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PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY UNDER GRADIENT CONDITIONS

II. A COMPUTER PROGRAM FOR THE DESIGN OF REVERSED-PHASE GRADIENT-ELUTION SEPARATIONS OF PEPTIDE AND PROTEIN SAMPLES

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SUMMARY

A commercially available computer program (BIOPREP) is described as an aid for developing preparative separations of peptide or protein samples using reversed-phase gradient elution. On the basis of four small-scale runs in the laboratory (with advice offered by the computer), experimental conditions for "touching-band" separations can be predicted. This in turn allows comparisons of the production rate of a purified product as a function of the gradient conditions and column dimensions. In this way, conditions can be selected that either maximize the production rate or provide an otherwise satisfactory separation.

INTRODUCTION

Currently reversed-phase gradient elution is widely used for the preparative purification of peptide and protein samples on either a laboratory[1] or a manufacturing[2] scale. The usual objective, especially at the R&D or pilot-plant stage, is to obtain a certain amount of purified product with the expenditure of minimum time and effort. Because of the complexity of these separations, and the strong dependence of production rate (g/h of purified material) on the separation conditions, the chromatographer is faced with the question of how much time should be spent on method development in order to minimize the time spent later in actually separating the sample.

The results described in Part I[3] can be used to guide the method development process, at least for the design of "touching-band"[a] separations. We can proceed

[a] "Touching-band" separations refer to the case where the sample is just large enough that the product band begins to overlap adjacent peaks (*e.g.*, yielding a 99.8% recovery of 99% pure product as in ref. 4); see the related discussion of touching-band separations in an isocratic mode[5,6].

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qualitatively (trial and error), or we can make use of accurate quantitative relationships for the selection of the best experimental conditions. In the latter instance, we can minimize the total number of experiments by using experimental data to calculate preferred conditions more precisely. However, this is tedious if carried out manually. A better approach is the use of a computer program to (a) execute the necessary calculations, (b) draw conclusions from prior experiments and (c) make recommendations and alert the user to potential problems as method development proceeds.

In this paper we describe an efficient approach to method development for the reversed-phase gradient-elution high-performance liquid chromatographic (HPLC) separation of peptide and protein samples. We also illustrate how this procedure can be simplified by the use of an appropriate computer program, BIOPREP. The resulting touching-band separations are suitable for the purification of multi-gram amounts of final product. Additional increases in production rate by the use of heavily overloaded (<99.8% recovery of product) separation are possible by the further trial-and-error adjustment of separation conditions. This will be examined in the future, but guidelines can be inferred in part from the results in ref. 4.

THEORY[a]

Our proposed method-development strategy can be summarized as follows:

(1) Carry out one or more initial runs with a small sample, varying the mobile phase composition (organic solvent, pH, additives) and column packing in order to maximize the selectivity. The objective is to achieve the largest possible value of the separation factor, α, between the product band and the adjacent earlier-eluting impurity.

(2) Use the initial run(s), typically carried out with wide-range gradients, to estimate favorable values of (a) the initial and final %B in the gradient, (b) gradient times for two additional exploratory runs and (c) flow-rate.

(3) Carry out two additional small-sample runs with the recommended gradient range from step 2, varying only the gradient time; these two runs can be used to define separation as a function of gradient conditions and column dimensions.

(4) Carry out a fourth run, using the same conditions as in step 3, but with a sample that is large enough to increase the bandwidth appreciably; this run can be used to estimate the column capacity w_s and loadability for the sample in question.

(5) With the data and conditions from steps 3 and 4, calculate the sample size and production rate for touching bands as a function of gradient time and column dimensions.

(6) Verify the predicted (best) separation from step 5 experimentally; fine-tune the conditions if necessary.

Step 1: maximizing α

The choice of preferred mobile and stationary phase conditions for a given sample is usually a matter of experience plus trial-and-error experimentation. In the absence of advance information, many workers will begin with a 5–70% water–

[a] A list of all symbols used in Parts I–III is included in ref. 3.

acetonitrile [0.1% trifluoroacetic acid (TFA)] gradient. In many instances[7–10], it will be possible to improve the initial separation considerably with these conditions (TFA–water–acetonitrile) by adjusting the gradient steepness. Alternatively, changes in the mobile phase pH, buffer or buffer concentration may be required to provide further improvements in band spacing.

