phase.

More surprisingly, it is the retention time of the most retained enantiomer that is most drastically changed by higher concentrations (Fig. 2). This resembles the "tag-along" effect, recently described by Ghodbane and Guiochon[17]. However, this would require that the first eluting compound blocks adsorption sites. Being less adsorbed, it could only do so by virtue of a numerical predominance, a situation that is unlikely for a racemate.

Another explanation would be that, superimposed on a more general retention mechanism, similar for both enantiomers, enantioselectivity is obtained through adsorption sites that interact nearly uniquely with one enantiomer. Accordingly, the more retained enantiomer shows an overload behaviour, independent of the first, and without displacement. In turn, this effect could serve as a measure of the chiral specificity of the interactions. This specificity seems to contrast with the broad range of compounds resolvable on a single protein phase, but it is significant that serum

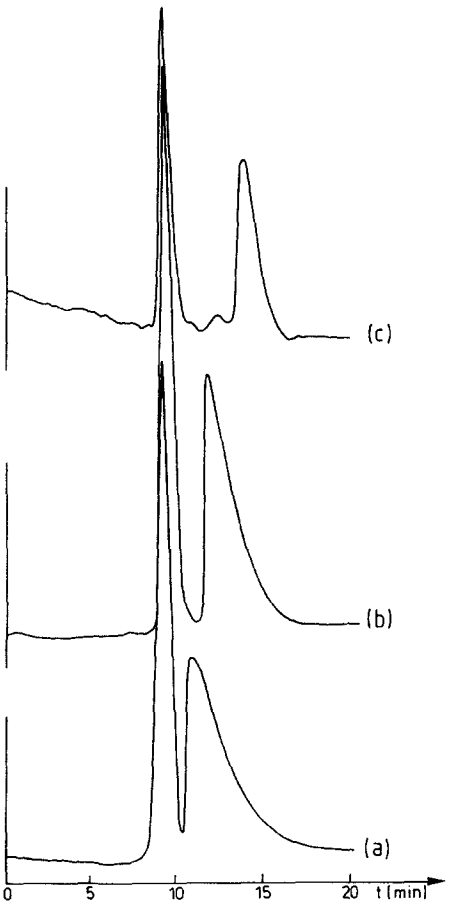


Fig. 1. Resolution of 3,5-dinitrobenzoylleucine on BSA-coated silica gel. Amounts injected: (a) 60; (b) 30; (c) 3.8 ng.

albumin is not an enzyme, with a unique substrate, but acts as a carrier of small molecules and is known to bind many ligands[18].

Apparently, our findings are not restricted to the type of compounds that we investigated, but apply also to other compounds such as DL-benzoin, as can be judged from Fig. 9 in ref. 9. Overloading was observed with relatively small samples (calculated to be below 12.5 μg per gram of silica gel), causing a decrease in resolution, but no comment was made on the unusual nature of the change in selectivity (α). The differential changes in capacity factors, discussed above, lead to changes in α that are dependent on sample size (Fig. 3). A similar case has been described for a preparative separation on a Pirkle phase[19], but in that study, covering a much larger sample load range (1/50 000), a 20% reduction in α also accompanied a 50% reduction in the capacity factor of the first-eluting enantiomer. In at least one instance (DL-3,5-dinitrobenzoylleucine), we found a 30% reduction in α, with only a negligible change in the capacity factor of the first-eluting enantiomer.

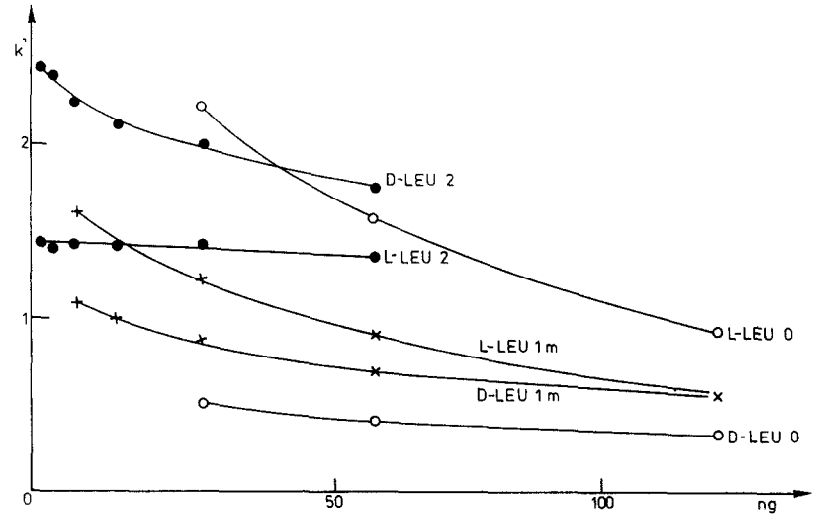


Fig. 2. Influence of amount injected on capacity factors. LEU 2 = 3,5-dinitrobenzoylleucine; LEU 1m = *m*-nitrobenzoylleucine; LEU 0 = benzoylleucine.

