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Note

Selection of optimum conditions for the preparative-scale separation of a dipeptide diastereomer mixture on the basis of measurements made in an analytical column

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Literature data[1–8] indicate that all liquid chromatographic modes are commonly employed for the analysis and preparative separation of peptides. Recently, the reversed-phase technique has been used particularly widely owing to its great flexibility in the selection of a suitable composition of the mobile phase. However, adsorption systems can also be used successfully for the separation of protected peptides. When using preparative chromatography, two problems must be solved: selection of the chromatographic system and selection of the sample size. Numerous papers describing the changes in chromatographic parameters with the variation of the sample size have been published, the most important of which were cited by Majors *et al.*[9]. At present, it can be stated that the effect of sample volume on the retention and shape of a peak has been established[10–12], whereas there is little information concerning the effect of sample size on the shape of a chromatographic band.

Investigations of mass overloading have shown that the capacity of the packing is higher for peaks with small k' values than for those with large k' values. This may suggest that it is advantageous to select a system with small k' values. As the sample size also depends on the distance between the peaks, it is necessary to consider whether the advantages resulting from lowering the k' value will offset the loss resulting from lowering of the resolution. It is most convenient to select the conditions for preparative separations on the basis of measurements carried out on an analytical column. In order to do so, the same eluent strength and selectivity of the mobile phase should be maintained in both the analytical and preparative columns. The efficiency of the two columns determines the allowable sample volume, whereas the mass of substances is of lesser significance. Under conditions of column overloading the peak widths are similar[12,13], as the band broadening resulting from non-linearity of the isotherm becomes a major factor compared with dispersion of mass in the bed[14].

EXPERIMENTAL

Adsorbents

Silica gel, d_p = 33 μm, obtained by repeated sedimentation of H60 gel (Mach-

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ery, Nagel & Co., F.R.G.), LiChrosorb Si 100, d_p = 10 μm (Merck, F.R.G.) and μBondapak C_{18} ODS, d_p = 10 μm (Waters Assoc., U.S.A.) were used.

Solvents

n-Hexane (analytically pure) (Reachim, U.S.S.R.), isopropoanol (analytically pure) (POCH, Poland), methanol and water were used as solvents.

Apparatus

The apparatus employed for preparative-scale liquid chromatography was equipped with a pump of output up to 270 cm^3/min and a UV-254 detector with a measuring vessel of 10 mm^3 capacity. A 300 × 17 mm I.D. column was dry packed by the tamping method. Sampling of the substance was performed by means of a valve with loops of different volumes. The 300 × 3.3 mm I.D. analytical columns were packed by the wet method and used with a KB 5113 analytical chromatograph (KABID, Poland).

Mixture to be separated

The main components of the post-reaction mixture were (1) N-(CHO–Phg)–DCU, (2) CHO–D-Phg–D-Leu–OMe + CHO–L-Phg–L-Leu–OMe and (3) CHO–D-Phg–L-Leu–OMe + CHO–L-Phg–D-Leu–OMe (DCU = dicyclohexylurea; Phg = phenylglycyl). For clarity, these components will be referred to by these numbers 1–3. For the purposes of the study it was necessary to isolate components 2 and 3 from the mixture.

RESULTS AND DISCUSSION

The conditions for the preparative separation were selected on the basis of the data obtained for an analytical column. Comparability of the two systems was achieved by using in both the analytical and preparative column silica gel of the same average particle size.

Selection of the mobile phase

The components of the mixture differed considerably with respect to polarity, which renders difficult the selection of an appropriate preparative system. Numerous systems were tested, but in all of them the results obtained were similar to those shown in Fig. 1.

It wass found that a change in the isopropanol concentration has a substantial effect on the resolution (R_s) between substances 2 and 3 and a small effect on the R_s value for substances 1 and 2.

The values of some retention parameters obtained for various mobile phases are given in Table I. It can be seen that decreasing the isopropanol concentration in the mobile phase results in an increased separation of the peaks, but also in rapid band broadening with an increase of column loading, which leads to an increase in fraction volume, and hence an increase in the time of a single separation. Eventually, a mobile phase containing 7.5% of isopropanol in *n*-hexane was chosen for further work, the capacity factor (k') for substance 3 being *ca.* 7.5.