Step 2: selecting favorable conditions for the next two runs

Gradient range. Experienced chromatographers will usually adjust the gradient range (select the best values of the initial and final %B) on the basis of an initial wide-range gradient. This is illustrated in Fig. 1 by a computer-simulated chromatogram[9] for a representative sample: interleukin-2 (IL-2), a 14000-Da protein, plus three impurities that correspond to oxidation or reduction products of the parent molecule. In order to minimize separation time, it is desired to select a gradient range that just brackets the compounds of interest, *i.e.*, the product (IL-2) and the immediately preceding impurity band (marked with an asterisk in Fig. 1).

Theory[11,12] plus experience gained through the use of computer simulation[8] (DryLab G; LC Resources, Lafayette, CA, U.S.A.) suggests the following equations for estimating best values of the initial and final values of %B (φ_i and φ_f) from an exploratory gradient run such as in Fig. 1:

$$\varphi_i = (\Delta\varphi/t_G)\,(t_g - t_0 - t_D) + \varphi_i^0 - 2/S \tag{1}$$

and

$$\varphi_f = (\Delta\varphi/t_G)\,(t_g - t_0 - t_D) + \varphi_i^0 + 0.01 \tag{2}$$

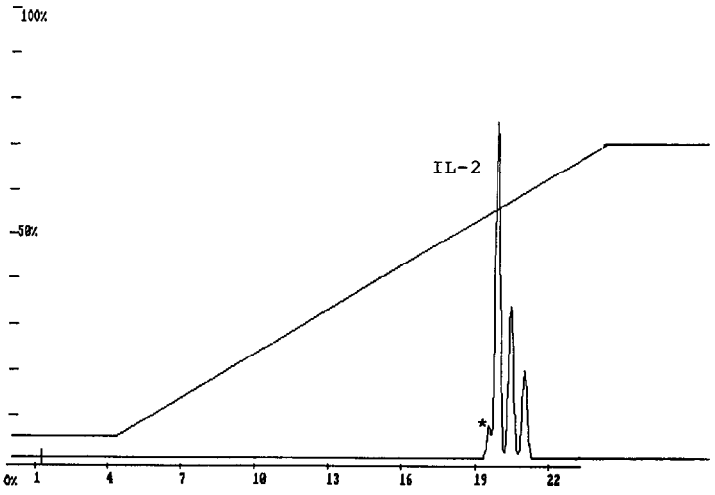

Fig. 1. Recreated chromatogram (using experimental data[9] plus DryLab G software[10]) for the separation of a mixture of desAla^{1}Ser^{125} IL-2 plus three oxidized/reduced impurities. Conditions: 25 × 0.46 cm I.D. C_3 column; 5–70% gradient of acetonitrile in water (0.1% TFA added in 20 min); flow-rate, 2 ml/min.

where t_g is the retention time of the product band, t_D is the dwell time of the HPLC equipment and φ_i^0 is the value of φ_i in the initial gradient run. The other symbols are standard terminology defined in the list of symbols in ref. 3.

Returning to the example in Fig. 1, the initial gradient is 5–70% B in a time of 20 min, the dead time t_0 is 1.28 min and the dwell time is 3.1 min. From the molecular weight of the product band (14 000 Da), $S \approx 32$ (eqn. 6 in Part I[3]). Eqns. 1 and 2 then give $\varphi_i = (0.65/20)(19.93 - 1.28 - 3.10) + 0.05 - (2/32) = 0.493$ and $\varphi_f = (0.65/20)(19.93 - 1.28 - 3.10) + 0.05 + 0.01 = 0.565$, *i.e.*, a 49.3–56.5% B gradient range is recommended.

