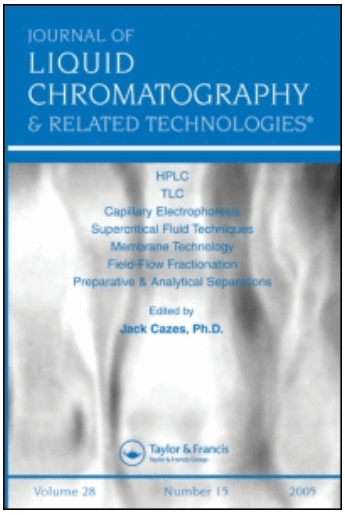

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COMPUTER SIMULATION IN HPLC METHOD DEVELOPMENT. REDUCING THE ERROR OF PREDICTED RETENTION TIMES

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ABSTRACT

Computer simulation uses two experimental HPLC runs to allow prediction of sample retention as a function of mobile phase composition or gradient conditions. The general approach is rigorous, but it is assumed that reversed-phase retention obeys the relationship log k' = log k_w - S Ø. Small deviations in this relationship can lead to error in predicted retention times. We have studied this error empirically for several different reversed-phase systems. This provides a basis for partially correcting these errors, and suggests recommendations for avoiding significant errors during computer simulation.

INTRODUCTION

Previous papers have described a new approach to method development for HPLC (1-6), so-called computer

simulation. The technique begins with two experimental runs for a sample that requires method development. In the case of an isocratic reversed-phase method, these initial runs can be isocratic separations with different values of mobile-phase %-organic, or gradient runs differing only in gradient time. In either case the resulting retention data are entered into a computer which has been programmed (7) to predict isocratic retention and separation as a function of %-organic. Now the operator can explore the effects of mobile-phase composition (%-organic) on the resulting separation. It is generally observed (6) that a change in %-organic leads to significant changes in band spacing for the majority of samples. When a promising %-organic value has been found, it can be confirmed experimentally. Computer simulation allows the further optimization of run conditions (column length, particle size, flowrate [8]), as well as the development of a final gradient elution method for the sample (9).

Previous work has shown that predictions of isocratic retention from gradient data and vice versa can be reasonably accurate (10-14) for the case of reversed-phase HPLC. However, these studies have dealt mainly with interpolation within a narrow range of mobile-phase composition. In computer simulation we desire to carry out extrapolative predictions, as well

as interpolative calculations. There are other practical problems associated with such simulations, for example, the accuracy of various experimental parameters that enter into these predictions. In this paper we will try to define these various sources of error within narrow limits, and to apply our findings to the use of computer simulation in HPLC method development.

THEORY

Predictions of retention using computer simulation are based on some expected dependence of solute retention time on mobile phase composition. For reversed-phase separations, it is generally observed that

$$\log k' = \log k_w - S\,\phi \quad , \qquad (1)$$

to a good approximation. Here k' refers to the solute capacity factor, k_w is the (extrapolated) value of k' for water as mobile phase, ϕ is the volume-fraction of organic in the mobile phase, and S is a constant for a given solute and mobile-phase organic solvent. Numerous papers have documented the validity of Eqn. 1, as well as modest deviations from this relationship (see discussion of Refs. 12 and 15). In general we can anticipate a slight curvature of plots of log k' vs ϕ,

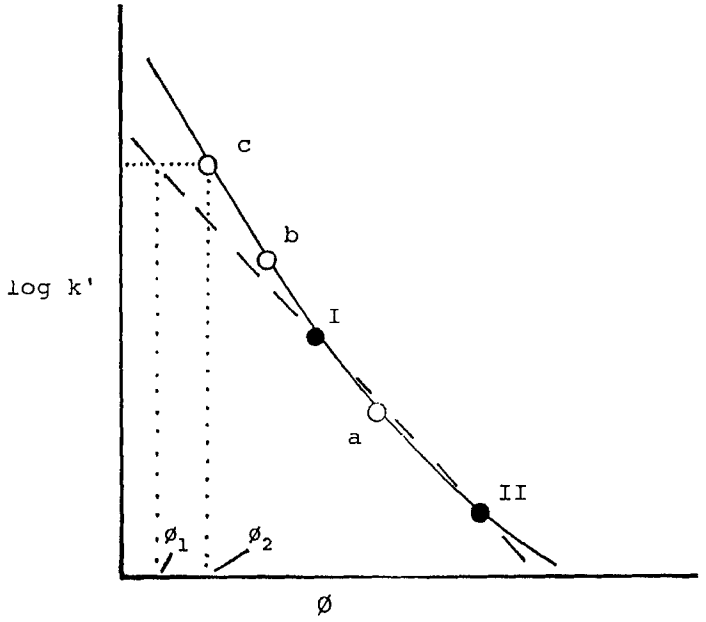

FIGURE 1. Illustration of deviations from linearity of log k' vs Ø plots. Effect on predictions of k' vs Ø. ●, experimental data points used for computer simulation; ○, data points to be predicted. See text for details.

as illustrated in Figure 1. One study (16) suggests that these curved plots of log k' can be linearized by plotting vs a spectroscopic function of the mobile phase: the so-called ET(30) index. If this is the case, then plots of log k' vs Ø should generally resemble plots of ET(30) vs Ø as in Figure 2, for either methanol/water (Fig. 2a) or acetonitrile/water (Fig. 2b) mobile phases.

