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ENANTIOMER RESOLUTION OF D- AND L-α-AMINO ACID DERIVATIVES BY SUPERCRITICAL FLUID CHROMATOGRAPHY ON NOVEL CHIRAL DIAMIDE PHASES WITH CARBON DIOXIDE

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SUMMARY

The rapid resolution of racemic N-4-nitrobenzoylamino acid isopropyl esters was accomplished without the loss of enantioselectivity by supercritical fluid chromatography (SFC) on novel chiral valine–diamide phases with carbon dioxide and a polar methanol modifier. In each stationary phase, a chiral moiety was anchored to the silica gel surface by a long decamethylene spacer. The enantioselectivity in SFC was comparable to that in liquid chromatography using 2-propanol–*n*-hexane. The time required for analysis was less than 5 min, and the range of enantiomer resolution (R_s) was 10.8–1.25. On using 2-propanol in place of methanol the separation was improved, but was accompanied by a decrease in column efficiency. The end-capping effect of the remaining surface silanols on enantiomer resolution is discussed.

INTRODUCTION

The liquid chromatographic (LC) resolution of enantiomers offers an intriguing approach to the solution of various stereochemical problems and facilitates the assessment of different metabolic processes that occur between enantiomers in drugs[1,2]. Recent progress in LC, especially with chiral stationary phases (CSPs), clearly demonstrates the variety of applications of this approach. Supercritical fluid chromatography (SFC)[3] should find more general acceptance for more rapid resolution, considering the longer time required for the performance of LC than gas–liquid chromatography[4,5]. Using SFC, the rapid resolution of enantiomers on various kinds of CSPs can be carried out without loss of enantioselectivity, owing to the fast solute diffusion[6–9].

Mourier *et al.*[6] have demonstrated that when used in conjunction with various alcohols as polar modifiers, a super- and subcritical carbon dioxide mobile phase can be used to effect the rapid resolution of racemic phosphine oxide on a classical Pirkle stationary phase. They also discussed a mechanism for the retention of achiral solutes in SFC with carbon dioxide[7]. We have conducted experiments to accelerate the resolution of racemic N-acylated amino acid esters through use of a combination of

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carbon dioxide and a polar modifier (methanol, acetonitrile and diethyl ether) with a chiral diamide phase (N-formyl-L-valylamino)propylsilica[8]. Carbon dioxide, with a polarity close to that of *n*-hexane, is an efficient mobile phase component in which diastereomeric hydrogen-bond associations occur. The separations thus effected occur in less than 4 min. Differences in the action of polar modifiers in SFC and LC resolution were also examined[8].

Novel CSPs used in this study contain a chiral valine–diamide moiety, anchored to the silica gel surface by a long decamethylene spacer (Fig. 1). On the remaining surface silanols, two different CSPs were made; one is exhaustively trimethylsilylated so as to remove bare silanols (CSP 1) and the another is not trimethylsilylated (CSP 2). The long spacer provides good accessibility for solute enantiomers to the chiral moiety without interaction with the silica surface. The CSPs depend entirely on two amide functionalities for entrapping enantiomers through hydrogen bonds and for the outstanding capacity they demonstrate in separating a wide range of enantiomers in LC[10]. On using the CSPs in SFC with polar modifiers, such as methanol and 2-propanol, more rapid enantiomer analysis of amino acid derivatives was achieved while maintaining the high enantioselectivity under LC conditions. The effects due to surface modification with trimethylsilylation on enantiomer resolution and the successful application of SFC resolution are discussed below.

EXPERIMENTAL

SFC was carried out with a Jasco Super-100 SF chromatograph, equipped with a Multi-320 multichannel UV detector, as described previously[8]. The chiral columns (25 cm × 4.6 mm I.D.), each packed with CSP 1 and 2 (Fig. 1), were prepared according to a recently reported procedure[10]. Liquid carbon dioxide was pumped into the column at a flow-rate of 5 ml/min. This flow-rate was measured at −5°C and corresponded to a column inlet pressure of 260 bar. Methanol and 2-propanol were used as polar modifiers and were delivered into the liquid carbon dioxide flow at flow-rates ranging from 10 to 200 μl/min. The column temperature was maintained at 40°C in a column oven. The carbon dioxide density was controlled through regulation of the column outlet pressure from 96 to 200 bar. Mobile phase equilibration was completed within 10 min under these conditions. All other chromatographic conditions were the same as previously reported[8]. The samples to be resolved were also the same as those in previous work[11].

