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Computer simulation as a tool for the rapid optimization of the high-performance liquid chromatographic separation of a tryptic digest of human growth hormone

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ABSTRACT

Computer simulation was used to optimize the separation of a tryptic digest of recombinant human growth hormone using reversed-phase high-performance liquid chromatography in a gradient mode. DryLab G/plus software modelled the retention behavior of the complex tryptic digest mixture as a function of gradient conditions, based on data from two experimental gradient runs. The theoretical optimum separation conditions were rapidly obtained and reproduced experimentally. Resolution did not simply increase as gradient steepness was decreased, rather, an intermediate gradient time provided maximum sample resolution. The simulation results also indicate that the method is reasonably rugged, with little change in the separation expected for different high-performance liquid chromatography systems, and changes in the separation can be compensated by a change in the gradient steepness. Computer simulation can also be useful to quickly reoptimize conditions for a new column, if it fails to provide the same separation.

INTRODUCTION

Peptide mapping by reversed-phase high-performance liquid chromatography (RP-HPLC) has become an important method for the characterization of recombinant DNA-derived proteins [1–4]. In this procedure, the protein is cleaved by a proteolytic enzyme, such as trypsin, to a number of peptides which are then separated by RP-HPLC. It has been shown in a variety of studies [5,6] that amino acid substitutions result in a substantial shift in the retention time of a given peptide. The map has been used to detect mistranslation events that result in the substitution of norleucine for methionine and to detect degradative processes such as deamidation, oxidation, and proteolysis [6,7]. Thus, reversed-phase peptide mapping is used for both the characterization of a novel protein product [8] as well as in quality control for the release of different production lots [2]. For these applications, it is important that the map is optimized so that the analyst is able to detect amino acid substitutions with a high probability [9].

The identification of minor new peptides is complicated by the fact that protein digests typically comprise a large number of "primary" peptides, plus a significant number of other reaction products present at lower concentrations. It is not unusual to see 50–100 distinct peaks in the final tryptic map chromatogram. The presence of lower level peptides can be related either to fragmentation of the enzyme during the digestion or to secondary cleavages of the primary peptides. The resolution of the primary peptides in such mixtures is usually a major consideration, but other peaks in the chromatogram are often of interest. For instance, the sep-

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aration of protein digests can be used to detect the presence of protein variants [3] usually at levels of 0.5 to 5%.

The complexity of protein digests and the variety of separation conditions that can be developed for HPLC in a gradient mode complicates the development of an optimized separation. A further problem is caused by changes in elution order that can occur with different gradient slopes, so that a longer gradient may give worse rather than improved resolution. A large number of experimental runs may be required before an acceptable chromatogram is obtained and during this time changes in column performance are common. The result is a considerable expenditure of time, materials and sample, with possible uncertainty in the final results. It is therefore rare that a final HPLC procedure has been "optimized" with respect to the goals of the separation (resolution, run time, etc.). The possibility that peaks of potential interest remain unresolved (and undetected) is also a concern.

An alternative to this traditional approach to HPLC method development for reversed-phase gradient elution is the use of computer simulation [10]. With this technique the data from two or three experimental runs are entered into a personal computer (PC), and separation can then be predicted as a function of gradient conditions. Further iterations via computer simulation are used to eventually arrive at an optimized separation, while minimizing the problems of trial-and-error experimentation.

Computer simulation has been applied previously for the HPLC separation of both peptides [10] and proteins [11–14] with the primary objective of resolving the major components of the sample. In the present study we have shown that the optimization of the tryptic map of recombinant DNA-derived human growth hormone (rhGH) is a complex task and that computer simulation can greatly aid this process. Another commonly encountered problem is a change in the separation when different operators or equipment are involved in a quality control program. This study explores the use of computer simulation to correct for changes in mobile phase composition, column-to-column variations and alterations in dwell volume. Computer simulation can also be used to readily reoptimize the separation for a given peptide, a feature which is particularly useful in the characterization of a protein variant.

EXPERIMENTAL

Equipment and materials

HPLC separations were carried out on an HP 1090 with autosampler (Hewlett-Packard, Palo Alto, CA, USA) and a diode array detector; the dwell volume was determined equal to 2.3 ml [15]. All solvents were of HPLC grade.