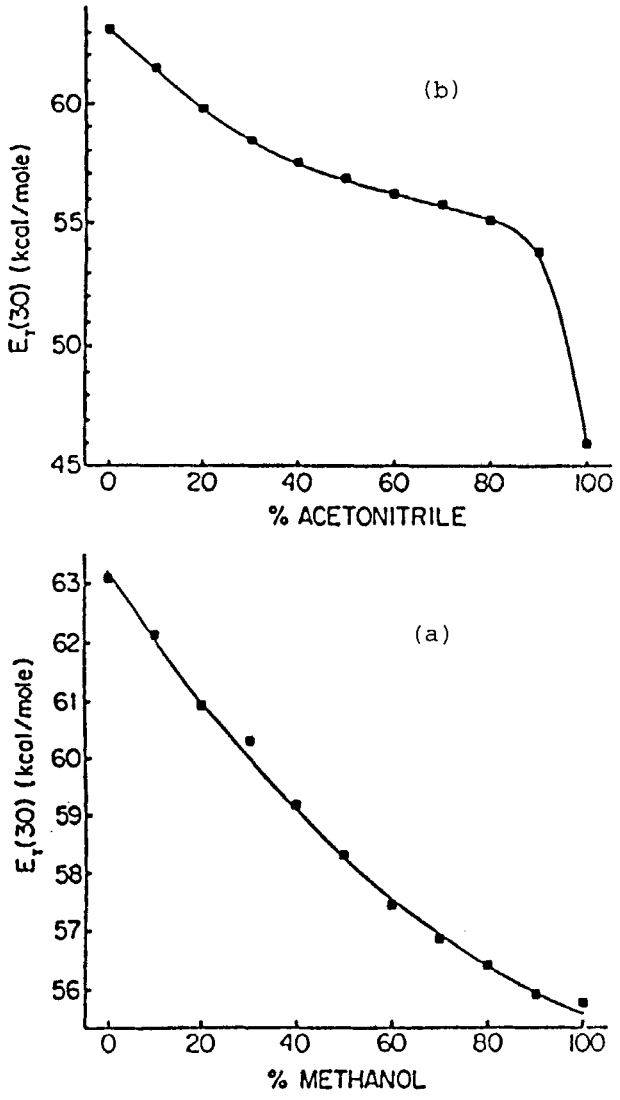

FIGURE 2. The function ET(30) as a function of mobile phase composition (reprinted from 16 with permission).

Whether isocratic retention is predicted on the basis of (experimental) isocratic or gradient data, the problem of curved log k' vs Ø plots is the same. In Figure 1 the experimental retention data for a given solute are indicated by the solid circles. The open circles indicate retention data that we desire to calculate; i.e., k' or retention-time values for a particular mobile phase composition. Beginning with Eqn. 1, we can construct a straight line through the experimental data points (I and II). In a first approximation, we can then estimate retention for other %-organic values (Ø) from this linear relationship. However, we see in Figure 1 that data-point (a) will be estimated slightly high, while data-points (b) and (c) will be estimated low. Greater extrapolation (e.g., point [c] vs [b]) leads generally to greater error.

A procedure for predicting isocratic retention from two experimental gradient runs has been described (12). The approach is based on a fundamental equation for retention time t_g in reversed-phase gradient elution, assuming that Eqn. 1 is obeyed (17):

$$t_g = (t_0/b) \log([2.3\ k_0\ b] + 1) + t_0 + t_D \quad . \quad (2)$$

Here, k_0 is the value of k' in the starting mobile phase, t_0 is the column dead-time, and t_D is the

dwell-time of the gradient system (11). The gradient parameter b is given as

$$b = V_m \Delta\emptyset\ S/t_G\ F \quad , \qquad (3)$$

where V_m is the column dead-volume, $\Delta\emptyset$ refers to the change in $\emptyset$ during the gradient, t_G is gradient time and F is mobile-phase flowrate. Given values of t_q for two gradient runs with different values of t_G (other conditions the same), it is possible to solve Eqns. 1-3 and obtain values of k_w and S for each solute. This then permits construction of linear log k' vs $\emptyset$ plots as in Figure 1.

