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CARBON DIOXIDE SUPERCRITICAL FLUID CHROMATOGRAPHY ON A CHIRAL DIAMIDE STATIONARY PHASE FOR THE RESOLUTION OF D- AND L-AMINO ACID DERIVATIVES

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SUMMARY

A supercritical carbon dioxide mobile phase, used in conjunction with polar modifiers, brought about the rapid optical resolution of racemic N-acetylamino acid *tert.*-butyl esters on chiral (N-formyl-L-valylamino)propylsilica without a loss of enantioselectivity. The time of analysis of the solute enantiomers was less than 4 min. Methanol, acetonitrile and diethyl ether were used as polar modifiers and their influence on the enantiomer resolution is discussed.

INTRODUCTION

A supercritical carbon dioxide mobile phase, containing polar modifiers, was found to be effective for the rapid resolution of enantiomeric amino acid derivatives on the chiral stationary phase (N-formyl-L-valylamino)propylsilica gel. Supercritical fluids have physical properties intermediate between those of liquids and gases; their viscosity is less than that of liquids, making possible the rapid diffusion of solutes in their mobile phase[1,2]. In the present study, an attempt was made to accelerate enantiomer resolution by a combination of the fast-diffusive supercritical carbon dioxide mobile phase and chiral stationary phase without a loss of efficiency and to demonstrate the relationship between resolution in the present supercritical fluid chromatography (SFC) and classical liquid chromatographic (LC) resolution in the action of polar modifiers.

Using our chiral stationary phase, the LC resolution of the enantiomers of N-acylated amino acid ester derivatives was successfully carried out with a non-aqueous mobile phase consisting of non-polar *n*-hexane and polar modifiers such as 2-propanol and diethyl ether[3–5]. The success of this resolution may be ascribed to molecular associations whose main driving force is the action of weak hydrogen bonds between the chiral moiety of the stationary phase and solute enantiomers. The sub- or supercritical state of carbon dioxide is virtually non-polar, as is *n*-hexane, and it can therefore provide effective media for diastereomeric associations as a mo-

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bile phase component. Its usefulness in conjunction with polar solutes is undoubtedly limited, even if the carbon dioxide physical state is modified. However, supercritical fluids have excellent solvating properties owing to their critical density and can thus accept various polar modifiers to facilitate the elution of polar solute enantiomers on chiral stationary phases[6–8]. Among these modifiers, polar solvents such as methanol and acetonitrile, which are not generally usable in liquid–solid chromatography owing to their limited solubility in the *n*-hexane diluent, can serve as polar modifiers in SFC. The polar modifiers used in the present chromatographic method were diethyl ether and others such as methanol and acetonitrile. The degree of enantioselectivity, measured in terms of the separation factor between two enantiomers, was compared with that obtained by LC.

In this work, SFC with polar modifiers on chiral packed columns was found to be a rapid means for enantiomer analysis, and much importance is attached to the effects of polar modifiers on enantiomer resolution rather than the contribution of the modification of the carbon dioxide physical state to the resolution.

EXPERIMENTAL

SFC was performed on a Jasco SUPER-100 SF chromatograph equipped with a MULTI-320 multi-channel UV detector, as previously reported[9], and LC on a laboratory-built liquid chromatograph. The chiral column, packed with (N-formyl-L-valylamino)propylsilica gel[3,4], was 4 mm I.D. and 25 cm long. LC was carried out using two of these columns linked in series, and SFC with a single column to avoid as far as possible a large pressure drop across the column, which would result in loss of resolution[10].

The delivery of liquid carbon dioxide (standard grade) was measured as gas at atmospheric pressure from the outlet of the restrictor. The actual gas flow was set at 2 l/min. Polar modifiers were added to liquid carbon dioxide, and the resulting mixtures were pumped into the column, maintained at 40–60°C in a column oven. The carbon dioxide density was controlled by regulating the column outlet pressure, ranging from 100 to 200 bar.

