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High-performance liquid chromatographic separation of the impurities in a pharmaceutical raw material with the aid of computer simulation

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Abstract

A complex pharmaceutical raw material was characterized by means of reversed-phase gradient elution. By varying gradient steepness and mobile-phase pH, it was possible to optimize band spacing so as to separate 16 impurities or degradation products from the drug substance. Computer simulation was useful in interpreting these complex chromatograms and determining the maximum number of peaks that could be separated in this way. A marginal separation of all 17 sample components could be obtained, but the resulting method was quite pH-sensitive and therefore not very rugged. As an alternative, a rugged method was developed that separates the drug substance from all other sample components. The present study also describes how present computer simulation software for isocratic separation can be used to predict resolution for gradient elution runs as a function of pH.

Keywords: Pharmaceutical analysis; Computer simulation; Retention prediction

1. Introduction

Raw materials for pharmaceutical and other formulations can be analyzed for possible impurities by means of HPLC. Regulatory agencies such as the FDA usually require that all impurities present in >0.1% w/w be separated and quantitated. Such samples may contain a dozen or more impurities, in which case the complete separation of the sample can be a formidable challenge. The possibility that one or more impurity peaks will overlap the product peak and go unnoticed is a further concern, especially since standards are not usually available for the various impurities. In the present investigation, computer simulation [1,2] was used to facilitate the development of a final HPLC procedure for a raw material analysis.

2. Experimental

2.1. Equipment and materials

The HPLC system was an HP 1090 Series II/HPLC-ChemStation (Pascal) (Hewlett-Packard, Waldbronn, Germany). The dwell volume [3] including the sample loop was 0.64 ml (manual injection). A photodiode-array detector was used with detection

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at 220 nm for the chromatograms shown in this paper. Peak characterization was also carried out using the HP-Spectra Library. A 11.9×0.40 cm Superspher 100 RP-18e column was used for all experiments. The column and all reagents were from Merck (Darmstadt, Germany).

2.2. Procedures

Initial experiments used gradient elution (1–20% B) (1% B was used to accelerate equilibration of the column). Solvent A was 20 m*M* NaH_2PO_4, pH 4.4 and solvent B was acetonitrile. The gradient time was varied (15 and 45 min) in order to determine the optimum gradient time (12 min). In subsequent experiments the gradient was constant (1–20% B in 12 min) and the pH of the solvent A was varied. All experiments used a flow-rate of 0.7 ml/min and a temperature of 35°C. Computer simulations were made using DryLab/Windows software (LC-Resources, Walnut Creek, CA, USA).

3. Results and discussion

3.1. Optimizing gradient steepness

Initial separation of the impurities in a raw material is best carried out with gradient elution, since the sample may contain components that elute very early or very late. This first run should use a wide gradient range (e.g. 0–100% B), but later runs can usually be shortened by adjusting the initial and final percentage of solvent B [4]. On the basis of the initial run, a final gradient range of 1–20% B was chosen. Further separation of the sample can be investigated by varying the gradient steepness or gradient time t_G. It is often found that band spacing or selectivity varies with gradient steepness, so that an optimum gradient steepness exists for a particular sample [2]. The determination of this optimum gradient steepness is most conveniently found by means of computer simulation [2].

Two experimental runs are performed with only gradient time varying; the information from these two runs is entered into the computer (DryLab software), and then different gradient conditions can be explored by computer simulation.

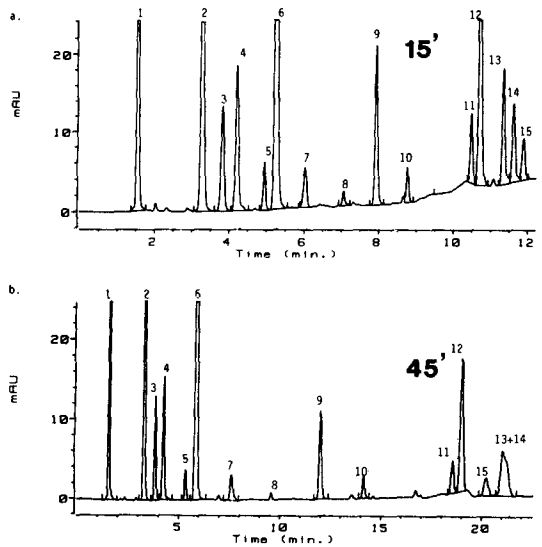

Fig. 1. Experimental chromatograms for the sample studied here. Two runs were initially used for computer simulation (to determine the optimum gradient time or steepness). Conditions: 1–20% B in 15 min (upper trace) and 45 min (lower trace), pH 4.40, respectively. Other conditions as in Section 2.