Gradient times. For the next two experimental runs, it is desirable to adjust the gradient time t_G so as to yield effective values of k' ($\bar{k}$) in the range 3–9, corresponding to a gradient steepness $b = 0.1$–0.3. Eqn. 5 in Part I[3] then permits estimates of appropriate values of t_G for the two runs:

$$\text{(run 1)} \quad t_G = 3.5 V_m \Delta\varphi \, S/F \tag{3}$$

$$\text{(run 2)} \quad t_G = 10.5 V_m \Delta\varphi \, S/F \tag{4}$$

Thus, the gradient time for run 1 is $3.5 \cdot 2.56 \cdot (0.565 - 0.493) \cdot 32/2 = 10.3$ min. Similarly, the gradient time for run 2 is three times this value (30.9 min). Again, the symbols in eqns. 3 and 4 are given in ref. 3.

The resulting chromatograms for these recommended conditions for the next two experimental runs are shown in Fig. 2 for (A) run 1 and (B) run 2. Reasonable resolution of the sample is observed (the result of favorable $\bar{k}$ values), and the gradient now brackets the product band IL-2 and the preceding impurity peak (marked with an asterisk). Similar tests of eqns. 1–4 for other peptide and protein samples gave comparable results to those in Figs. 1 and 2 (if there are later bands in the chromatogram that would not be eluted by the recommended gradient range, a steep gradient segment can be added to the end of the recommended gradient).

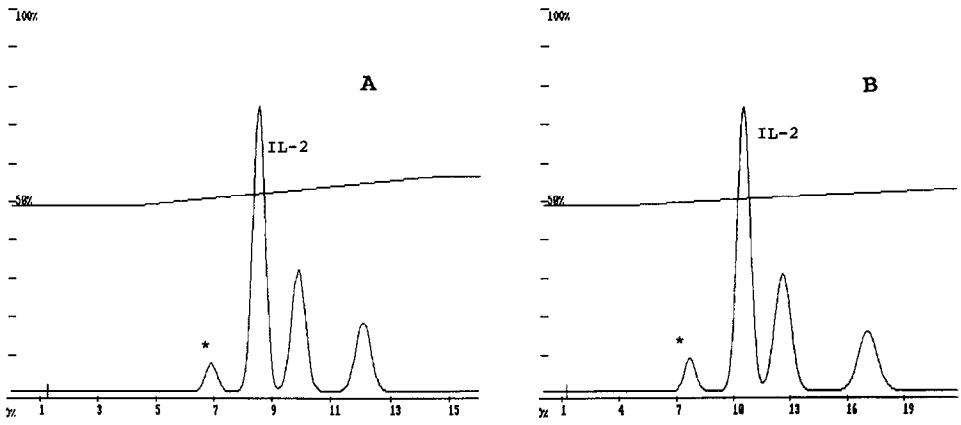

Fig. 2. Recreated chromatogram (using experimental data[9] plus DryLab G software[10]) for the separation of a mixture of desAla[1]Ser[125] IL-2 plus three oxidized/reduced impurities. Conditions as in Fig. 1, except: (A) 49.3–56.5% B gradient in 10.3 min; (B) the same in 30.9 min.

Flow-rate. In earlier papers[4,13] it was concluded that the column plate number in touching-band separations has a significant effect on production rate. That is, if the column length and/or flow-rate are varied while holding the column pressure below some upper limit (*e.g.*, 2000 p.s.i.), the best choice of flow-rate is one that will give a small-sample resolution of $R_s \approx 1.7$.

At this point we wish to select a flow-rate that is roughly optimum in terms of production rate. Resolution in gradient elution (for a small sample) is given by[11,12]

$$R_s = (1/4)\,(\alpha-1)N_0^{1/2}[\bar{k}/(\bar{k}+1)] \tag{5}$$

If we ignore the possible variation of α with gradient steepness b, the optimum value of $\bar{k}$ will be about 3[a]. The sample resolution for this value of $\bar{k}$ ($R_{s\,\mathrm{opt}}$) can then be related to the resolution R_s' of our initial run (as in Fig. 3) as

$$R_{s\,\mathrm{opt}} = 0.75[\bar{k}+1)/\bar{k}]\; R_s' \tag{6}$$

Likewise, N_0 can be assumed to vary with flow-rate F as

$$N_0 \approx \text{constant}/F \tag{7}$$

for conditions typical of preparative HPLC[b]. The optimum flow-rate F_2 relative to the initial flow-rate F_1 (as in Fig. 1) is then

$$F_2 = (R_{s\,\mathrm{opt}}/1.7)^2\; F_1 \tag{8}$$

Eqn. 8 follows from eqns. 5–7, with the preferred value of R_s (for a small sample) being 1.7, *i.e.*, the same as for isocratic runs[4]. The optimum flow-rate F_2 in subsequent runs is also limited by the maximum column pressure that we are willing to accept. For the moment it is assumed that we are not going to change the column length.