If the relationships summarized in Figure 2 are generally valid for reversed-phase HPLC, it should be possible to correct for the errors illustrated in Figure 1 (deviations of the solid and dashed plots). Thus, if a value of k' is desired for mobile phase of composition $\emptyset_2$ in Figure 1, an accurate value can be obtained from Eqn. 1 using the value $\emptyset_1$ instead of $\emptyset_2$. That is, we can define a value of $\emptyset_1$ that is equivalent to $\emptyset_2$ in terms of Eqn. 1 and the true (solid) curve. It further follows from Figure 2 and the discussion of Ref. 15 that this value of $\emptyset_1$ will be the same for every solute in the sample. That is, a separation calculated from Eqn. 1 with the value $\emptyset = \emptyset_1$ will be in good agreement with

an experimental separation with $\emptyset = \emptyset_2$. This relationship, which is discussed further in the Appendix, has important implications.

EXPERIMENTAL

A Du Pont Model 8800 HPLC system was used with a fixed-wavelength detector and heated column compartment. Other conditions are identical to those described in Ref. 10. Analysis of the resulting retention data was facilitated by the use of DryLab 4,5 software from LC Resources Inc. (San Jose, CA).

RESULTS AND DISCUSSION

Correction of Eqn. 1 for Non-linear log k' vs Ø Plots

Retention data were collected for three different systems, involving two sample-mixtures plus mobile phases based on both methanol/water and acetonitrile/-water. Tables 1-3 summarize isocratic retention as a function of %-organic for each system. Additional data were available for various dialkyl phthalates as a function of %-acetonitrile from the study of Ref. 11. This combined data set was then used to explore values of the quantity $(\emptyset_2-\emptyset_1)$ defined in Figure 1. Once it

TABLE 1. Retention Times for Nitro-compound Sample in Methanol/water Mobile Phases*.

compound	retention time (min) for varying Ø							
Ø =	0.30	0.40	0.45	0.50	0.55	0.60	0.65	0.70
nitrobenzene	17.66	9.45	7.10	5.38	4.16	3.40	2.95	2.45
benzene	30.29	13.98	9.80	7.02	5.10	3.95	3.26	2.59
2,6-dinitrotoluene	21.70	12.27	9.31	7.02	5.36	4.31	3.66	2.97
2-nitrotoluene	40.67	18.06	12.44	8.65	6.16	4.68	3.82	2.97
4-nitrotoluene	43.16	19.13	13.17	9.13	6.48	4.91	3.98	3.07
3-nitrotoluene	47.27	20.86	14.31	9.86	6.96	5.22	4.20	3.22
toluene	57.05	27.10	18.98	13.12	9.22	6.89	5.26	4.06
2-nitro-1,3-xylene	111.17	39.64	24.72	15.52	10.04	6.96	5.35	3.78
4-nitro-1,3-xylene	105.36	39.36	24.92	16.13	10.62	7.45	5.66	4.06
m-xylene	154.79	61.90	40.24	25.67	16.61	11.42	8.36	5.76

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* Column, 25x0.46-cm, 5-μm Zorbax C8; flowrate, 2 mL/min; 35°C; t_o = 1.2 min.

TABLE 2. Retention Times for Nitro-compound Sample in Acetonitrile/water Mobile Phases. Other conditions as in Table 1.

compound	retention time (min) for varying Ø						
Ø =	20%	25%	30%	35%	40%	45%	50%
nitrobenzene	22.7	15.5	11.1	7.78	5.92	4.60	3.71
2,6-dinitrotoluene		17.8	13.1	9.34	7.17	5.53	4.49
benzene	51.1	30.9	19.3	12.27	8.41	5.99	4.49
2-nitrotoluene	49.4	29.9	19.3	12.27	8.71	6.32	4.83
4-nitrotoluene	51.1	30.9	20.0	12.78	9.03	6.53	4.97
3-nitrotoluene	51.1	33.6	21.5	13.61	9.54	6.85	5.18
toluene	55.8	37.6	25.1	16.17	11.50	8.25	6.22
2-nitro-1,3-xylene	112.7	61.1	35.6	21.1	13.87	9.35	6.70
4-nitro-1,3-xylene	122.8	64.8	37.1	21.4	13.87	9.35	6.70
1,3-xylene	142.0	80.4	48.4	28.9	18.86	12.58	8.97

TABLE 3. Retention times for steroid sample in acetonitrile/ water mobile phases.*

compound	retention time (min) for varying Ø				
Ø =	0.20	0.30	0.35	0.40	0.50
hydrocortisone	10.67	2.82	1.94	1.53	1.13
prednisone	11.18	2.94	2.00	1.53	1.13
cortisone	12.18	3.16	2.12	1.61	1.13
corticosterone	---	4.62	2.82	2.00	1.30
cortexoloneone	---	5.89	3.56	2.50	1.58
dexamethasone	---	7.09	4.11	2.78	1.67

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* Column, 8x0.46-cm, 3-μm C8 Golden Series (Du Pont); flowrate, 2 mL/min; 35°C.

becomes possible to predict values of this quantity for a given case, we can then use Eqn. 1 to more accurately predict data points such as a-c in Figure 1; i.e., by substituting the value $Ø_1$ for $Ø_2$ in such calculations.