Analytical-reagent grade solvents were used for all chromatographic runs. N-Acetylated racemic and L-enantiomeric amino acid *tert.*-butyl esters, prepared according to the procedure previously described[11,12], were dissolved in analytical-reagent grade chloroform to make *ca.* 2% chloroform solutions, 5 μl of which were injected into the column. The nominal hold-up time under the supercritical conditions was measured from the frontal solvent peak.

The solute elution was monitored at two wavelengths, 225 and 235 nm, where all the amino acid derivatives examined for confirmation of enantiomer resolution absorbed. The absorption ratios obtained for the separated enantiomers were, of course, identical. The elution order of the solute enantiomers was identified by determining the retention of the corresponding L-enantiomer.

RESULTS AND DISCUSSION

All the amino acid derivatives to be separated had the structure of the N-acetyl O-*tert.*-butyl ester, which gave the most effective liquid chromatographic resolution

on the present chiral stationary phase[5]. The supercritical carbon dioxide mobile phase, containing methanol as a polar modifier, was effective for the separate elution of each of these enantiomers. The methanol was delivered to the carbon dioxide effluent at 0.5 ml/min. In contrast, classical LC with *n*-hexane containing 1% methanol (soluble at this concentration in *n*-hexane at ambient temperature) as the mobile phase virtually failed to provide any enantiomer resolution, although two solutes gave minimal peak splits. Retention data, obtained under supercritical conditions

TABLE I

COMPARISON OF SFC AND LC WITH PROTIC MODIFIERS FOR THE RESOLUTION OF RACEMIC N-ACETYLAMINO ACID *tert.*-BUTYL ESTERS ON THE CHIRAL STATIONARY PHASE (N-FORMYL-L-VALYLAMINO)PROPYLSILICA GEL

SFC conditions: carbon dioxide gas flow from the pressure restrictor outlet, 2 l/min; column outlet pressure, 200 bar; column temperature, 60°C; methanol modifier delivery rate, 0.5 ml/min; column, 25 × 0.4 cm I.D.; detection, UV at both 225 and 235 nm. LC conditions: column temperature, 40°C; flow-rate, 1 ml/min; column, two 25 × 0.4 cm I.D. columns linked in series; detection, UV at 254 nm. t_R = 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 0.5 min under the above SFC conditions; α = separation factor (k' of the L-enantiomer/k' of the D-enantiomer); Abu = α-aminobutyric acid.

Amino acid	*SFC using carbon dioxide–methanol*			*LC*					
				Methanol–n-hexane (1:99)			*2-Propanol–n-hexane (4:96)*		
	t_R	k'	α	t_R	k'	α	t_R	k'	α
Leu	1.30	1.60 1.90	1.19				11.63	2.42 2.93	1.21
Ile	1.29	1.58 1.77	1.12				10.54	2.10 2.53	1.20
Val	1.17	1.34 1.52	1.13				11.80	2.47 2.94	1.19
Abu*	1.40	1.80 1.95	1.08				13.77	3.05 3.59	1.18
Phe	1.58	2.16 2.37	1.10	48.00	9.00 9.20	1.02	12.88	2.79 3.28	1.18
α-Phenyl-Gly	1.68	2.36 2.56	1.08	52.10	9.85 –	1.00	14.42	3.24 3.61	1.11
S-Benzyl-Cys	1.95	2.90 3.12	1.08	39.98	7.33 7.46	1.02	11.83	2.48 2.77	1.12
N-*tert.*-Butyl-Trp	2.76	4.52 5.04	1.12*	33.80	6.04 –	1.00	12.38	2.64 3.04	1.15
O-Benzyl-Tyr	3.18	5.36 5.96	1.11*	45.60	8.50 –	1.00	22.07	5.49 6.07	1.11
O-*tert.*-Butyl-Ser	1.07	1.14 1.33	1.17				7.79	1.29 1.50	1.16
O-*tert.*-Butyl-Glu	1.65	2.30 2.60	1.13				11.83	2.48 2.72	1.10
Pro	1.79	2.58 –	1.00				12.82	2.77 –	1.00

* Peak asymmetry factors[13] calculated with the first eluted peaks were 1.23 for the tryptophan derivative and 1.38 for the tyrosine derivative.

with a column outlet pressure of 200 bar at 60°C, were compared with those obtained under the above LC conditions and are shown in Table I.