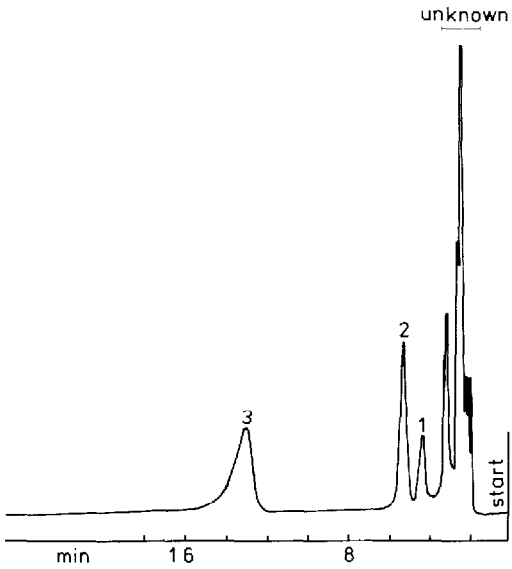

Fig. 1. Separation of diastereoisomers 1–3 on an analytical scale. Separation conditions: column 300 × 3.3 mm I.D.; stationary phase, LiChrosorb Si 100, d_p = 10 μm; mobile phase, *n*-hexane–isopropanol (92.5:7.5); UV detector.

Selection of sample size

In order to facilitate the selection of sample size on the basis of data obtained for an analytical column, the so-called "capacity factor" of the peak front was introduced. The capacity factor was calculated as $a = (V_F - V_0)/V_0$, where V_F is the retention volume of the peak front determined by the tangent to the ascending part of the peak and V_0 is the dead volume of the column. The value of a permits a comparison of the results obtained with columns of different sizes.

Initially, it was established that over a relatively broad range the column efficiency does not influence the peak widths on overloading of the packing with the

TABLE I

RETENTION PARAMETERS OBTAINED FOR MOBILE PHASES CONTAINING VARIOUS AMOUNTS OF ISOPROPANOL IN *n*-HEXANE

Isopropanol concentration (%)	*Relative selectivity* $\alpha_{1/2}$	$R_{s3/2}$	*Distance between peaks 2 and 3 determined on the baseline for the analytical system (multiple of* V_0*)*	*Approach rate of peak front of component 3 to the start (mg/a*)*	*Total volume of mobile phase (multiple of* V_0*)*
15	3.3	2.2	0.9	3.3	–
7.5	4.0	4.2	4.3	1.8	11.7
3	4.4	5.1	11.0	0.5	–
2	4.2	5.1	19.0	0.4	35.8

* $a = (V_F - V_0)/V_0$.

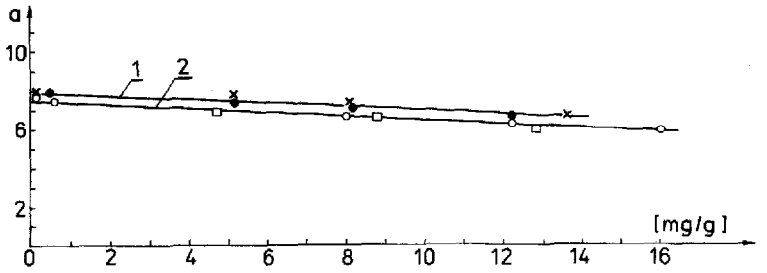

Fig. 2. Plot of $a = (V_F - V_0)/V_0$ *versus* mass of sample mixture. Mobile phase as in Fig. 1. Curve 1: column, 300 × 17 mm I.D.; volume injected, V_i = 5 ml; efficiency, (×) N_1 = 500, (●) N_2 = 230. Curve 2: column, 300 × 3.3 mm I.D.; V_i = 200 μl; efficiency, (○) N_1 = 370, (□) N_2 = 1000. (N = Number of theoretical plates.)

separated mixture (Fig. 2). Subsequently, the dependence of a on the mass loading of the column with samples of various concentration was plotted (Fig. 3). It was found that with a mixture to be separated it is disadvantageous to use the maximum sample volume (9 ml) for the analytical column. An increase in the sample volume from 3.4 to 5.1 ml did not decrease mass overloading (Fig. 3). In the case discussed, the optimum sample volume is considerably smaller than the maximum; hence, the possible difference in the efficiencies of the analytical and preparative columns (packed with the same gel) had no effect on the determination of sample size.