Using data as in Table 1, we can select various mobile-phase pairs as our experimental starting point; i.e., solid circles of Figure 1. For example, 50 and 65% methanol/water data can be used to define k_w and S in Eqn. 1 for each solute. Now we can predict values of k' for each solute in other mobile phases; e.g., 30 or 45% methanol/water. Finally, these calculated values of k' or retention time can be compared with the actual values given in Table 1. We carried out this procedure using different starting mobile phases for each of the data sets of Tables 1-3 and Ref. 11. Such calculations

are normally tedious, but the use of DryLab 5 software (7) greatly facilitated this study.

For each simulation (e.g., using 50 and 65% methanol as starting values) and a given value of $\phi \neq 0.50$ or 0.65, we found some error in the resulting prediction. We assumed that these errors were mainly due to the failure of Eqn. 1 (as in Fig. 1). Our present analysis (above and Appendix) suggests that the use of the value ϕ_1 in place of ϕ_2 (Fig. 1) should give a better prediction of retention time for all solutes in the sample. Therefore, we used a trial-and-error substitution of values of ϕ_1 for each ϕ_2 in order to determine $(\phi_2-\phi_1)$ for ϕ as a function of the ϕ-values (I and II) used to construct plots as in Figure 1.

On the basis of a large number of such simulations, we came to the following generalization. The quantity $(\phi_2-\phi_1)$ is determined mainly by the magnitude of the extrapolation or interpolation from the nearest experimental data-point. For example, let ϕ_I and ϕ_{II} refer to the ϕ-values used to construct the plot of Figure 1. The value of $(\phi_2-\phi_1)$ for data-points a-c will be determined by the following quantities: (a) $\phi_a-\phi_I$; (b) $\phi_I-\phi_b$; and (c) $\phi_I-\phi_c$ (in this case, ϕ_a, ϕ_b, and ϕ_c, are all closer to ϕ_I than to ϕ_{II}). The size of $(\phi_2-\phi_1)$ also differs, depending on whether ϕ for a data point is less than ϕ_I or greater than ϕ_{II}. These

TABLE 4. Values of $(\phi_2-\phi_1)$ as a function of $(\phi_I-\phi_2)$ or $(\phi_2-\phi_{II})$. See discussion in text.

$(\phi_I-\phi_2)$	$(\phi_2-\phi_1)$	$(\phi_2-\phi_{II})$	$(\phi_2-\phi_1)$
-0.10*	-0.01	-0.10*	-0.01
-0.05*	-0.005	-0.05*	-0.005
-0.00	0.00	0.00	0.00
0.05	0.01	0.05	0.005
0.10	0.02	0.10	0.01
0.15	0.03	0.15	0.015
0.20	0.06	0.20	0.02
0.25	0.09	0.25	0.025

---

* interpolated value; e.g., point-a of Figure 1.

relationships are summarized in Table 4. In each case, the value of ϕ_2 of interest is compared with ϕ for the experimental data-point (I or II) that is closest in value.

For example, if the experimental data points are 50 (I) and 65% (II) methanol/water, and it is desired to predict k' for 40% methanol/water (ϕ_2), we proceed as follows. First, 40% is closer to 50% (data-point I) than to 65%. Next, the value of $(\phi_I-\phi_2)$ is determined: 50% - 40% = 0.10. The corresponding value of $(\phi_2-\phi_1)$ from Table 4 is 0.02, which means that ϕ_1 = 0.40-0.02 = 38%. This value of ϕ_1 is used in Eqn. 1 to calculate values of k' for each solute in the sample (for $\phi = \phi_2$ = 40%). The resulting predictions of retention time are in this way corrected for the

curvature of plots of log k' vs Ø as in Figure 1. Additional examples of this approach are given in the following discussion.

Accuracy of Predicted Values of Retention Time t_R

Experimental Isocratic Data. The approach of Table 4 was applied to a large number of simulations for the prediction of t_R as a function of Ø, using various values of $Ø_I$ and $Ø_{II}$ (experimental values). It was found (as expected) that interpolation is more reliable than extrapolation, and that extrapolation begins to fail beyond a certain point. These results are summarized in Table 5, which gives the average error in predicted values of t_q and α as a function of $Ø_I - Ø_2$ or $Ø_2 - Ø_{II}$.*

Table 5 indicates that interpolation (negative values of $[Ø_I - Ø_2]$ or $[Ø_2 - Ø_{II}]$) is generally quite accurate. Retention time is predicted within better than ±3% (CV) on average, and values of α are good to about ±1%. Extrapolation becomes less accurate when $Ø_2$ is outside the range $Ø_I < Ø < Ø_{II}$ by more than 5%. It is probably inadvisable to extrapolate data by more than 10%, because values of α become increasingly unreliable -- even with correction of Eqn. 1 as in the Appendix. The reasons for this probably reflect several

TABLE 5. Error in predicted values of retention time t_R and separation factor α as a function of extent of extrapolation or interpolation.