Fig. 1 shows the chromatograms for the two initial runs: 1–20% B in periods of 15 and 45 min, respectively. A total of 14 impurity peaks and the drug substance (peak no. 6) with areas >0.1% of the total peak area can be recognized in these two runs. The peaks are numbered 1–15 in each chromatogram of Fig. 1. Note that in the 45-min run peaks 13 and 14 are coeluting and the separation sequence of the last three peaks changes from 13–14–15 in the 15-min gradient to 15–13–14 in the 45-min gradient. The matching of compounds between the two runs in Fig. 1 was carried out automatically by means of the peak-tracking option of the DryLab software. Once the data from Fig. 1 are entered into the computer, a resolution map can be requested for this system (Fig. 2). Fig. 2 shows that the best separation (as measured by the least resolved or 'critical' band-pair) occurs for a gradient time of 9–15 min (arrow in Fig. 2). The 'optimum' gradient time was chosen as 12 min, in order to achieve a more rugged method (i.e. one less affected by gradient time or a change in gradient equipment). The predicted (simulated) separation for a 12-min gradient is shown in Fig. 3a and compared with the actual chromatogram in Fig. 3b. Good

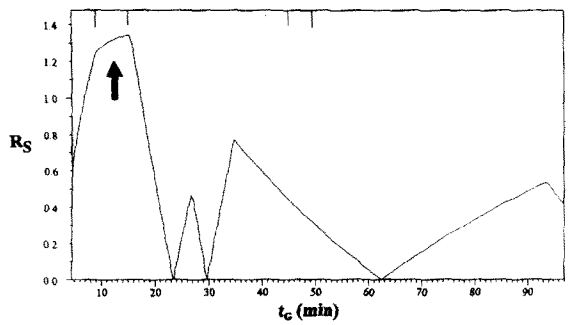

Fig. 2. Resolution map from separations of Fig. 1 (DryLab computer simulation). Arrow indicates preferred gradient time t_G for maximum resolution R_s.

agreement is observed between the two chromatograms; retention times agree within an average of 0.1 min. Note that a different sample was used for the separation of Fig. 3 vs. the sample used in Fig. 1.

3.2. Effect of pH on separation

The selection of a gradient steepness that provides maximum resolution of the sample often results in a complete separation. For a sample whose initial composition is unknown, however, this must be verified by other means. One approach is to change separation conditions so as to change selectivity. The effect of the eluent pH on the separation of weak acids and bases in reversed-phase chromatography is quite strong and can be used to modulate selectivity, as was shown by Horvath et al. [5]. The chemical structure of the present raw material suggested that a change in pH would be useful for this purpose [6,7]. Separations were therefore carried out at pH values of 4.0, 4.4 and 5.0, without change in the other conditions (1–20% B in 12 min). A different sample was used for these three runs (shown in Fig. 4). When developing a routine HPLC procedure for unknown samples such as this, it is useful to examine several samples, since some impurities may be present in one sample but not in another. The pH 4.4 chromatogram of Fig. 4 for this new sample shows the appearance of an additional impurity peak (no. 10a), with a total of 16 major peaks.

The separation at pH 4.0 in Fig. 4 gives a total of 17 major peaks. A new component (no. 5a) has been resolved from the original peak no. 5. In addition, this change in pH from 4.4 to 4.0 has resulted in a

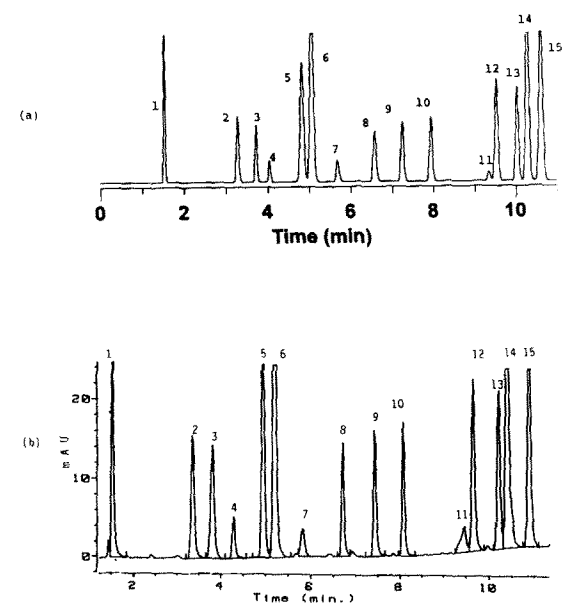

Fig. 3. Separation of present sample with an optimum gradient time (12 min): (a) DryLab simulation; (b) experimental run. Other conditions as in Fig. 1.