Considering the above results and the solubility of the sample in the mobile phase, in the first stage a sample of 75 ml containing 260 mg of compounds was separated in a 330 × 17 mm I.D. column (40 g of silica gel) to obtain two fractions.

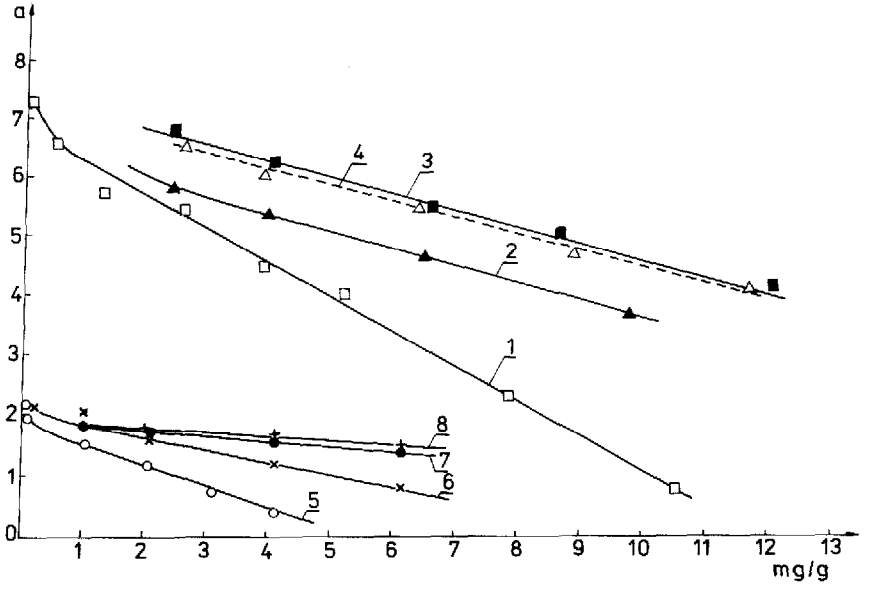

Fig. 3. Plot of $a = (V_F - V_0)/V_0$ *versus* mass of substance. Column, 300 × 3.3 mm I.D.; mobile phase, as in Fig. 1. Curves 1–4, substance 3; curve 5–8, substance 2. Curves 1 and 5, V_i = 200 μl; 2 and 6, V_i = 1.5 ml; 3 and 7, V_i = 3.4 ml; 4 and 8, V_i = 5.1 ml.

The total volume of the mobile phase consumed was 11.7 V_0, the volume of fraction II with component 3 was 5.5 V_0 and that of fraction I with component 2 was 4.7 V_0. The purity of the fractions was checked by high-performance liquid chromatography (HPLC) (Fig. 4a). Fraction II was found to contain only component 3 from the reaction mixture.

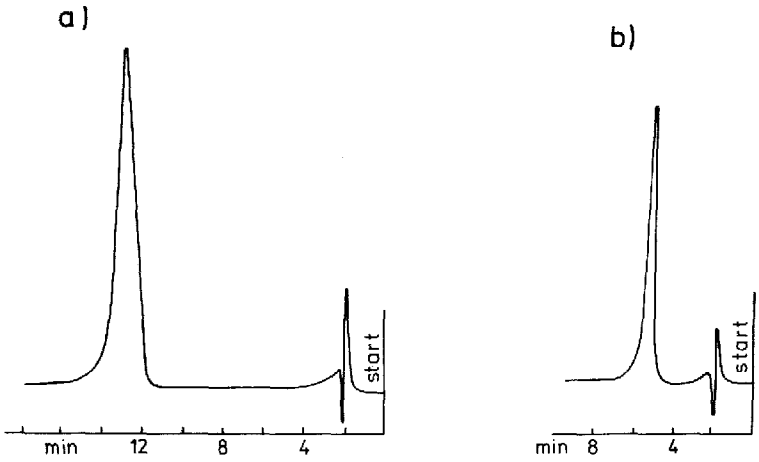

Fig. 4. Control of the purity of diastereoisomers separated on a preparative scale. Separation conditions as in Fig. 1. (a) Chromatogram of fraction II with compoonent 3; (b) chromatogram of fraction I weith component 2.