The physical state of carbon dioxide was modified for the tyrosine derivative, the retention of which was greatest among the solutes examined. The density of the carbon dioxide effluent has been reported to control solute retention[1,2]. A reduction in the column outlet pressure at a constant carbon dioxide effluent flow, owing to the decrease in carbon dioxide density, increased the solute retention and was responsible for greater enantioselectivity. However, when the outlet pressure was reduced from 200 to 100 bar, a constant delivery of carbon dioxide effluent became difficult, resulting in a wavy baseline. This behaviour was marked with carbon dioxide effluent containing aprotic modifiers, such as diethyl ether and acetonitrile, as described below. Thus, 200 bar was used as the minimal outlet pressure. The other operating parameter that modifies the carbon dioxide density is the column temperature. When this was raised from 40 to 60°C at a constant delivery rate of methanol, it enhanced the elution without any loss in enantioselectivity. A typical chromatogram of racemic N-acetyl-O-benzyltyrosine *tert.*-butyl ester, obtained under the above optimized conditions, is presented in Fig. 1.

Previous LC data obtained with 2-propanol instead of methanol are also included in Table I for comparison with those obtained by SFC. SFC resolution of solute enantiomers was achieved in as little as 4 min. This is faster than resolution by LC with 2-propanol, although it was necessary to reduce the time for analysis under the LC conditions to half because two linked chiral columns had to be used. The enantioselectivity obtained with the carbon dioxide–methanol mixture was modest and comparable to that obtained in the present LC. In all SFC experiments, the elution order of enantiomers was such that the L-enantiomer was always followed by the D-enantiomer. This was also observed in LC.

It seems reasonable to conclude, at least under the LC conditions, that replacement of 2-propanol with the more polar solvent methanol reduced the enantioselectivity because of the possible more competitive association of methanol with

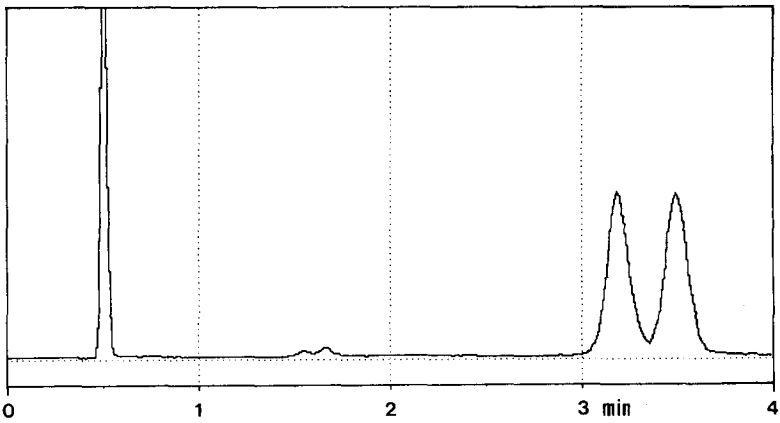

Fig. 1. Optical resolution of the racemic N-acetyl-O-benzyltyrosine *tert.*-butyl ester with the supercritical carbon dioxide mobile phase containing methanol as modifier on the chiral stationary phase. SFC conditions as in Table I.

solute–chiral stationary phase interactions. However, supercritical conditions were not as sensitive as the LC conditions when methanol was used as the modifier and provided modest resolutions, comparable to those obtained by LC with the *n*-hexane–2-propanol mixture.