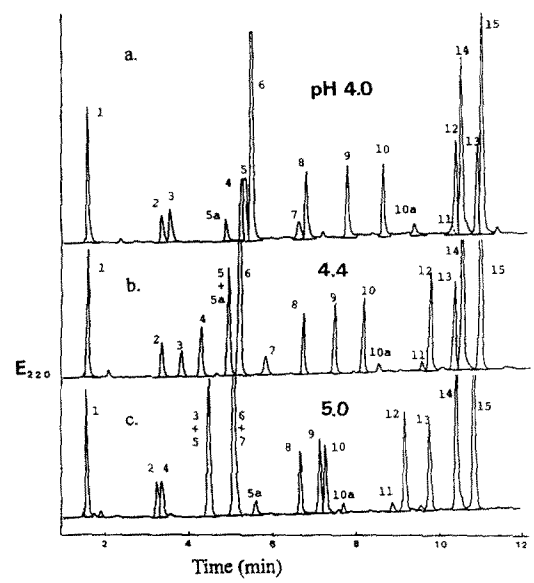

Fig. 4. Separation of present sample with mobile-phase pH varied. Other conditions as in Fig. 3 (1–20% B in 12 min). These runs were used for computer simulation with varying pH. One should note the numerous band spacing changes as a function of pH: peaks nos. 8–10 are moving closer, peaks nos. 11–15 are moving apart as pH increases from 4.0 to 5.0.

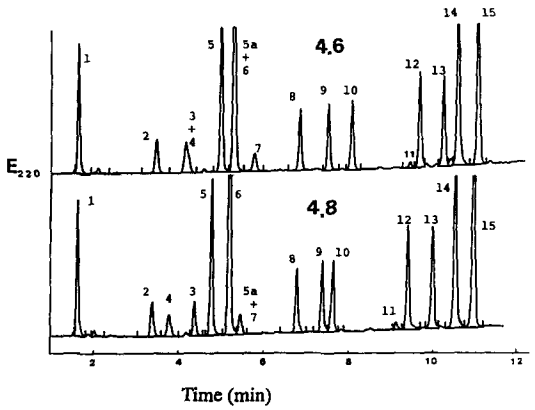

Fig. 5. Separation of present sample with mobile phase pH varied. Other conditions as in Fig. 4. These runs were used to evaluate the accuracy of computer predictions and to verify peak tracking.

number of other changes. Peak tracking for the three runs of Fig. 4 is somewhat complicated because of the large changes in band spacing, the similar size of some of the peaks, and the small size of other peaks. For these reasons, use was made of the diode-array spectra of these bands as a further aid in peak-tracking. When minor impurity peaks overlap larger peaks, however, it can be difficult to determine with certainty which bands overlap which. As will be shown, computer simulation can be used to minimize ambiguities of this kind.

Retention time predictions

The selection of an optimum pH for the separation of the present sample is in principle possible by means of computer simulation. Although the present DryLab software does not allow the direct optimization of pH for gradient runs, we have found that the DryLab software for isocratic separation can be used instead. Several retention models are available for this purpose, e.g., for variation of pH, temperature, buffer concentration, ternary-solvent composition, etc. The most suitable of these modes for application to gradient runs is that used for ternary-

Table 1
Accuracy of predicted retention times for a change in pH

Peak	Retention times (min)			
	pH 4.6		pH 4.8	
	Experimental	Calculated[a]	Experimental	Calculated[a]
#1	1.57	1.50	1.57	1.50
#2	3.40	3.28	3.32	3.25
#3	4.09	3.88	4.32	4.11
#4	4.09	3.82	3.73	3.52
#5	4.91	4.72	4.72	4.55
#5a	5.20	5.00	5.13	4.97
#6	5.20	5.04	5.40	5.24
#7	5.68	5.48	5.40	5.21
#8	6.74	6.68	6.72	6.65
#9	7.41	7.28	7.32	7.18
#10	7.96	7.82	7.57	7.33
#10a	[b]		[b]	
#11	9.34	9.22	9.07	8.99
#12	9.56	9.46	9.34	9.26
#13	10.12	10.06	9.92	9.86
#14	10.45	10.46	10.44	10.39
#15	10.92	10.84	10.87	10.77
Average error				
in t_R	0.13 min		0.13 min	
in Δt_R	0.07 min		0.06 min	