Procedure

Gradient separations were carried out using 0.1% trifluoroacetic acid (TFA)–water as solvent A and 0.08% TFA–acetonitrile as solvent B at a flow-rate of 1.0 ml/min. The column temperature was 40°C and 100 μl of 1.0 mg/ml sample was injected. Two 15 × 0.46 cm I.D., 5-μm Nucleosil C_{18} columns with 100-Å pores (Alltech, Deerfield, IL, USA) from different manufacturing lots were used.

Sample

Tryptic digestion. A sample of rhGH (Genentech) South San Fransisco, CA, USA) was exchanged into 100 m*M* sodium acetate–10 m*M* Tris–1 m*M* calcium chloride, pH 8.3 with a final concentration of approximately 1 mg/ml. Samples were incubated at 37°C for a total of 4 h with the addition of trypsin (1:100 ratio by weight of trypsin to substrate) at time $t = 0$ and $t = 2$ h. The digest was stopped by the addition of phosphoric acid to a final pH of 2–3. The sample was stored at 2–8°C.

Digestion of rhGH by trypsin results in the release of 21 peptides. These peptides are sequentially numbered from the N-terminus (T1) through to the C-terminus (T21).

Software

The computer simulation software used in the present study is DryLab G/plus from LC Resources (Walnut Creek, CA, USA). It requires an IBM-compatible PC with 640K of RAM memory; a math coprocessor was used for fast computation.

RESULTS AND DISCUSSION

Previous work [10,16] has shown that the separation of protein digests by reversed-phase gradient elution can be quite sensitive to gradient conditions. In this study the least resolved pair of peaks in a given separation will be defined as a "critical

peak pair". A resolution factor (R_s) equal to 1.5 would result in baseline resolution of a peak pair. A change in gradient slope can often be used to alter the peak spacing within the chromatogram, leading to major improvements in the resolution of critical peak pairs. By varying gradient steepness for different parts of the chromatogram (multi-segment gradients), it may be possible to achieve a separation that is better than any linear (single-segment) gradient [11–14].

Resolution of major components in the hGH digest

Gradient separations were initially carried out from 0–60 % B in 30 and 120 min, in order to obtain the necessary data for computer simulation. These chromatograms are shown in Fig. 1.

Peak tracking. It is necessary to match the peaks between the two chromatograms (referred to as peak tracking [17]) before beginning computer simulation, as summarized in Table I for all peaks with areas >0.5% of the total sample. In Table I the peak areas show that there are three unresolved doublets in the 30-min run, and peaks 9 and 10 are reversed in the 120-min run. The assignment of the various peaks is indicated in Table I, including two minor "unknown" peaks. Peak tracking as in Table I can be done manually, but we have also used recently developed software to accomplish this by computer [18]. The ease of peak tracking for protein digests is very much dependent on the reproducibility of peak areas from run to run (about ±5% in the runs of Table I), which in turn depends on the quality of the baseline (*i.e.*, blank gradient). A complete digestion of the protein with minimum formation of artifacts, such as non-specific cleavages or contamination with fragments of the protease, is also desirable.

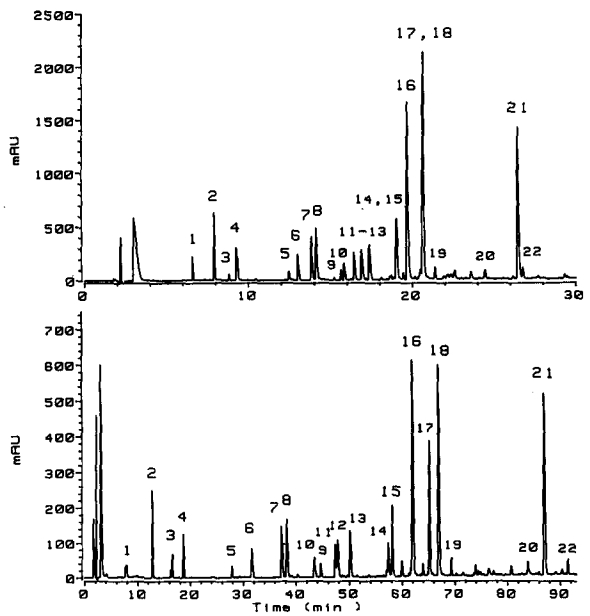

Fig. 1. The experimental separations of the tryptic digest of rhGH used as input for computer simulation. These were performed on a 5-μm Nucleosil C_{18} column (15 × 0.46 cm I.D.) with a mobile phase A of 0.1% TFA–water and a mobile phase B of 0.08% TFA–acetonitrile. The flow-rate was 1 ml/min and the column temperature was 40°C. The 100-μg samples were loaded in 100-μl of digest buffer and monitored at 220 nm. One separation (top) was a linear gradient from 0–60% B in 30 min and the other separation (bottom) was a linear gradient from 0–60% B in 120 min.