$\phi_I-\phi_2$ or $\phi_2-\phi_{II}$	error in prediction (CV)	
	t_R	α
-0.10**	1.3%	1.0%
-0.05**	2.3	0.7
0.00	(0)	(0)
0.05	2.5	1.1
0.10	3.0	2.5
0.15	4.6	3.2
0.20	8	6
0.25	17	14

** interpolated value; e.g., point-a of Figure 1.

---

* Larger errors were found for the case of k' > 25. Large k'-values are not usually of interest in isocratic method development, so these errors are omitted from the average values of Table 5. Data of Table 5 are after correction as in Table 4.

factors. First, the reliability of Eqn. i-I (Appendix) for precise extrapolation is probably questionable. The study of Ref. 16 emphasizes correlations in terms of the correlation coefficient r, which tells us little about the accuracy of such extrapolations. Second, error in values of k' for $\phi = \phi_I$ and ϕ_{II} translate into error in the slope S of Eqn. 1. Such errors have an increasing effect for larger extrapolations. Finally,

error in the assignment of values of t_o and t_D are also involved. This is discussed below.

A few examples from the data-base of Table 5 will illustrate the conclusions we have reached. We will use the nitro-compound sample of Table 2 with acetonitrile/-water as mobile phase, and values of ϕ_I and ϕ_{II} (experimental ϕ-values) equal to 40 and 50% acetonitrile. These comparisons for three different values of ϕ_2 are shown in Table 6. In the first comparison, experimental data for mobile phases containing 40 and 50% acetonitrile/water are used to predict retention times for 45% acetonitrile/water. This involves interpolation of Eqn. 1, which is seen to be good for both the uncorrected ($\phi = \phi_2$) and corrected ($\phi = \phi_1$) cases. Values of t_R agree with the experimental values within $\pm$0.2 min or better than 2% for the corrected predictions, and are only slightly poorer for the uncorrected simulations.

Similar comparisons are offered in Table 6 for predictions based on extrapolation rather than interpolation: ϕ_2 = 30% and 20%. Agreement between predicted and experimental retention data is seen to worsen in going from 45% to 30% to 20% acetonitrile, as expected from our preceding discussion. In both cases the corrected predictions are in better agreement with experimental values than are the uncorrected data. The

TABLE 6. Comparisons of experimental and predicted Retention times for nitro-compound sample and acetonitrile/water as mobile phase, using data of Table 2. $\emptyset_I$ = 40% and $\emptyset_{II}$ = 50%.

compound	retention times (min)		
	predicted* (no corrn.)	predicted** (corrected)	experimental
$\emptyset_2$ = 45% ($\emptyset_1$ = 45.5%)			
nitrobenzene	4.64	4.54	4.60
benzene	5.63	5.50	5.53
2,6-dintrotoluene	6.07	5.88	5.99
2-nitrotoluene	6.42	6.23	6.32
4-nitrotoluene	6.63	6.44	6.53
3-nitrotoluene	6.96	6.75	6.85
toluene	8.39	8.14	8.25
2-nitro-1,3-xylene	9.55	9.21	9.35
4-nitro-1.3-xylene	9.55	9.21	9.35
1,3-xylene	12.91	12.44	12.58
$\emptyset_2$ = 30% ($\emptyset_1$ = 28%)			
nitrobenzene	10.1	11.3	11.1
benzene	12.0	13.4	13.2
2-nitrotoluene	16.7	19.2	19.3*
2,6-dinitrotoluene	17.0	19.7	19.3*
4-nitrotoluene	17.5	20.0	20.0
3-nitrotoluene	18.7	21.5	21.5
toluene	22.3	25.6	25.1
2-nitro-1,3-xylene	30.4	35.7	35.6
4-nitro-1,3-xylene	30.4	35.7	37.1
1,3-xylene	41.3	48.5	48.4
$\emptyset_2$ = 25% ($\emptyset_1$ = 22%)			
nitrobenzene	13.4	15.9	15.5
benzene	15.8	18.7	17.8
2-nitrotoluene	23.6	29.0	29.9
4-nitrotoluene	24.6	30.4	30.9
2,6-dintrotoluene	24.6	30.8	30.9
3-nitrotoluene	26.5***	32.8	33.6
toluene	31.5***	38.8	37.6
2-nitro-1,3-xylene	45.5***	58.1	61.0
4-nitro-1,3-xylene	45.5***	58.1	64.8
1,3-xylene	61.7***	78.6	80.4

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* Eqn. 1, $\emptyset = \emptyset_2$; ** Eqn. 1, $\emptyset = \emptyset_1$; *** k' > 25

combination of a large value of ($\phi_I - \phi_2$) with large values of k' (*** in Table 6) gives poor results for both corrected and uncorrected simulations.