Fraction I contained 66.7% of component 2. The second stage of the work, *i.e.*, isolation of component 2 in an adsorptive system, was more difficult. This component had been separated from the preceding one using silica gel with *n*-hexane–isopropanol (98:2) by injecting 0.53 mg/g of pure compound, *i.e.*, 0.8 mg/g of fraction I, at a volume not exceeding the σ value of the peak. The elution volume was 11 V_0. Hence this stage was six times longer than the stage during which elution of component 3 and concentration of component 2 occurred. No other component except component 2 was found in fraction I by HPLC (Fig. 4b).

Component 2 can also be separated by reversed-phase chromatography on ODS with water–methanol (45:55) (Fig. 5). The resolving power of this system is greater than that of the adsorptive sysem, but because of the weak solubility of the sample its output is similar to that in the first stage. It was not been employed, however, because of the large amounts and the high coast of ODS required for packing the columns.

The results of the study reported here led to the following conclusions. The column efficiency (calculated on the basis of an analytical sample) plays no essential role after overloading of the column, a fact that was made use of in establishing the size of the preparative-scale sample on the basis of data obtained for an analytical column. It is necessary to judge if there is advantage in increasing the R_s value by increasing the k' value, considering the fact that at high k' values the output with time is decreased.

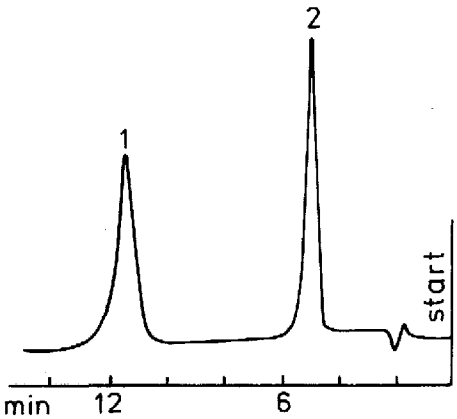

Fig. 5. Separation of components 1 and 2 by employing the ODS column (100 × 4 mm I.D.) with methanol–water (45:55) as the eluent.

In solving problems of preparative-scale separations it is advisable to consider whether further dilution of a sample is worthwhile. There comes a moment where further dilution does not decrease the mass overload, and an increase in sample volume only leads to a poorer separation and increases the fraction volume. Although, according to some studies, reversed-phase systems can be more overloaded than adsorptive systems, the output ultimately depends on sample solubility.

REFERENCES

1 J. Richey, *Int. Lab.*, January/February (1983) 50.
2 M. T. W. Hearn, *Advan. Chromatogr.*, 20 (1982) 1.
3 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 1 (1981) 103.
4 B. Makuch, A. Arendt and A. F. Kołodziejczyk, *Pol. J. Chem.*, 55 (1981) 701.
5 M. T. W. Hearn, M. T. W. Hearn, E. L. Harris, G. J. Bethell, W. S. Pancoch and J. A. Ayess, *J. Chromatogr.*, 218 (1981) 509.
6 R. S. Ward and A. Pelter, *J. Chromatogr. Sci.*, 12 (1974) 570.
7 T. F. Gabriel, J. Michalewsky and J. Meienhofer, *J. Chromatogr.*, 129 (1976) 287.
8 C. A. Bishop, D. R. K. Harding, L. J. Meyer, W. S. Hancock and M. T. W. Hearn, *J. Chromatogr.*, 192 (1980) 222.
9 R. E. Majors, H. G. Barth and C. H. Lochmüller, *Anal. Chem.*, 54 (1982) 323R.
10 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 119 (1976) 467.
11 B. Coq, G. Cretier and J. L. Rocca, *J. Chromatogr.*, 186 (1979) 457.
12 P. Gareil and R. Rosset, *Analusis*, 10 (1982) 397.
13 A. W. J. de Jong, H. Poppe and J. C. Kraak, *J. Chromatogr.*, 148 (1978) 127.
14 A. W. J. de Jong, J. C. Kraak, H. Poppe and F. Nooitgedacht, *J. Chromatogr.*, 193 (1980) 181.