Inaccurate peak matching or a failure to account for all the peaks of interest can occur for chromatograms as complex as those of Fig. 1. In order to insure that errors of this kind have been avoided, a third experimental run with an intermediate gradient time (*e.g.*, 60 min) was carried out. Comparison of the resulting chromatogram with that predicted by computer simulation can uncover certain errors. As seen in Table II and Fig. 2, all major

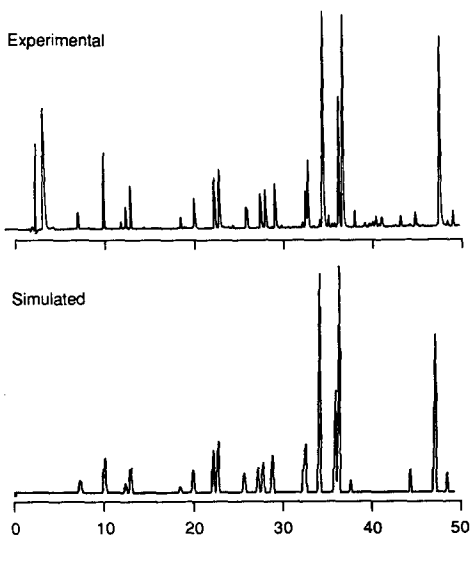

Fig. 2. Comparison of experimental and computer-simulated rhGH tryptic digest separations for 0–60% B in 60 min. Other conditions as in Fig. 1.

TABLE I

TRACKING OF THE MAJOR PEAKS IN THE TWO RUNS OF FIG. 1

Compound[a]	Run 1 (30 min)		Run 2 (120 min)	
	Retention time (min)	Area	Retention (min)	Area
1 T7	6.6	87	7.9	93
2 T14	7.9	245	12.8	238
3 T14c	9.3	225[b]	16.5	68
4 T12	9.3	225[b]	18.7	151
5 T10a	12.5	47	27.9	44
6 T13	13.0	152	31.6	164
7 T20–21	13.8	247	37.2	242
8 T15	14.1	304	38.2	306
9 T8	15.6	46	44.6	49
10 T17–18–19	15.8	99	43.4	99
11 T2	16.5	159	47.3	148
12 T18–19	16.9	186	47.8	199
13 T1	17.4	236	50.1	241
14 T10bc	19.1	429[b]	57.3	163
15 T11	19.1	429[b]	58.1	269
16 T4	19.7	1113	61.9	1131
17 T10	20.2	1527[b]	65.1	562
18 T6–16	20.2	1527[b]	66.8	1040
19 T6–16c	21.4	63	69.3	58
20 Unknown	24.5	72	83.9	80
21 T9	26.5	951	86.9	990
22 Unknown	26.8	73	91.3	87

[a] The tryptic peptides are numbered from the amino terminus so that T1 contains residues 1 to 7. The letters a, b and c refer to peptide fragment [8]. The numbers refer to the peak assignment in Fig. 1.

[b] These peaks were not resolved and the total peak area was recorded.

peaks are accounted for, and the predicted retention times match those for the 60-min gradient within an average error of ±0.1 min (±0.3%). We can therefore assume that no errors in peak matching have occurred in the assignments of Table I.

Optimizing gradient steepness. Computer simulation requires entry into the computer of the run conditions, retention times and peak areas from the experiments of Fig. 1. Correction for differences in column plate number can also be made by entering the actual resolution of a peak pair for a specific gradient time. Peak pair 11/12, (R_s = 0.87 in the 120-min run), was selected in this case and the resulting average plate number for the present separations of the rhGH digest was N = 3600. The adjusted column plate number allows the simulation of chromatograms which will closely match experimental runs.