If the flow-rate for the next two runs (as in Fig. 2) is changed, the gradient time must be varied inversely, in order to maintain optimum values of $\bar{k}$ ($3 < \bar{k} < 9$). At this point we have chosen the most favorable conditions for further separation as in Fig. 2: initial and final %B, flow-rate, gradient times (differing by a factor of 3) for the next two (small sample) runs.

Step 3: second and third small-sample runs

Having established favorable conditions for further separations, two small-sample runs under these conditions are carried out (as in Fig. 2). From these two runs we can determine the separation characteristics of our sample as defined by the sample parameters S and k_w for both the product band and the adjacent, early-eluting impurity (see discussion in refs. 10 and 12). It is thus possible to estimate small-sample retention times (t_g) and bandwidths (W_0) as a function of gradient time. When values of S are different for the two bands, α will vary with gradient time, in turn leading to

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[a] Unreported data.

[b] For example, mol. wt. = 1000–30 000, 10–20-μm particles, 25-cm columns, pressures of 1000–2000 p.s.i. These conditions correspond to $n \approx 1$; see ref. 13.

a marked increase in resolution (and production rate) for some value of t_G. This can be of critical importance in maximizing the production rate for a given sample (see Part III).

Steps 4 and 5: fourth run with a large sample; estimation of production rate and sample size vs. gradient time and column dimensions

One of the latter small-sample runs (as in Fig. 2) with optimum gradient conditions is now repeated with a larger sample[a]. If the width W of the product band is measured, a value of W_{th} can be determined from eqn. 1 in Part I[3]. Eqn. 9 in Part I[3] (assume $z \approx 0.6$) can then be used to determine values of W_{th} for other sample sizes and gradient times (which determine a value of b). This in turn permits the estimation for a given gradient time of (a) the sample size that will result in touching bands and (b) the production rate corresponding to that gradient time. The production rate can then be mapped against gradient time and a "best" gradient time can be selected.

This treatment can be extended to the case of longer or wider columns, using various relationships from preceding papers[3,4,13].

Step 6: verification of optimum run

The best conditions arrived at in the above fashion can be evaluated in a final experimental run. The predicted separation should be close to that observed, although small adjustments in sample size may be needed at this point.

EXPERIMENTAL

Equipment

The HPLC system was a Beckman System Gold liquid chromatograph equipped with a Model 126 programmable solvent module, a Model 166 programmable UV detector module (Beckman Instruments, San Ramon, CA, U.S.A.), a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector and a Model A-318 precolumn filter (Upchurch Scientific, Oak Harbor, WA, U.S.A.).

Reagents

Solvents were HPLC-grade acetonitrile (ACN) (American Burdick & Jackson, Muskegon, MI, U.S.A.), triethylamine (TEA) (J. T. Baker, Phillipsburg, NJ, U.S.A.) and HPLC/spectro grade trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.). A Milli-Q Plus system (Millipore, Bedford, MA, U.S.A.) was used for water purification and filtration. Solvents were degassed by helium sparging prior to and during use. Cytochrome *c* type V from bovine heart, type XVIII from dog heart and type III from horse heart were obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification.

Column

A 25 × 0.46 cm I.D. Zorbax BioSeries Protein PLUS column was used, packed

[a] It is possible to estimate a convenient sample size based on the approximate constancy of w_s values for proteins (10–20 mg for a 15 × 0.46 cm I.D. column) (eqn. 8 values from Table II in ref. 3).

with nominal 6-μm particles bonded with dimethylpropylchlorosilane (C_3) (DuPont, Wilmington, DE, U.S.A.).