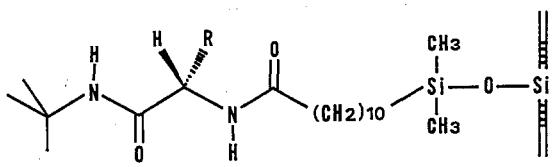

Fig. 1. Chiral diamide-bonded silica (R = isopropyl). The remaining surface silanols on CSP 1 were exhaustively trimethylsilylated. CSP 2, packing not trimethylsilylated.

RESULTS AND DISCUSSION

A series of racemic N-4-nitrobenzoylamino acid isopropyl esters were found to be completely resolved on CSP 1 with a carbon dioxide–methanol mixture, as shown in Table I. LC data obtained with a 2-propanol–*n*-hexane mixture are also included in Table I for comparison with those obtained by SFC. The enantioselectivity in SFC was comparable to that in LC. In the previous study, the enantioselectivity was found to be not so markedly diminished as that in LC when polar methanol was used as a modifier (LC with a possible methanol–*n*-hexane mixture virtually failed to provide any enantiomer resolution of the amino acid derivatives on the classical chiral diamide phase[8]). In LC, maximum enantiomer separability could be induced by separating the chiral moiety from the silica gel surface via a long spacer and by exhaustive trimethylsilylation of the remaining surface silanols[10,12]. Only in this way can diastereomeric hydrogen-bond association in the chiral moiety assume a significant role in determining the enantioselectivity and retentivity of a solute enantiomer[7]. Stated differently, solute retention is determined by the particular degree of hydrogen-bond

TABLE I

COMPARISON OF SFC AND LC WITH ALCOHOL MODIFIERS IN THE RESOLUTION OF RACEMIC N-4-NITROBENZOYLAMINO ACID ISOPROPYL ESTERS ON CSP 1

SFC conditions: liquid carbon dioxide delivery rate, 5 ml/min; column outlet pressure, 200 bar; column temperature, 40°C; methanol modifier delivery rate, 20 μl/min, except for the tryptophan derivative (100 μl/min); column, 25 × 0.46 cm I.D.; detection, UV at 255 nm. LC conditions: mobile phase, 1% (v/v) 2-propanol in *n*-hexane; column temperature, ambient (*ca.* 24°C); flow-rate, 1 ml/min; detection, UV at 254 nm.

Amino acid	*SFC (carbon dioxide–methanol)*				*LC (2-propanol–n-hexane)*		
	t_{RD}*	k'_D**	α***	R_s§	t_{RD}*	k'_D**	α***
Leu	2.69	1.99	2.32	10.75	8.32	1.15	2.69
Val	1.71	0.90	1.69	4.26	6.30	0.64	2.02
Ala	2.00	1.22	1.94	6.54	9.46	1.48	1.89
Phe	2.64	1.93	1.85	7.39	7.96	1.08	2.12
Ile	1.83	1.03	1.74	4.67	5.94	0.67	2.04
Asp	1.55	0.72	1.24	1.25	6.00	0.69	1.29
Glu	2.16	1.40	1.74	5.53	7.50	1.12	1.74
Ser	4.10	3.56	1.34	3.38	20.43	4.15	1.25
Thr	3.17	2.52	1.32	3.78	12.17	2.43	1.30
Tyr	5.37	4.97	1.83	8.02	12.22	2.45	1.80
Cys	5.31	4.90	1.37	4.14	15.40	3.33	1.29
Met	2.99	2.32	1.94	7.90	11.99	1.95	1.93
Trp	11.44	11.71	1.42	6.30	54.08	13.99	1.69

* t_{RD} = Retention time of the first-eluted D-enantiomer.

** k' = Capacity factor for SFC (calculated with nominal hold-up time, determined by the frontal solvent peak, which was a constant 0.89 min under the above SFC conditions).