The next step is to generate a resolution map, as shown in Fig. 3 for the present sample. Here the resolution (R_s) of the most-overlapped or critical peak pair is plotted against gradient time for a starting mobile phase of 0% B. The numbering on the plot in Fig. 3 identifies the critical peak pair for each value of % B/min, and it is seen that different peaks become "critical" for a change in gradient time. For example, for gradient times between 40 and 200 min, the critical peak pairs are 9/10, 11/12, and 14/15. Segments of simulated chromatograms illustrating the critical peak pairs at several gradient times in the resolution map are shown in Fig. 4. An examination of Figs. 3 and 4 shows that sample resolution does not simply increase as gradient steepness (time) is decreased. Peaks 9 and 10, for example, co-elute (R_s = 0) for a gradient time of 45 min, with peak 10 eluting first for shorter gradient times and peak 9 eluting first for longer gradient times (see Fig. 4). Alternatively, peaks 11 and 12 are found to be well resolved at shorter gradient times and actually decrease in resolution with increasing gradient time, until they co-elute at a 180-min gradient time. In most cases, there will be an intermediate gradient time that provides maximum resolution of the initial peak pair.

Maximum resolution is indicated for a gradient time of 74 min (R_s = 1.3, %B/min = 0.6) or 330 min (R_s = 2.0, %B/min = 0.1). Normally the steeper gradient would be preferred (0.6% B/min, 74 min), because of the considerably shorter run time and narrower peaks for easier detection. The predicted and experimental chromatograms for the optimal linear gradient are shown in Fig. 5. The experimental retention times match predicted values within an average error of ±0.2 min (±0.7%). The observed resolution for the critical peak pairs 11/12 and 14/15 was R_s = 1.1 *vs.* 1.3 predicted. The slightly lower experimental resolution can be attributed to a decrease in the column plate number during the one month that elapsed between the runs of Fig. 1 and the run of Fig. 5. This emphasized the fact that changes in performance can be expected during continued use of the column (see later discussion).

Initial mobile phase composition (%B). Often it is found that the maximum possible resolution is de-

TABLE II

CONFIRMING THE PEAK ASSIGNMENTS OF TABLE I

Experimental and predicted retention times for a 0–60% B gradient in 60 min.

Band	Retention times (min)		
	Calc.	Expt.	Difference
1	7.3	7.0	0.3
2	10.1	9.8	0.3
3	12.4	12.3	0.1
4	13.0	12.8	0.2
5	18.5	18.4	0.1
6	20.0	19.9	0.1
7	22.2	22.2	0.0
8	22.7	22.7	0.0
9	25.7	25.7	0.0
10	25.8	25.8	0.0
11	27.3	27.3	0.0
12	27.9	27.9	0.0
13	29.0	29.0	0.0
14	32.4	32.4	0.0
15	32.6	32.7	−0.1
16	34.2	34.3	−0.1
17	36.0	36.1	−0.1
18	36.4	36.5	−0.1
19	37.8	37.9	−0.1
20	44.5	44.6	−0.1
21	47.3	47.3	0.0
22	48.7	48.8	−0.1
Av. error		±0.1 min (±0.3%)	

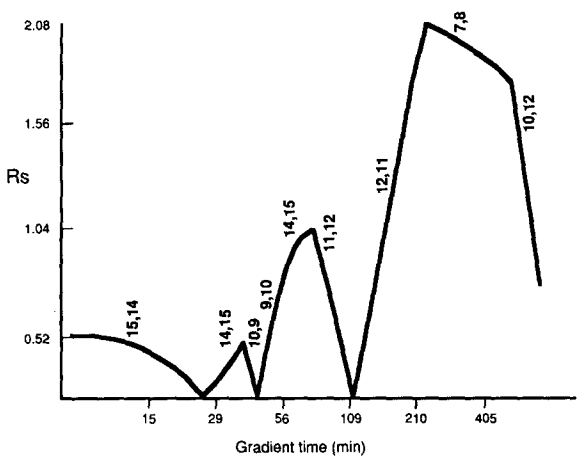

Fig. 3. The relative resolution map generated using the retention time data from the chromatograms in Fig. 1. The resolution (R_s) of the predicted critical peak pair is plotted *vs.* gradient time. The identity of the critical peak pair is indicated by numbers above the line. The column used in this separation had an N = 3600.