Chromatographic conditions

Mobile phase A was prepared by combining 50 ml of ACN, 950 ml of water, 1.392 ml of TEA and 1.742 ml of TFA. Mobile phase B was prepared by combining 200 ml of water, 800 ml of ACN, 1.392 ml of TEA and 1.892 ml of TFA. Because each mobile phase contained a certain percentage of both ACN and water, actual gradient programs were designed to give the acetonitrile (%B) compositions indicated in the various figure captions, *i.e.*, a 30–38% B gradient begins at 30% (v/v) acetonitrile and ends at 38% (v/v) acetonitrile (with all other mobile phase components in proportion). The temperature of the column was maintained at 30°C with an oven. The detection wavelength was 220–240 nm for small samples and 254 nm for large samples.

Software

The BIOPREP program is available from the Medical Products Department of DuPont or from LC Resources.

RESULTS AND DISCUSSION

Development of a method for the purification of a cytochrome c sample using the BIOPREP program as a guide

The BIOPREP program is based on the preceding discussion under Theory, and the following example parallels that general treatment. Thus, when it is stated that "BIOPREP recommends ...", the recommendation is based on the Theory section; that is, there are no "black-box" features in the BIOPREP program; for further details, see ref. 14.

As an illustration of our computer-assisted approach, we shall describe the development of a preparative HPLC procedure for the purification of a sample of crude bovine cytochrome *c* (BCc; mol. wt. 13 300 Da). The sample was formulated as a mixture of BCc with smaller amounts of two "impurities", horse cytochrome *c* (HCc) and dog cytochrome *c* (DCc). The separation of this mixture by reversed-phase isocratic HPLC was previously reported by Terabe *et al.*[15].

For an unknown sample, the usual approach is to begin with an exploratory separation using a broad-range gradient. BIOPREP can be requested to suggest conditions for this initial run, *e.g.*, 5–60% acetonitrile–water (0.1% TFA) for a peptide or protein sample (this will elute most peptides and proteins). The resulting separation for the cytochrome *c* sample is shown in Fig. 3. The column packing was DuPont Zorbax BioSeries Protein PLUS as 5-μm particles in a 25 × 0.46 cm I.D. column. BIOPREP allows the user to enter data from this exploratory separation (Fig. 3) for an estimate of the optimum run conditions for later runs required by BIOPREP. These data from Fig. 3 are summarized in Table I.

On continuing computer simulation with the aid of BIOPREP, we are advised (eqn. 6) that there is potentially excess resolution for a maximum production rate. That is, a higher flow-rate (eqn. 8) or a shorter column is advisable. At this point we are asked to define a maximum column pressure; 2000 p.s.i. is our choice for the present example. The larger the allowable column pressure, the greater is the potential

Fig. 3. Initial separation of cytochrome *c* mixture (85% BCc, 10% HCc and 5% DCc) by reversed-phase gradient elution. Conditions: column, 25 × 0.46 cm I.D. 5-μm DuPont Zorbax Bioseries Protein PLUS; 5–60% acetonitrile in water (plus TEA and TFA) gradient in 20 min; flow-rate, 1 ml/min; ambient temperature; 5 μg injected (small sample).

production rate. Given this maximum pressure, BIOPREP next recommends conditions for two more small-sample runs on the starting (small-diameter) column: gradients of 30 to 38% B in 10 and 30 min, at 2.5 ml/min (all other conditions remaining the same as those used for the run in Fig. 3). These latter runs will allow BIOPREP to map retention as a function of gradient steepness, which is a parameter of major importance in the maximization of production rate.

TABLE I

SEPARATION DATA FROM INITIAL EXPLORATORY RUN IN FIG. 3 (CYCTOCHROME *c* SAMPLE) FOR ENTRY INTO BIOPREP

Parameter	*Value*	*Parameter*	*Value*
System dwell volume	2.3 ml		
Column length	25 cm	Gradient time	20 min
Column I.D.	0.46 cm	Bandwidth Y^a	0.24 min
Flow-rate	1.0 ml/min	Sample mol. wt.	14000 Da
Initial B concentration	5.0%		
Final B concentration	60.0%		
Retention times	First impurity 16.43 min, product band 16.92 min		

[a] Baseline bandwidth W_b.