*** α = separation factor (k' of the L-enantiomer/k' of the D-enantiomer).

§ R_s = resolution $[2\Delta T/(W_D + W_L)$, where Δt is the difference between t_{RD} and t_{RL} (retention time of the L-enantiomer) and W is the peak width in units of time measurement].

association and therefore, for certain cases of weak hydrogen-bonding solutes, it is difficult to vary the solute retention in response to changes in modifier concentration in LC. In SFC, however, the physical state of carbon dioxide can be adjusted so as to facilitate the retention of less polar solute enantiomers. In this separation study, the column temperature was decreased from 60 to 40°C and the rate of application of methanol modifier from 500 to 20 μl/min, in contrast to the conditions used in the previous SFC study[8]. The time required for enantiomer analysis was less than 5 min, except for the tryptophan derivative, which showed the highest retention of all the amino acid derivatives, being 11.44 min for the first eluted D-enantiomer, even at a methanol flow-rate of 100 μl/min in SFC. Fig. 2a illustrates a typical chromatogram of a racemic N-4-nitrobenzoylleucine isopropyl ester with a high resolution (R_s = 10.8), and Fig. 2b is a chromatogram obtained with a very short analysis time (less than 2 min). In all SFC experiments, the D-enantiomer was always eluted before the L-enantiomer, as in LC.

Mourier and co-workers[6,7] found that 2-propanol, being less polar than methanol, acts more effectively as a modifier in a supercritical carbon dioxide mobile phase, at least with respect to the magnitude of the separation factor. The use of 2-propanol instead of methanol on CSP 1 resulted in a greater separation factor. Resolution data for neutral amino acid derivatives with the 2-propanol modifier are listed in Table II. For instance, at a 2-propanol flow-rate of 40 μl/min, which gave essentially the same retention of the first cluted D-enantiomer as did methanol, the separation factor of the valine derivative was increased from 1.69 to 2.15. Such an effect appears reasonable considering the possibly more competitive association of the more polar methanol compared with the less polar 2-propanol for hydrogen bonds between the chiral moiety and solute enantiomers. With the polar methanol, the column efficiency was greater. The valine derivative indicated an HETP value of 0.165 mm for SFC under the above conditions and 0.076 mm on using the methanol modifier. The average HETP value for all amino acid derivatives resolved was 0.07 mm with the

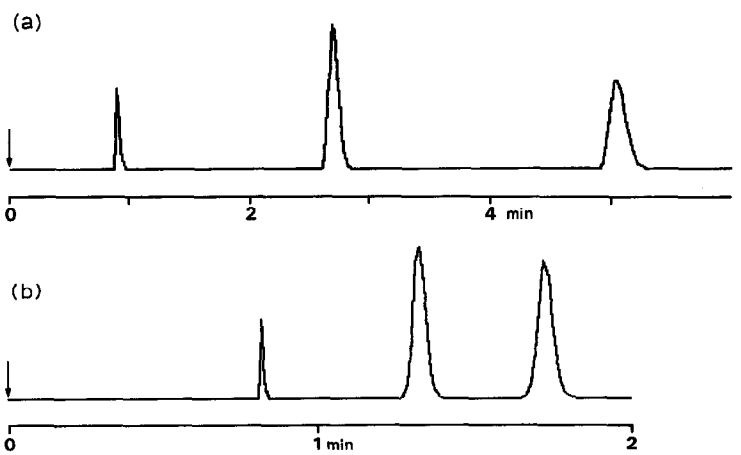

Fig. 2. Optical resolution of racemic N-4-nitrobenzoylleucine isopropyl ester with supercritical carbon dioxide containing methanol as the modifier. (a) SFC conditions as in Table I; (b) SFC on CSP 2 at a methanol flow-rate of 200 μl/min. Other conditions as in Table I.

TABLE II

SFC WITH 2-PROPANOL MODIFIER FOR RESOLUTION OF NEUTRAL N-4-NITROBENZOYLAMINO ACID ISOPROPYL ESTERS ON CSP 1

SFC conditions as in Table I, although the modifier and delivery rate are different.