Experimental Gradient Data. Two initial gradient elution runs can be used in place of two isocratic runs for predicting sample retention as a function of ϕ. In this case the gradient time for the two runs must be different, by a factor of two or more. Errors due to a failure of Eqn. 1 (as in Fig. 1) have the same effect on the accuracy of predicted isocratic data as for the case of two starting isocratic runs. However, in the latter case all compounds in the sample are eluted at a given value of ϕ in each run (by definition). In gradient elution each compound moves through the column with a different average value of ϕ, which can be determined as follows. First, the average value of k' during gradient elution is related to the gradient-steepness parameter b (Eqn. 3) as

$$\bar{k} = 1/2.3\ b \quad . \qquad (4)$$

Second, the average value of ϕ during elution of a given compound is then obtained from Eqn. 1 as

$$\bar{\phi} = (\log k_w - \log \bar{k})/S \quad . \qquad (5)$$

The values of $\bar{k}$ for the two experimental gradient runs will be in proportion to the ratio of gradient times t_G for the two runs (Eqn. 3), leading to corresponding differences in $\bar{\phi}$ for each run.

The effect of curvature of log k' vs ϕ plots (Fig. 1) on predicted retention data will be the same as for the case of isocratic separation. Errors caused by this effect can be corrected in the same way as for initial isocratic runs. That is, values of $\bar{k}$ and $\bar{\phi}$ for each gradient run replace k' and ϕ for each of the two isocratic runs in Figure 1. Because $\bar{\phi}$ will be different for each solute in a given gradient run, this correction (Table 5) must be applied to each individual solute; i.e., values of $(\phi_2-\phi_1)$ will vary for each solute, unlike the case for isocratic experimental data discussed above (where $\phi_2-\phi_1$ is constant for all bands).

Table 7 shows some comparisons of predicted and experimental retention times for similar examples as in Table 6, but using two gradient runs for the experimental retention data that precede computer simulation. The predicted values are not corrected for the failure of Eqn. 1, but the same trends as in Table 6 can be seen. In this case, $\bar{k}$ for the two gradient runs ranges from $3 < \bar{k} < 4$ for the first run (t_G = 15 min) to $9 < \bar{k} < 12$ for the second run (t_G = 45 min). For the three isocratic runs the k' range is as

TABLE 7. Comparisons of experimental and predicted retention times for nitro-compound sample and acetonitrile/water as mobile phase, using two experimental gradient runs as input to computer simulation. Gradient times of 15 and 45 min, gradient from 5 to 100% acetonitrile, and other conditions as in Table 2.

compound	retention times (min)					
Ø =	50%		30%		20%	
	expt.	pred.	expt.	pred.	expt.	pred.
nitrobenzene	3.71	3.60	11.1	11.3	22.7	22.0
benzene	4.49	4.36	13.2	13.0	----	24.1
2,6-dinitro-toluene	4.49	4.63	19.3	19.2	51.2*	40.3
2-nitrotoluene	4.83	5.00	19.3	19.6	49.4*	41.6
4-nitrotoluene	4.97	5.18	20.0	19.9	51.1*	43.8
3-nitrotoluene	5.18	5.45	21.5	21.1	51.1*	44.1
toluene	6.22	6.59	25.1	23.9	55.8*	47.8
2-nitro-1,3-xylene	6.70	7.37	35.6*	31.95	112.7*	69.8
4-nitro-1,3-xylene	6.70	7.37	37.1*	31.95	122.8*	69.8
1,3-xylene	8.97	9.93	48.4*	39.5	142.0*	83.3

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* $k' > 25$

follows: 50% acetonitrile, $2 < k' < 7$; 30% acetonitrile, $8 < k' < 32$; 20% acetonitrile, $17 < k' < 67$ (predicted values of k'). From this it follows that the closest agreement between experimental and predicted t_R-values should be found for 50% acetonitrile, followed by 30% and (worst) 20%. Compounds that are more retained should show even poorer agreement for the 30% and 20% acetonitrile mobile-phases. These trends are observed

in Table 7. Correction of predicted retention times according to Table 5 is expected to improve the agreement in Table 7 significantly, but this requires modification of the DryLab 4 software (not completed at this time).

Predictions of Retention in Gradient Runs. The DryLab G software (9) allows us to begin with two experimental gradient runs, then we can predict retention and separation in corresponding gradient runs where conditions have been changed: gradient time, flowrate, column dimensions, etc. The initial optimization of retention using DryLab G proceeds by varying gradient time, gradient range ($\Delta \emptyset$) and gradient shape (segmented vs linear). We will consider only the variation of gradient time here. Preliminary data for the application of the DryLab G software to the separation of the steroid sample of Table 3 is summarized in Table 8. The 15-min and 45-min runs were used as input data for computer simulation, and retention times for 30-min and 90-min gradients were predicted. Experimental data are compared with these simulations in Table 8. As expected, the 30-min gradient (which involves interpolation) shows good agreement between experimental and calculated retention. Retention times for the 90-min run (extrapolation) are

