Journal of Chromatography, 389 (1987) 256–260
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 312

Note

Use of microcolumn liquid chromatography with a chiral stationary phase for the separation of low-resolution enantiomers

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(First received October 13th, 1986; revised manuscript received November 26th, 1986)

Packed microcolumn liquid chromatography (LC), as developed by Ishii and co-workers[1–3], has been shown to be an effective method for separations that require a large number of theoretical plates. Indeed, plate numbers (n) exceeding 100 000 have been reported by a number of research groups[4,5]. There are at least two important cases where these extraordinarily high plate numbers are essential. The first is in the separation of very complex mixtures containing a large number of peaks. In this case, greater efficiency generally results in an increase in the number of peaks resolved. In the second case, only a few peaks may be present but they may be very difficult to resolve from one another (*i.e.*, low selectivity, α). A prime example of the second case is the LC separation of many enantiomers on chiral stationary phases (CSPs). Although there are few select compounds that have appreciable α values on a particular CSP, there are many more which have low α values and for which enantiomeric resolution is difficult or impossible on current, commercially available CSPs. Indeed, packed microcolumn LC containing an appropriate CSP may prove to be a valuable analytical tool for the LC resolution of many of these difficult-to-separate enantiomers.

Although chiral mobile phase additives have been used successfully in reversed-phase microcolumn LC[6], this is the first report, to our knowledge, of a microcolumn CSP separation. A well characterized, hydrolytically, stable, β-cyclodextrin bonded phase (first developed in our laboratory) was used as the CSP[7,8]. Racemic dansyl amino acids were used as test solutes because of the extensive amount of separation data available for these compounds.

EXPERIMENTAL

A Shimadzu Model LC-6A liquid chromatograph was used in the constant-pressure mode. The detector was a UV spectrophotometer (Shimadzu SPD-2AM) with a custom-designed flow cell (0.02 μl in volume) set at 254 nm. The chromatograms were obtained by using a chart recorder, Linear 1200. The flow cell was constructed by stripping the polyimide external coating from fused silica tubing; PTFE tubing was connected between the end of the column and the flow cell. A 0.2-μl internal sample loop valve (VICI Valco Instrument, Houston, TX, U.S.A.) was used for sample injection. Vespel ferrules (Alltech, Houston, TX, U.S.A.) were used to connect the fused-silica tubing and stainless-steel union.

0021-9673/87/$03.50

Dansyl DL-amino acids (Kit No. Dan-DL-15) were obtained from Sigma (St. Louis, MO, U.S.A.) and used without purification. The mobile phases were generally filtered through a 0.45-μm membrane filter before use. The mobile phases consisted of HPLC grade acetonitrile and water, purchased from Fisher Scientific (Raleigh, NC, U.S.A.). The aqueous portion of the mobile phase consisted of 0.5% triethylammonium acetate (pH = 7.1 or pH = 4.1). The flow-rate was 1.5 μl/min. The pressure drop for a 190-cm column (acetonitrile–water, 95:5, mobile phase) was 160 atm and that for an acetonitrile–water (80:20) mobile phase was 190 atm. The enantiomeric compounds were dissolved in methanol before injection. All separations of the enantiomeric compounds were carried out at room temperature.

Column preparation

The packing material was 5-μm spherical silica gel to which β-cyclodextrin was bonded through an eight-atom spacer[9]. The column was made from fused-silica tubing (250 μm I.D., 350 μm O.D.) (Polymicro Technologies, Phoenix, AZ, U.S.A.). Fused-silica tubing was selected as the column material because of its flexibility, inert surfaces, and good mechanical strength. The stationary phase was packed in slurry form. This packing technique has been previously reported[10,11]. As in all microcolumn LC, the theoretical plate number varies tremendously with the mobile phase composition. For example, n = 35 000 with acetonitrile–water (95:5) and n = 120 000 with acetonitrile–water (85:15) for dansyl DL-valine as the test solute.

RESULTS AND DISCUSSION

Fig. 1 shows the capacity factors (k') of the investigated dansyl amino acids as a function of percent acetonitrile in the mobile phase. All previous separations on β-cyclodextrin bonded phases were done in the reversed-phase mode and with mobile phases with a high percentage of water or buffer. Indeed, increasing proportions of buffer in the mobile phase to levels higher than that shown in Fig. 1 will also produce higher retention. This mobile phase region was investigated before. Previous reports indicated that increasing the concentrations of organic modifier in the mobile phase reduced both retention and resolution. Resolution values (R_s) eventually went to zero at sufficiently high levels of organic modifier[12,13]. In this study, however, it was found that further increases in the modifier concentration eventually caused a reversal in retention behavior, and therefore a minimum in the retention behavior. As the concentration of acetonitrile increased to very high levels, both retention and enantiomeric resolution increased. For example, when the mobile phase composition was acetonitrile–buffer (85:15), the solute was not retained thereby producing the expected sharp peak at the void volume and no separation. When the mobile phase composition was acetonitrile–buffer (95:5) there was retention and baseline resolution of enantiomers. This observation is important for at least two reasons: first, the presence of minima indicates that the retention mechanism(s) at high buffer concentrations (*e.g.*, inclusion complex formation) may differ from that at high acetonitrile concentrations. The fact that chiral recognition also increases at high acetonitrile concentration (see Fig. 2) indicates that the retention increase stems largely from interaction with the β-cyclodextrin and not with the achiral support (*i.e.*, residual silanol groups) or the linkage chain. One possibility is a "normal-phase" adsorption