Amino acid	*Modifier delivery rate (μl/min)*								
	40			*70*			*100*		
	t_{RD}	k'_D	α	t_{RD}	k'_D	α	t_{RD}	k'_D	α
Val	1.68	0.88	2.15	1.63	0.78	1.33	1.54	0.71	1.16
Ala	1.93	1.11	2.02	1.84	1.04	1.90	1.76	0.96	1.83
Phe	2.43	1.78	1.81	2.33	1.58	1.88	2.17	1.40	1.84
Leu	2.49	1.87	2.36	2.37	1.63	2.31	2.22	1.50	2.16

methanol modifier. It is therefore evident that the resolution of a series of enantiomers of the amino acid derivatives can be achieved with the carbon dioxide–methanol mixture. The enantiomer resolution (R_s) exceeded 3.4, except for the aspartic acid derivative (R_s = 1.25). The largest R_s, 10.8, was obtained with the leucine derivative.

Fig. 3 shows the influence of the 2-propanol content on the retention of the first eluted D-enantiomer of the (a) leucine and (b) alanine derivatives and on the separation factors of these derivatives. Comparison of trimethylsilylated CSP 1 with non-trimethylsilylated CSP 2 in Fig. 3 indicates that CSP 2 causes a greater increase in retention with decreasing 2-propanol content. In both instances a greater enantiomer retention initially led to a slight increase in the separation factor, and then a marked decrease ensued. In contrast, CSP 1 caused less change in the retention and separation factors. It is particularly significant that no marked decrease in the separation factor occurred in a region of lower 2-propanol content for CSP 1. With CSP 1, the supercritical mobile phase gave fairly symmetrical peaks, whereas CSP 2 gave a larger peak asymmetry factor, *i. e.*, a greater degree of peak tailing, with an increase in enantiomer retention. Peak asymmetry factors[13], as determined from the first eluted peak, are given in Fig. 3. The retention behaviour of the two enantiomeric derivatives with changing methanol content were similar to those observed with changing 2-propanol content, as can be seen from Fig. 4. The retention enhancement with a decrease in the modifier content was, however, smaller with methanol than with 2-propanol as the modifier. At methanol flow-rates exceeding 50 μl/min, either derivative provided greater separation factors on CSP 2 than on CSP 1. Fig. 2b shows rapid resolution of the leucine derivative on CSP 2 at a flow-rate of 200 μl/min.

The results of a previous LC study on CSP 1 clearly demonstrate that the separation factor becomes larger after trimethylsilylation of the remaining surface silanols of the CSPs[10,12]. However, under SFC conditions, the separation factor decreased rather than increased with trimethylsilylation in experiments with higher modifier contents. This tendency was more pronounced with the leucine than the alanine derivative when the 2-propanol modifier was used. As reported by Mourier *et al.*[7], the retention effect, as discussed in connection with non-polar–non-polar chromatography[14], may contribute at least to some extent to SFC when using the CSP discussed here. When conducting non-polar–non-polar chromatography with octade-