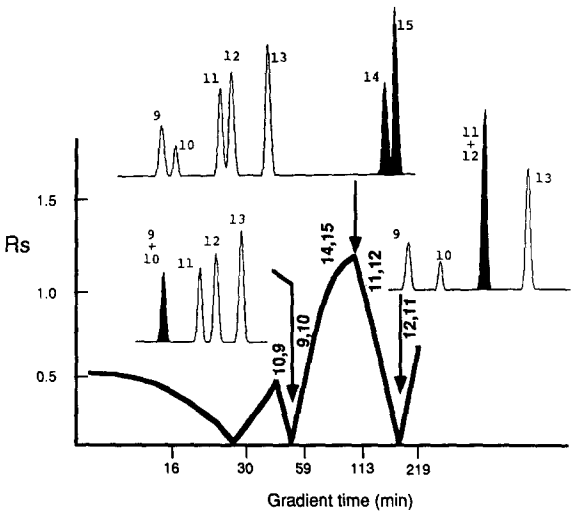

Fig. 4. The relative resolution map with critical peak pairs illustrated at several gradient times by relevant segments of simulated chromatograms. An $R_s > 1$ for 14, 15 at a gradient time between 70 and 110 min results in good resolution of the critical peak pair and indicates a region in the resolution map where the gradient time would be optimal for good resolution of all the peptide peaks.

pendent on the choice of the initial mobile phase %B (see Figs. 4–6 of ref. 19).

The best choice of the initial mobile phase is easily addressed via computer simulation. Resolution maps were generated for 2, 5, and 7.5% B as starting mobile phase, similar to the map of Fig. 5 for 0% B. The maximum possible resolution for run times of less than 5 h was found to vary from R_s = 1.25 to 1.28 between 0 and 5% B ($R_s < 0.7$ for 7.5% B). These data make it clear that the initial mobile phase concentration should be $\leqslant$5% B, but resolution is essentially the same for 0–5% B. Run time decreases from 74 min for 0% B to 65 min for 5% B, favoring a higher value of %B. Changes in the retention of early peaks are shown in Fig. 6 as a function of %B, with experimental chromatograms shown for comparison. These data suggest that beginning the gradient with 2% B will avoid any problems with the early elution of initial peptide peaks, since in comparative mapping applications it is important that the polar peptides are retained sufficiently for separation, so that any alteration in these peptides can be detected. These conditions could also be an advantage because the time required for column equilibration is often less when the initial

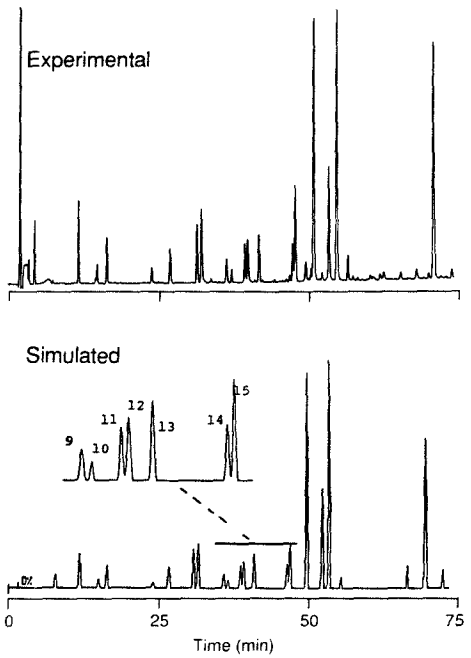

Fig. 5. A comparison of the predicted optimal linear gradient in a minimum run time *vs.* the experimental run. Conditions: 0–47% B in 74 min; other conditions as in Fig. 1.