Separation of Fig. 5 (see text for details).
[a] Calculated using DryLab isocratic ternary-solvent model (Eq. 1) with experimental input data from three runs of Fig. 4.
[b] Peak no. 10a missing from this sample.

solvent simulation. This mode is based on a quadratic relationship for the retention factor k ($k = [t_R - t_o]/t_o$) vs. the volume fraction x of one binary-solvent mobile phase (e.g. 50% methanol–water) that is combined with a second binary-solvent mixture (e.g. 40% acetonitrile–water):

$$k = ax^2 + bx + c \quad (1)$$

Data for the three runs of Fig. 4 (pH 4.0, 4.4, 5.0) were entered into the ternary-solvent mode of DryLab (as $x = 40$, 44 and 50% B), following which retention could be predicted as a function of pH. As a check on the accuracy of Eq. 1, two additional runs were carried out at pH 4.6 and 4.8 (Fig. 5). Table 1 compares actual vs. predicted retention times for these latter two runs.

Retention times predicted using Eq. 1 (using the DryLab software) were found to be acceptably accurate: average error in predicted values of t_R = 0.13 min; average error in retention time difference (Δt_R) = 0.06 min. Since the average peak width in the runs of Figs. 4 and 5 is about 0.2 min, this corresponds to an average error in predicted resolution values of (0.06/0.2)=0.3 units in t_R. This is acceptable for method development based on computer simulation.

Peak tracking

The ability to predict retention as a function of pH (as in Table 1) provides a stringent check on the proper assignment of bands in each chromatogram of Fig. 4 (peak tracking). If an error in assignment had been made for the data used as input to computer simulation, a significant discrepancy in the comparisons of Table 1 would have resulted. No such discrepancy is observed in Table 1, confirming that the peaks are assigned properly in the five chromatograms of Figs. 4 and 5.

Optimizing pH

Although it has been shown that DryLab can accurately predict isocratic retention as a function of pH [4], the isocratic model used here (Eq. 1) cannot predict peak widths for gradient elution and therefore cannot predict resolution as in Fig. 2. Whereas peak widths remain relatively constant in gradient elution

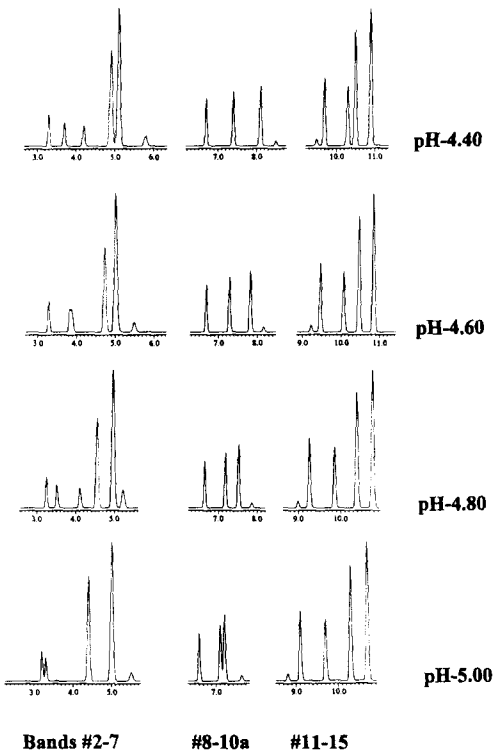

Fig. 6. Simulation of chromatograms for 'critical peak groups' of the present sample as function of pH. Other conditions as in Fig. 4 and Fig. 5. These simulations should be compared with the experimental data of Fig. 4 and Fig. 5. A value of the plate number N was assumed for each peak group: N=22 000 for peak nos. 2–7, N=100 000 for peak nos. 8–10a, and N=150 000 for peak nos. 11–15. Differences in composition among these samples led to differences in peak heights for simulated vs. experimental chromatograms and should be ignored.

for both early- and late-eluting peaks, peak width in isocratic elution increases with increasing retention time. We have overcome this problem by restricting computer simulation for gradient runs to groups of adjacent peaks. Within each group, isocratic peak widths will be roughly constant, approximating the case of gradient elution. This is illustrated in Fig. 6 for three groups of peaks: nos. 2–7, nos. 8–10a and nos. 11–15. In each case, data for the input runs (e.g., for pH 4.4 or 5.0) were used to determine an 'apparent' isocratic plate number N, that would provide the correct peak widths and resolution. For peak nos. 2–7, a value of N=22 000 was selected;

similarly, values of $N=100\,000$ and $N=150\,000$, respectively, were selected for groups nos. 8–10a and nos. 11–15. Comparison of the simulations of Fig. 6 with the experimental runs of Fig. 4 and Fig. 5 shows generally good agreement (as expected from the comparisons of Table 1).