TABLE 8. Comparisons of experimental and predicted retention times for steroid sample (Table 3) and methanol/water as mobile phase.*

compound	retention times t_g (min)**					
t_g =	15 min	45 min	30 min		90 min	
	(expt)	(expt)	(expt)	(calc)	(expt)	(calc)
prednisone	12.71	25.83	19.51	19.61	42.37	43.84
cortisone	12.84	26.20	19.76	19.86	43.09	44.59
hydrocortisone	13.18	26.92	20.30	20.41	44.22	45.69
dexamethasone	13.89	29.29	21.84	21.94	49.21	51.02
corticosterone	14.13	29.72	22.20	22.29	49.74	51.51
cortexolone	14.29	30.38	22.60	22.69	51.26	53.16

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* Column, 25x0.46-cm, 5-μm Zorbax C8; 5-100% methanol gradient, 2 mL/min; 35°C; t_D = 2.75 min, t_0 = 1.2 min
** DryLab G simulations (9) for calculated values

in error by 1.5-2.0 min, but relative retention is predicted quite well. Further work with the DryLab G program is required in order to determine the effectiveness of correction using Table 5.

The gradient chromatograms of Table 8 are shown in Figure 3. Starting with the data for the 15-min and 45-min runs, the DryLab G program can provide a relative resolution map of resolution (for a 10,000-plate column) vs gradient time, as shown in Figure 4. Maximum resolution (R_s = 1.7) is predicted for a gradient time of 40-50 min, and this is confirmed in the chromatograms of Figure 3 (45-min gradient best). The steroid sample does not require gradient elution, but can be separated

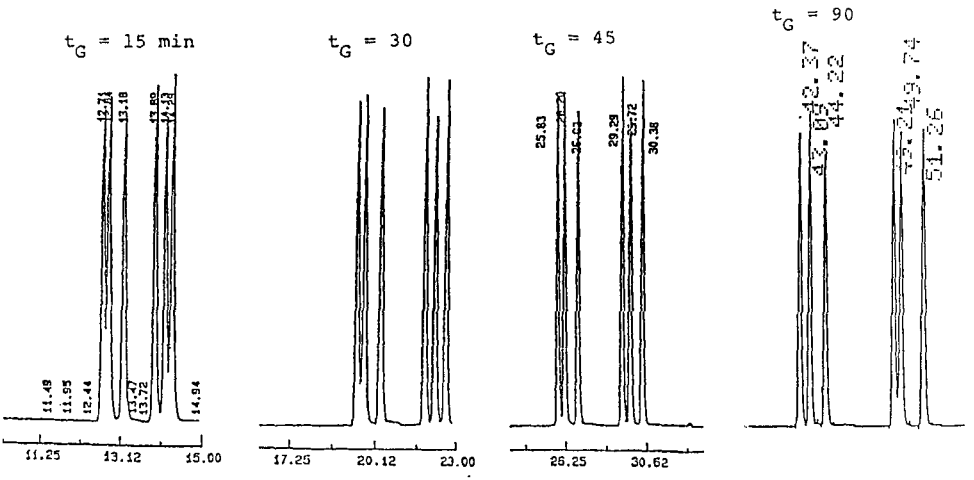

FIGURE 3. Gradient-elution separation of mixture of six steroids as a function of gradient time t_G. See Table 8 for details.

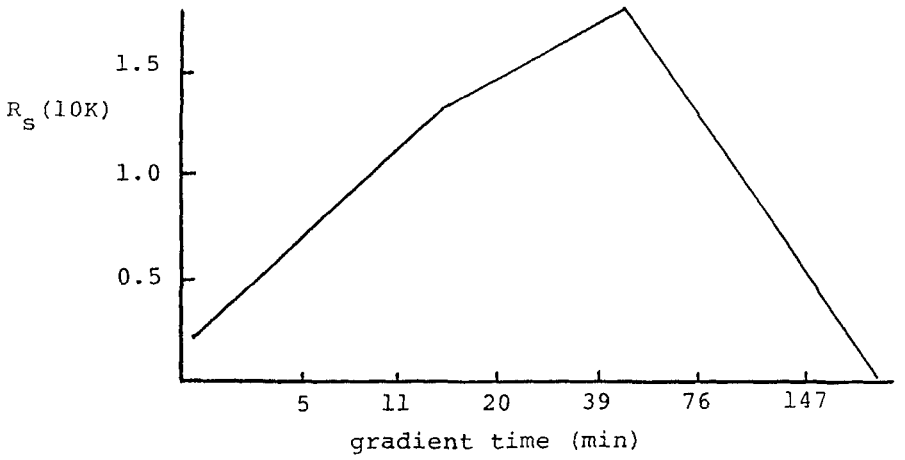

FIGURE 4. Relative-resolution map for gradient-elution separation of steroid mixture of Table 8. Calculated using DryLab G software (9).

isocratically. The examples of Figures 3 and 4 are therefore mainly for illustration.