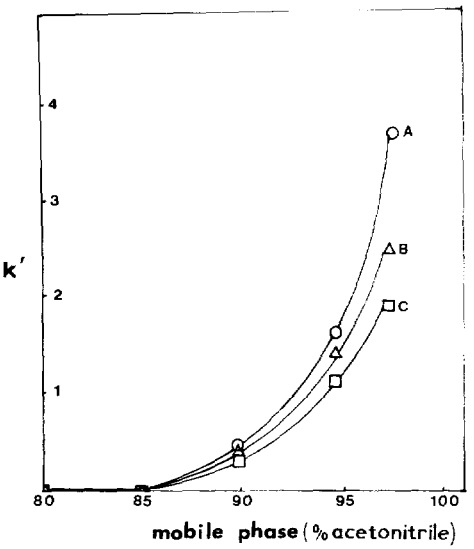
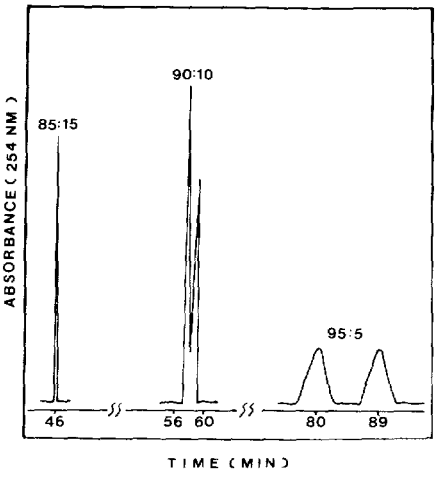


Fig. 1. Influence of percentage acetonitrile on the capacity factors of some dansyl amino acids. A = Dansyl D-tryptophan; B = dansyl D-leucine; C = dansyl D-phenylalanine.

to the external hydroxyls of the β-cyclodextrin molecule. Second, high efficiencies in microcolumn LC are very dependent on mobile phase composition. In general the highest efficiencies are observed at high concentrations of organic modifier.

Table I summarizes some of the separation data for a variety of racemic dansyl amino acids. As can be seen, excellent resolutions have been achieved for various DL



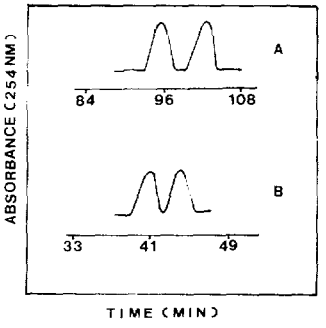


Fig. 2. Enantiomeric separation of dansyl butyric acid as a function of the percentage of acetonitrile in the mobile phase. The α values were 0.0, 1.12, and 1.23 for mobile phase compositions of 85:15, 90:10, and 95:5, respectively.

Fig. 3. Enantiomeric separation of dansyl tryptophan and dansyl aspartic acid. See Table I for experimental conditions. A = Dansyl DL-tryptophan; B = dansyl DL-aspartic acid.

TABLE I

OPTICAL RESOLUTION OF THE ENANTIOMERS OF DANSYL AMINO ACIDS

Amino acid	*k'*		α	R_s	*Mobile phase*
	L	D			
Tryptophan*	3.22	3.65	1.14	1.68	97:3**
Aspartic acid*	0.98	1.20	1.22	1.20	95:5***
Glutamic acid*	0.81	0.96	1.19	1.20	95:5***
Valine	2.35	3.27	1.39	2.83	97:3**
Butyric acid	0.90	1.11	1.23	1.67	95:5**
Serine	0.90	1.06	1.18	1.50	95:5**
Norleucine	0.80	0.99	1.23	1.75	95:5**
Threonine	0.61	0.80	1.31	1.86	95:5**
Phenylalanine	1.49	1.86	1.25	2.33	97:3**
Methionine	0.57	0.79	1.39	1.78	95:5**
Leucine	1.14	1.15	1.28	2.20	95:5**
Norvaline	0.81	1.01	1.25	2.00	95:5**

* Could not be separated on ordinary 25-cm LC columns of the same packing.

** Numbers represent the volume percent of acetonitrile to buffer (pH 7.1). Data obtained using a 190-cm β-cyclodextrin bonded microcolumn.

*** Numbers represent the volume percent of acetonitrile to buffer (pH 4.1). Data obtained using a 120-cm β-cyclodextrin bonded micro column.

pairs with R_s values greater than 2.00 in several instances. Dansyl DL-valine gave the highest resolution (R_s = 2.83). No separation gave a resolution less than 1.2. Previous reports[12] indicated that dansyl tryptophan, aspartic acid and glutamic acid could not be resolved on standard analytical β-cyclodextrin bonded phase columns at *high water concentrations*. Of these three only dansyl DL-tryptophan could be partially resolved on a standard analytical β-cyclodextrin column (R_s = 0.7) at high acetonitrile concentrations. All three were well resolved on the microcolumn, however. Fig. 3 shows a typical separation of some of these "difficult-to-separate" compounds. These chromatograms were obtained under isocratic conditions. The peak identification was made by separately injecting the dansyl derivatives of the optically pure D- and L-amino acids. In all cases, the L isomer of the dansyl amino acid elutes before the D isomer.

CONCLUSIONS

Both retention and enantiomeric resolution tend to increase at very high organic modifier concentrations (on β-cyclodextrin bonded phases). This reversal in retention behavior indicates a possible change in the separation mechanism and in chiral recognition. Microcolumn separations of many hard-to-resolve enantiomers are particularly effective when using mobile phases with high volume percentages of organic modifier.

ACKNOWLEDGEMENT

Support of this work by the Department of Energy, Office of Basic Energy Science (DE-AS0584ER13159) is gratefully acknowledged.

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