The simulation of a total gradient chromatogram as the sum of peak groups (as in Fig. 6) is generally tedious and probably not worthwhile. However, for many samples the critical peak pairs (as pH or some other variable is changed) will be found in only one or two peak groups (so-called 'critical peak group'). Over the range $4<pH<5$, critical peak-pairs are found either in peak-group nos. 2–7 or nos. 11–15 of the present sample (see Fig. 4 and Fig. 5). For a case such as this, a resolution table can be requested for each 'critical' peak group. Table 2 summarizes these tables, from which the resolution for the entire sample can be determined. Sample resolution is equal to the smaller of the two peak-group R_s values (last column in Table 2). Table 2 suggests that marginal resolution ($R_s = 1.0 - 1.2$) could be obtained either for pH 4.05 or pH 4.70. However, either of these two separations would be adversely affected by small errors in mobile-phase pH (±0.1 unit) and would not be suitable for use as a robust assay method.

An alternative approach is to select conditions that provide good resolution of the major component (peak no. 6). The optimum pH for the separation of peak no. 6 can be obtained by examining a resolution table for peak group nos. 2–7, where peak no. 6 is flagged as the peak of interest. Computer simulation for this case suggested that peak no. 6 can be separated at pH 4.76 with $R_s = 1.7$ and the method is suitably rugged (changes in pH of ±0.1 unit can be tolerated). The experimental chromatogram for this case is shown in Fig. 7a and compared with the predicted separation of peak-group nos. 2–7 in Fig. 7b. There is generally good agreement between the two chromatograms, confirming the value of these conditions for acceptable separation of peak no. 6.

Table 2
Resolution tables for peak-groups nos. 2–7 and nos. 11–15

pH	Predicted resolution for each group		
	Bands nos. 2–7[a]	Bands nos. 11–15[b]	Sample resolution[c]
4.0	0.7	0.9	0.7
4.05	1.3	1.1	1.1
4.10	0.9	1.2	0.9
4.15	0.2	1.3	0.2
4.20	1.2	0.6	0.6
4.25	1.3	0.0	0.0
4.30	1.1	0.7	0.7
4.35	0.6	1.3	0.6
4.40	0.0	1.9	0.0
4.45	0.6	2.1	0.6
4.50	0.6	2.3	0.6
4.55	0.1	2.4	0.1
4.60	0.3	2.5	0.3
4.65	0.7	2.6	0.7
4.70	1.2	2.7	1.2
4.75	0.6	2.8	0.6
4.80	0.2	2.9	0.2
4.85	1.0	3.0	1.0
4.90	0.9	3.1	0.9
4.95	0.5	3.2	0.5
5.0	0.0	3.3	0.0

[a] Calculated with $N=22\,000$.
[b] Calculated with $N=150\,000$.
[c] Smaller of two resolution values for two band-groups.

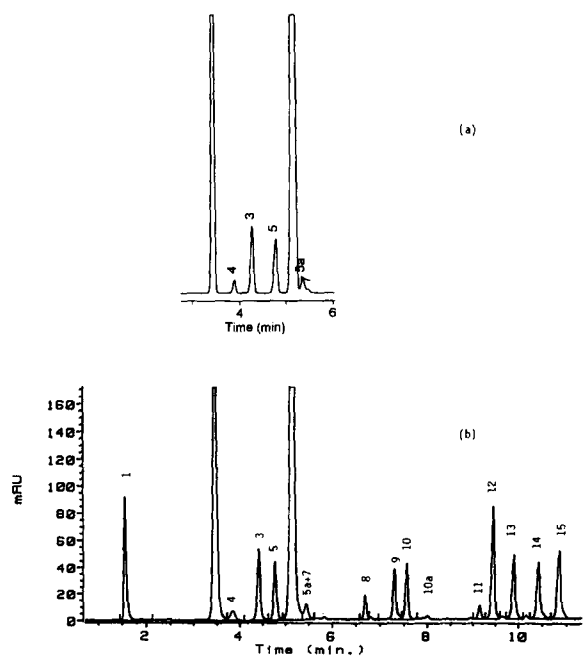

Fig. 7. Separation of sample at pH 4.76 for best separation of major band no. 6. Other conditions as in Fig. 4. (a) Predicted separation of peaks nos. 2–7. (b) Experimental separation.

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