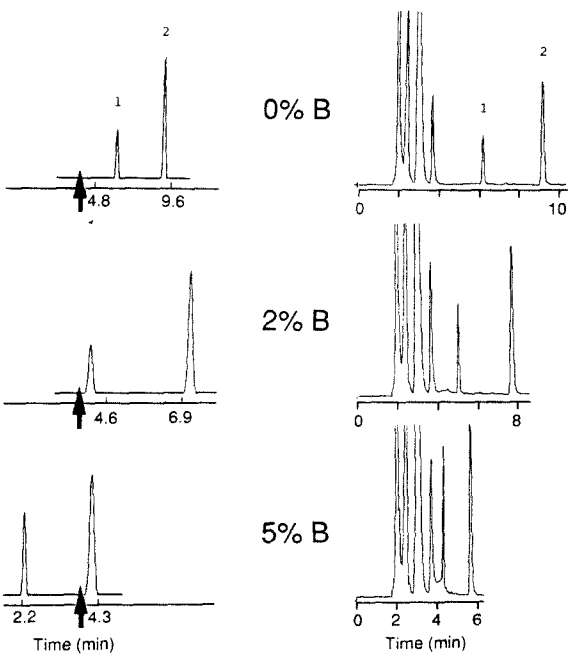

Fig. 6. The effect of starting mobile phase composition of the separation of early rhGH tryptic peptide peaks. The arrows on the simulated chromatograms (left) indicate the minimum retention time for peaks before the void peaks in the experimental runs (right) would interfere with detection. Other conditions as in Fig. 2.

mobile phase contains some organic solvent (*e.g.*, 2% B *vs.* 0% B).

Use of segmented gradients. The run time for the separation in Fig. 5 can be further shortened by noting that peaks eluting after the last critical pair (14/15) are all well resolved. This suggests the use of a steeper gradient after the elution of the latter peaks at 48 min. Computer simulation allows the evaluation of a number of 2-segment gradients, with an improved separation shown in Fig. 7. The run time is reduced from 74 min in Fig. 5 to 53 min. The experimental chromatogram (top) compares well with the predicted separation (bottom), and retention times agree within ±0.2 min or ±0.7% (average error). The total effort required for the development of an optimized separation such as this should be about two days for experimental runs plus a few hours of computer simulation.

Other workers have shown [20] that changes in the column, mobile phase pH and/or concentration of an ion-pair reagent, etc. may provide greater control over the separation of polar peptide mixtures compared to the variation of gradient steepness. This may be true for some samples, but changes in gradient conditions, especially when guided by computer simulation, will usually provide adequate separation of all but the most polar peptides. Hydrophobic ion-pairing reagents can be used in such cases [21].

Correction for changes in separation

The use of silica-based columns with low-pH mobile phases can lead to a loss of bonded phase and changes in sample retention [22]. Differences in the reversed-phase retention of peptide and protein samples are also found for columns from different production batches [11–13]. Once an optimized separation has been developed for a given sample (as in Fig. 7), it is not uncommon to find a loss of resolution for one or more critical peak pairs at some later time.

Column-to-column variations. These were studied by using an identical column (see Experimental) from a different manufacturing lot. Runs were car-

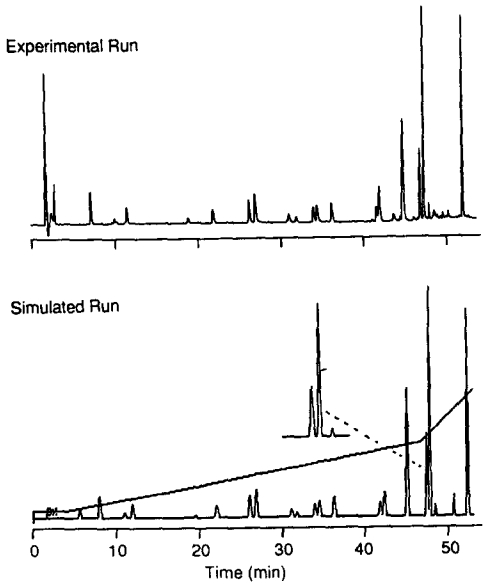

Fig. 7. The total run time of the optimal separation of rhGH tryptic peptides in Fig. 5 was reduced to 53 min by a segmented gradient of 2–32% B in 48 min, then 32–47% B in 5 min. The predicted chromatogram is compared to the actual run.

ried out from 0–60% B in gradient times of 60 and 240 min. It was found that the new column was slightly more efficient (N = 5000 *vs.* 3600 for the previous column), so a somewhat better separation was expected–other factors being equal. Confirmatory runs at 30 and 120 min were also made, with a average agreement between experimental and predicted retention times of ±0.2–0.3% (data not shown).