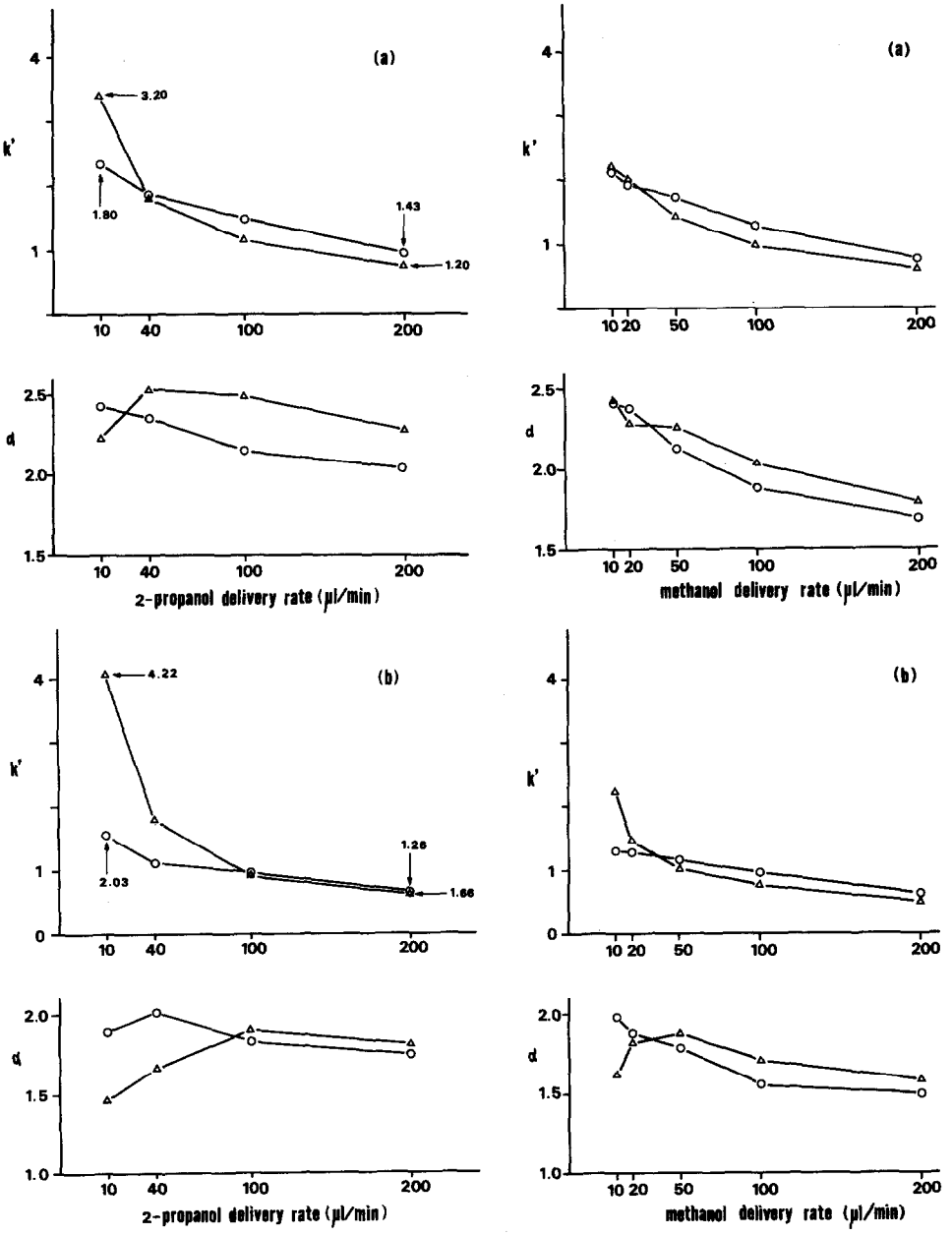

Fig. 3. Influence of 2-propanol delivery rate on k' of the first-eluted D-enantiomer of racemic (a) leucine and (b) alanine derivatives and on α. SFC conditions as in Table I, but with different modifier and delivery rate. ○, For CSP 1, trimethylsilylated packing; △, for CSP 2, packing not trimethylsilylated. Peak asymmetry factors (A_s), calculated from the first-eluted peak, are indicated by arrows.

Fig. 4. Influence of methanol delivery rate on k' of the first-eluted D-enantiomer of racemic (a) leucine and (b) alanine derivatives and on α. SFC conditions as in Table I, but with different delivery rate. ○, For CSP 1; △, for CSP 2.

cylsilica as the stationary phase and *n*-hexane as the mobile phase, the solute retention increases with decrease in the concentration of polar alcohol modifier in the mobile phase. Should the above contribution of the non-polar–non-polar interaction to the enantiomer retention become apparent on the trimethylsilylated surface of CSP 1, this would possibly lead to greater increase in retention than that observed on CSP 2 and a decrease in the separation factor through intervention of a non-chiral retention process other than the aforementioned hydrogen-bond association. The leucine derivative, having an isobutyl side-chain, would probably exert a greater influence on CSP 1 than the alanine derivative with only a methyl side-chain. This influence of surface modification on retention and enantioselectivity will be examined in further detail.

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