The two runs recommended by BIOPREP are shown in Fig. 4. On entering the retention data for these two runs into BIOPREP, the computer recommends[a] a fourth experimental run with a larger sample, *i.e.*, 0.5 mg, with the same conditions as in Fig. 4A. This experimental run is shown in Fig. 5. The sample weight and width of the product band (BCc) are entered into BIOPREP, and the program is now able to

[a] Based on an estimated w_s value for the column; 10–20 mg of a protein sample for a 15 × 0.46 cm I.D. column. This fourth run should give a significant increase in W but maintain $R_s > 1$.

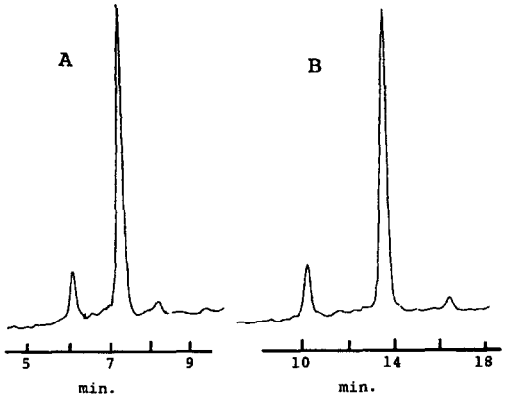

Fig. 4. The subsequent two small-sample runs for cytochrome *c* sample. Conditions: column as in Fig. 3; 30–38% acetonitrile in water in (A) 10 min and (B) 30 min; flow-rate 2.5 ml/min; pressure, 2000 p.s.i.; 5-μg sample.

predict the sample size and production rate for touching-band separation as a function of gradient time. This information, summarized in Table II, includes a 1-min column equilibration with 30% acetonitrile–water (recommended by BIOPREP[a]).

According to Table II, the maximum production rate is predicted for a gradient time of about 2 min. We arbitrarily chose a 2.5-min gradient with our 25-cm column (production rate 14 mg/h for a 25 × 0.46 cm I.D. column).

The conditions that we have selected allow for injection of a 0.8-mg sample. Assuming that we need to purify a larger amount of the product (BCc), we can automate the separation for repetitive injections as described in ref. 17. The resulting separation (three injections in series) is shown in Fig. 6. Touching-band separation is observed, confirming the predictions of the BIOPREP software. Continuous sample

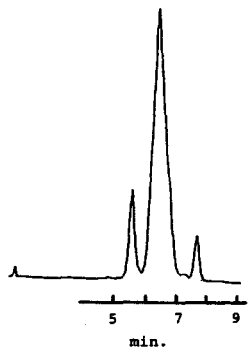

Fig. 5. Separation of a large sample (0.53 mg) of cytochrome *c* sample under conditions of the 10-min run of Fig. 4A.

[a] The recommended column equilibration time is based on 15 column volumes for a full-range (5–100% B) gradient, and proportionately less for a narrow-range gradient[16].

TABLE II

SAMPLE SIZE AND PRODUCTION RATE AS A FUNCTION OF GRADIENT TIME AND COLUMN LENGTH FOR SEPARATION OF CYTOCHROME *c* SAMPLE

Gradient time (mm)	*Sample size (mg)*	*Production rate (mg/h)*[a]
1.6	0.66	15
2.3	0.76	14
3.2	0.79	11
6.6	0.73	5.8
13.5	0.62	2.6
27.5	0.50	1.1

[a] Includes 1 min of column-regeneration time.

injections would be expected to result in the purification of 14 mg/h of BCc (taking column equilibration into account).

For an increased production rate, columns of larger diameter can be used. BIOPREP provided estimates of the optimum sample size and flow-rate for columns of other dimensions, based on constant values of w/w_s and mobile phase velocity u. Thus, a 2-in. I.D. column with a flow-rate of 300 ml/min is predicted to yield 2 g/h of purified product. These scale-up predictions are easy to calculate manually, but BIOPREP offers a more convenient alternative.