The resolution map of an hGH digest for the new column showed the same essential features as before, but minor differences existed which had a significant effect on separation. This is illustrated in Fig. 8, where the separation of peaks 7 through 15 are compared by computer simulations for the two columns. Fig. 8A shows the separation of the sample on the original column with the optimized conditions of Fig. 5. Under the same separation conditions as (A) it is apparent that the resolution of peaks 11 and 12 is not as good (R_s = 0.9 *vs.* 1.3) for the new column (B). The higher N-value of the new column tends to hide part of these differences in the two columns, as illustrated in Fig. 8C which shows the theoretical separation on the original column if the column had the same selectivity but with N = 5000 (R_s for bands 11/12 = 1.5). That is, the differences in the separations are due to differences in selectivity for the two columns.

Computer simulation can be repeated with a new column so as to reoptimize the separtion. When this was attempted with the new column, it was found that a minor change in the original gradient (2–28% B in 37 min, then 28–46% B in 13 min *vs.* 2–32% B in 48 min, then 32–47% B in 5 min) increased the resolution of the critical pair to R_s = 1.1 *vs.* R_s = 0.9. This is somewhat less than found for the original column (R_s = 1.3), but is probably adequate for the present application.

Separation using a different HPLC system

The use of different HPLC equipment can result in a change in separation when using gradient elution [23,24]. Therefore, a procedure that is intended for use by others should be evaluated for system dependence as a part of method development. The primary cause of system-to-system differences in

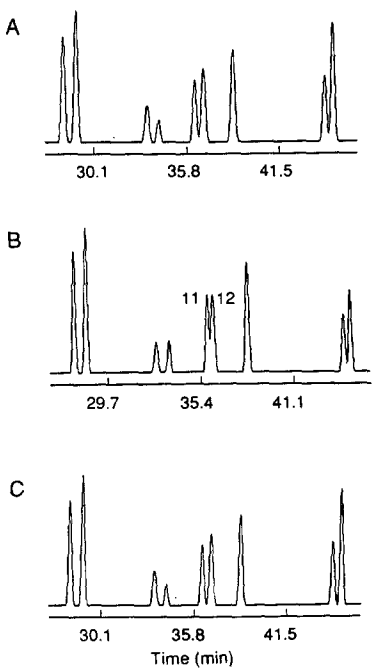

Fig. 8. The effect of a change in column efficiency on the separation of rhGH tryptic peptides. Computer simulations show: A, an optimized separation of peaks 7–15 using the first column and N = 3600 (conditions, Fig. 5); B, the same separation with a second column and N = 3600; C, the same separation with a first column and N = 5000.

gradient separation is a difference in *dwell volume* (V_D) (the volume from the gradient mixer to the column inlet). The effect of a change in dwell volume is usually to shift all peaks in the chromatogram to higher or lower retention times, in some cases with a change in relative retention. This can lead to two kinds of problems: (a) loss in resolution for early-eluting peaks and (b) confusion as to the identity of various peaks in the chromatogram.

Computer simulation can be used to evaluate system-to-system reproducibility, as illustrated in Fig. 9. The chromatogram labeled "V_D = 2.3 ml" corresponds to our optimized separation (Fig. 7) carried out on the present HPLC system (HP 1090 with autosampler, V_D = 2.3 ml). Other HPLC systems can often have dwell volumes as large as 10 ml [23], depending on the system components. Fig. 9 also shows a computer simulation for a system having a much larger dwell volume (V_D = 10 ml). The data of Fig. 9 demonstrate that an increase in V_D results in the later elution (by 2–8 min) of all peaks, and peaks 1–4 are more spread out for V_D = 10 ml. However the relative retention of peaks 5–22 is unchanged, and the resolution of critical peak-pairs (9/10, 11/12, 14/15) remains the same. These predictions are confirmed in the experimental runs on two different HPLC systems, where the dwell volumes have been adjusted to match those of Fig. 9 (data not shown).