Given the large change in the capacity factor with the Pirkle phase, the change in α could be ascribed to subtle differences in the non-linear behaviour that occurs for both enantiomers. On BSA, however, the fact that the two enantiomers behave more independently suggests that at least one of the interactions is more chirally specific in comparison with the Pirkle phase.

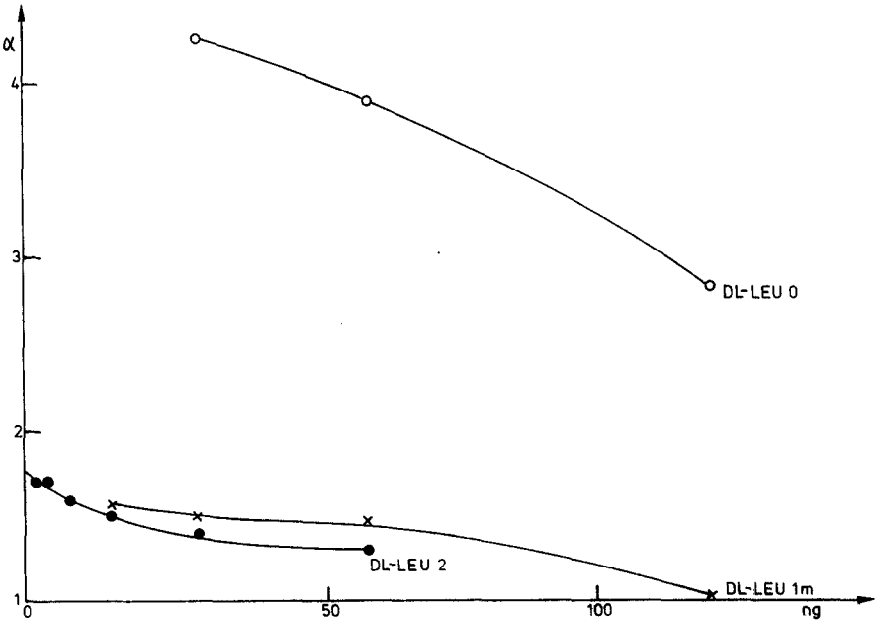


Fig. 3. Influence of amount injected on selectivity. Abbreviations as in Fig. 2.

Unsuspected overload effects at low sample loadings may help to explain why the resolution of some compounds varies with the origin or mode of preparation of the albumin phase[9], without having to assume structural changes in the albumin. Possibly, a quantitative description of overload effects could yield more information on the reversals in elution order described above, and would allow different adsorption sites of BSA, BSA fragments or proteins in general to be recognized. It should be obvious that micro-LC is best suited for such investigations.

ACKNOWLEDGEMENTS

We thank the Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw — IWONL, the Nationaal Fonds voor Wetenschappelijk Onderzoek — NFWO and the Ministerie voor Wetenschapsbeleid for financial support to the laboratory. We thank RSL-Alltech for the gift of a sample of wide-pore silica. We thank W. Pirkle for some of the leucine derivatives that were used in introductory experiments.

REFERENCES

1 W. H. Pirkle, T. C. Pochapsky, G. S. Mahler and R. E. Field, *J. Chromatogr.*, 348 (1985) 89.
2 W. Hinze, T. Riehl, D. Armstrong, W. DeMond, A. Alak and T. Ward, *Anal. Chem.*, 57 (1985) 237.
3 J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.
4 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 237 (1982) 473.
5 M. Verzele and C. Dewaele, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 280.
6 M. Verzele, M. De Weerdt, C. Dewaele, G. De Jong and N. Lammers, *LC · GC Mag. Liq. Gas Chromatogr.*, 4 (1986) 1162.
7 M. De Weerdt, C. Dewaele and M. Verzele, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 553.
8 J. Vindevogel, G. Schuddinck, C. Dewaele and M. Verzele, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 317.
9 P. Erlandsson, L. Hansson and R. Isaksson, *J. Chromatogr.*, 370 (1986) 475.
10 P. Erlandsson and S. Nilsson, paper presented at the *11th International Symposium on Column Liquid Chromatography, Amsterdam, 1987.*
11 M. Verzele, C. Dewaele, M. De Weerdt and S. Abbott, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, in press.
12 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 264 (1983) 63.
13 S. Allenmark and S. Andersson, *J. Chromatogr.*, 351 (1986) 231.
14 J. E. Eble, R. L. Grob, P. E. Antle and L. R. Snyder, *J. Chromatogr.*, 384 (1987) 25.
15 P. Larsson, M. Glad, L. Hansson, M. Mansson, S. Ohlson and K. Mosbach, *Adv. Chromatogr.*, 21 (1983) 41.
16 K. Unger, *Porous Silica*, Elsevier, Amsterdam, 1979, p. 15.
17 S. Ghodbane and G. Guiochon, *J. Chromatogr.*, in press.
18 C. Chignell, in G. Fasman (Editor), *Handbook of Biochemistry and Molecular Biology, Vol. II, Proteins*, CRC Press, Boca Raton, FL, 3rd ed., 1976, p. 554.
19 V. Meyer, *Chromatographia*, 24 (1987) 639.