A higher degree of enantiomer resolution was achieved by using a supercritical carbon dioxide mobile phase containing acetonitrile (delivery of 0.5 ml/min), as is evident from Table II. This modifier, as well as methanol, has a limited solubility in *n*-hexane. *n*-Hexane containing 2% of acetonitrile (a concentration close to its maximum solubility at ambient temperature) was not capable of eluting the solutes within a reasonable time. SFC resolution with the acetonitrile modifier was comparable to that with the aprotic modifier diethyl ether (delivery rate 2 ml/min to the carbon dioxide effluent) from the standpoint of the enantioselectivity observed, as can be seen from Table II. SFC with these aprotic modifiers was carried out under the same conditions as those for the above SFC, except for the column temperature, which was maintained at 40°C instead of 60°C owing to the stable delivery of the carbon dioxide effluent. It is likely that aprotic modifiers such as these disrupt enantioselective associations on chiral stationary phases to a lesser extent than protic modifiers. In LC, the liquid mobile phase containing aprotic stronger solvent components such as diethyl ether was also observed to bring about a degree of enantioselectivity exceeding that of a mobile phase containing 2-propanol. The data for this observation

TABLE II

COMPARISON OF SFC AND LC WITH APROTIC MODIFIERS FOR THE RESOLUTION OF THE RACEMIC SOLUTES ON THE CHIRAL STATIONARY PHASE

SFC and LC conditions as in Table I, except for the delivery rate of polar modifiers and the column temperature. Modifier delivery rate: acetonitrile, 0.5 ml/min; diethyl ether, 2 ml/min. Column temperature, 40°C.

Amino acid	*SFC*						*LC using diethyl ether–n-hexane (80:20)*		
	Carbon dioxide–acetonitrile			*Carbon dioxide–diethyl ether*					
	t_R	k'*	α	t_R	k'**	α	t_R	k'	α
Leu	1.27	1.65	1.35	1.06	1.72	1.36	14.01	3.12	1.39
		2.23			2.33			4.33	
Val	1.25	1.60	1.31	0.96	1.46	1.28	14.99	3.41	1.38
		2.10			1.87			4.69	
Phe	1.68	2.50	1.20	1.31	2.36	1.20	15.84	3.66	1.31
		3.01			2.82			4.80	
α-Phenyl-Gly	1.79	2.73	1.17	1.36	2.49	1.17	15.54	3.57	1.23
		3.18			2.92			4.38	
S-Benzyl-Cys	1.87	2.90	1.16	1.35	2.46	1.17	14.21	3.18	1.27
		3.35			2.87			4.05	
N-*tert.*-Butyl-Trp	2.55	4.31	1.20***	1.94	3.97	1.22	19.35	4.69	1.32
		5.15			4.85			6.20	
O-Benzyl-Tyr	2.95	5.15	1.21	2.11	4.41	1.22	21.25	5.25	1.31
		6.25			5.36			6.85	

* Nominal hold-up time, 0.48 min.
** Nominal hold-up time, 0.39 min.
*** Peak asymmetry factor, 1.08.

are also shown in Table II. However, these resolutions were not improved as much as was expected from the separation factor observed, owing to bad peak tailing. In contrast, the supercritical mobile phase gave fairly symmetric peaks, not only with the most polar methanol but also with the least polar diethyl ether. These characteristics of aprotic modifiers in the supercritical carbon dioxide effluent and the less competitive association with solute–chiral stationary phase interactions should make SFC of interest.

CONCLUSIONS

The supercritical carbon dioxide mobile phase used in conjunction with polar modifiers provided rapid and satisfactory separations of racemic amino acid derivatives on a chiral packed column. The enantioselectivity observed depended markedly on the selection of stronger solvent components, as was also the case when *n*-hexane was used as the LC mobile phase. The degree of enantioselectivity given by SFC did not always correspond to that obtained by LC with mobile phases containing the same modifier, as was noted when methanol was used as the modifier. This influence of the polar modifier on enantioselectivity and the occurrence of good peak shapes are both effects that should be investigated further.

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