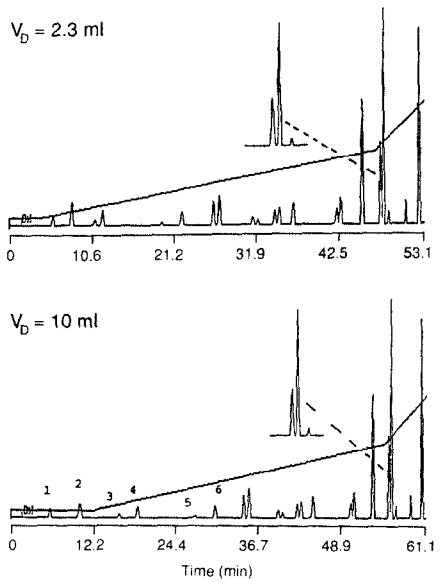

Fig. 9. A simulation of the effect of equipment dwell volume (V_D) on the optimized separation (as shown in Fig. 7) of the rhGH tryptic digest.

Optimization of the separation of a specific peptide

Computer simulation lends itself to a variety of other separation goals, such as maximizing the resolution of a given peak in the chromatogram for subsequent preparative isolation. Computer simulation can be used to explore such possibilities, as illustrated by peak 11 of the rhGH digest. The separation of Fig. 5 and 7 has been optimized in terms of a maximum resolution for the poorest-resolved peak pair in the sample. As a result, the resolution of peak 11 is R_s = 1.3. If peak 11 is the only component of interest, however, its separation from adjacent peaks can be considerably improved. In this case it is useful to generate a *partial resolution map*, where the resolution of peak 11 from adjacent peaks is plotted *vs.* gradient time (Fig. 10, above). The latter map shows that a resolution of R_s = 2.2 can be achieved for peak 11 in a run time of only 25 min. The predicted separation in Fig. 10, (below) shows that it is possible to greatly improve the separation of peak 11 when other components of the sample can be ignored. Advance knowledge of the appearance of the chromatogram by computer simulation will also greatly aid in the isolation of the correct peak(s) in a preparative run.

CONCLUSIONS

Computer simulation was used to optimize gradient conditions for the RP-HPLC separation of a tryptic digest of human growth hormone. Twenty two major bands were resolved almost to baseline (R_s = 1.3) in a run time of 53 min. The effort required for method development was relatively minor: four experimental runs plus a few hours of computer time.

Changes in separation due to a change in column or HPLC equipment were also studied via computer simulation. It was found possible to correct for column-to-column differences so as to maintain acceptable separation. Even less time was required for reoptimization because the validity of the computer simulation had already been established. Similarly, computer simulation indicated that the present separation should be consistent when using different

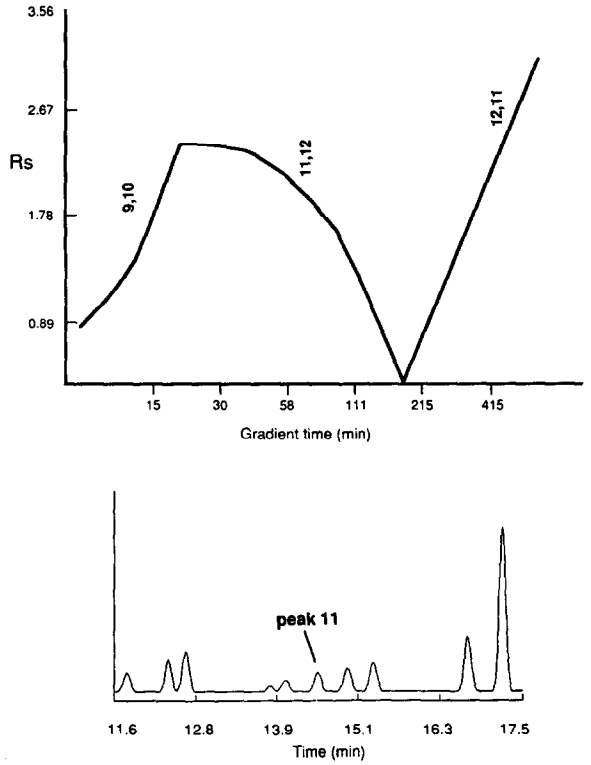

Fig. 10. The optimized separation of peak 11 of the rhGH tryptic digest sample. The partial resolution map for peak 11 is shown above and the predicted separation for 0–60% B in 25 min is shown below. Other conditions as in Fig. 1.

HPLC systems. This was verified experimentally. Computer simulation can also be used to optimize the separation of a single sample component, prior to its isolation or purification from other peaks in the chromatogram.

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