CONCLUSIONS

On the basis of the preceding discussions of touching-band separation, it is possible to develop systematically a reversed-phase gradient elution procedure for maximum production (g/h) of purified peptide or protein products. This requires attention to several aspects of the separation: (a) optimum values of α and $\bar{k}$, which can

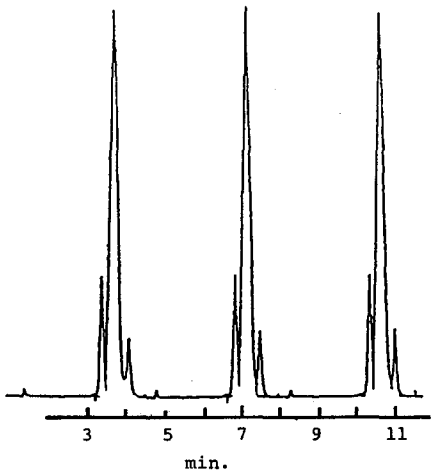

Fig. 6. Repetitive separations of cytochrome *c* sample with touching bands (conditions predicted by BIOPREP). Conditions as in Fig. 4, except 2.5-min gradient, 1-min re-equilibration with 30% B and 0.8-mg sample.

be varied by changing the gradient steepness; (b) an optimum value of N, which can be varied by changing the flow-rate (or column length and particle size, in some instances); (c) column dimensions, which allow a small-column separation to be scaled up.

This approach forms the basis of a commercially available computer program (BIOPREP) that is described here. Its application to the separation of a mixture of cytochrome *c* variants is shown as one example. BIOPREP allows the user to take advantage of our present knowledge of gradient-elution preparative HPLC so as to arrive quickly at reversed-phase conditions that are roughly optimum for the touching-band separation of a given peptide or protein sample. Further adjustment of the conditions can then be used to fine-tune the final separation and/or increase the production rate at the expense of product recovery.

REFERENCES

1 J. Rivier, R. McClintock, R. Galyean and H. Anderson, *J. Chromatogr.*, 288 (1984) 303.
2 E. P. Kroeff, R. A. Owens, E. L. Campbell, R. D. Johnson and H. I. Marks, *J. Chromatogr.*, 461 (1989) 45.
3 G. B. Cox, L. R. Snyder and J. W. Dolan, *J. Chromatogr.*, 484 (1989) 409.
4 L. R. Snyder, J. W. Dolan and G. B. Cox, *J. Chromatogr.*, 483 (1989) 63.
5 L. R. Snyder, G. B. Cox and P. E. Antle, *Chromatographia*, 24 (1987) 82.
6 L. R. Snyder and G. B. Cox, *LC · GC, Mag. Liq. Gas Chromatogr.*, 6 (1988) 894.
7 J. L. Glajch, M. A. Quarry, J. F. Vasta and L. R. Snyder, *Anal. Chem.*, 58 (1986) 280.
8 B. F. D. Ghrist, B. S. Silverman and L. R. Snyder, *J. Chromatogr.*, 459 (1989) 1, 25 and 43.
9 M. Kunitani, D. Johnson and L. Snyder, *J. Chromatogr.*, 371 (1986) 313.
10 J. W. Dolan, L. R. Snyder and M. A. Quarry, *Chromatographia*, 24 (1987) 261.
11 L. R. Snyder, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, p. 207.
12 L. R. Snyder and M. A. Stadalius, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 4, Academic Press, New York, 1986, p. 195.
13 L. R. Snyder and G. B. Cox, *J. Chromatogr.*, 483 (1989) 85.
14 *BIOPREP Users' Manual*, Medical Products Department, E. I. DuPont de Nemours & Co., Wilmington, DE, 1989.
15 S. Terabe, H. Nishi and T. Ando, *J. Chromatogr.*, 212 (1981) 295.
16 J. W. Dolan and L. R. Snyder, *LC · GC, Mag. Liq. Gas Chromatogr.*, 5 (1987) 971.
17 G. Franke and F. Verillon, *J. Chromatogr.*, 450 (1988) 81.