Effect of Error in Values of t_0 and t_D

Effect of t_0. The use of initial isocratic data to predict isocratic retention for other values of Ø requires a value of the column dead-time t_0. Exact values of t_0 are not easy to determine, as shown by numerous studies summarized in Ref.18. Furthermore, t_0 actually varies with Ø (e.g., 12). Under these circumstances, the question arises as to whether the (inevitable) errors in values of t_0 can seriously affect predicted values of t_g. A preceding analysis for the case of gradient elution (12) suggests that this is not the case. Error in t_0 can affect predicted values of k', but the dependence of values of t_R on assumed values of t_0 is much less serious. This is illustrated for the data of Table 9, where the simulations of Table 6 are repeated -- but with an assumed value of t_0 = 1.4 min, instead of the correct value of 1.2 min. Errors in t_0 that are larger than this (17%) are unlikely, if reasonable care is taken to measure t_0 (see discussion of Ref. 7). It is seen in Table 9 that the resulting change in t_R for this 17% change in t_0 is generally minor, and changes in band-spacing (the more important prediction) are not significant.

TABLE 9. Effect of error in t_o on predicted values of retention time. Data of Table 6.

compound	predicted retention time (min)					
ϕ_1 =	45.5%		28%		22%	
t_o =	1.2	1.4	1.2	1.4	1.2	1.4
nitrobenzene	4.54	4.52	11.3	11.5	15.9	16.5
benzene	5.50	5.49	13.4	13.6	18.7	19.2
2,6-dinitrotoluene	5.88	5.87	19.2	19.5	29.0	29.9
2-nitrotoluene	6.23	6.22	19.7	20.1	30.4	31.3
4-nitrotoluene	6.44	6.42	20.0	20.4	30.8	32.0
3-nitrotoluene	6.75	6.74	21.5	21.8	32.8	33.8
toluene	8.14	8.12	25.6	25.9	38.8	39.7
2-nitro-1,3-xylene	9.21	9.19	35.7	36.2	58.1	59.6
4-nitro-1,3-xylene	9.21	9.19	35.7	36.2	58.1	59.6
1,3-xylene	12.44	12.43	48.5	49.0	78.6	80.6

Effect of t_D. The dwell-time of a particular HPLC system can be measured easily and precisely (7). It has been shown (3) that error in values of t_D (or gradient dwell volume V_D) of as much as 10% have a negligible effect on relative resolution maps -- generally the most useful result of computer simulation. Table 10 provides a further comparison of the effect of a large change in t_D on resulting computer simulations. A large (27%) error in t_D for this example is seen to significantly affect predicted values of t_R. A value of t_D that is 27% too low leads to values of t_R that are low by as much as 20%.

TABLE 10. Effect of a change in t_D on computer-simulated values of retention time t_R. DryLab 4 simulations for nitro-compounds of Table 1.*

compound	predicted retention times (min)					
Ø =	50%		30%		20%	
t_D =	5.5	4.0	5.5	4.0	5.5	4.0
nitrobenzene	3.6	5.0	11.3	13.1	22.0	22.2
benzene	4.4	5.9	13.0	14.7	24.1	24.2
2,6-dinitrotoluene	4.6	6.4	19.2	20.3	40.3**	36.6
2-nitrotoluene	5.0	6.8	19.6	20.6	41.6**	37.7
4-nitrotoluene	5.2	7.0	19.9	20.9	43.8**	38.7
3-nitrotoluene	5.5	7.3	21.1	22.0	44.1**	39.6
toluene	6.6	8.5	23.9	24.5	47.8**	42.9
4-nitro-1,3-xylene	7.4	9.4	32.0**	30.9	69.8**	57.6
2-nitro-1,3-xylene	7.4	9.4	32.0**	30.9	69.8**	57.6
1,3-xylene	9.9	12.1	39.5**	37.2	81.3**	97.5

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* Conditions as in Table 1, except 5-100% methanol/water gradients, with t_G = 15 and 45 min for experimental data (input to DryLab 4).

** $k > 25$

CONCLUSIONS

Method development for reversed-phase HPLC procedures is most easily carried out by initial optimization of solvent strength (%-organic in the mobile phase). This is readily achieved by carrying out two initial experimental runs in either an isocratic or gradient mode, followed by computer simulation to map

retention and resolution as a function of %-organic. Computer simulation for this purpose can be quite accurate, but extrapolation from the initial experimental data is subject to error in certain cases. The main source of error is the curvature of log k' vs %-organic plots (computer simulation assumes a linear relationship).

The present study combines a large experimental data base with computer simulations to examine errors in predicted retention times. It is found that curvature of log k' vs %-organic plots can be corrected for, to some extent. Resulting predictions appear accurate when interpolation between experimental data is used, or for modest extrapolations (5-10%v organic) from experimental data. Recommendations are made which should ensure accurate results in most cases. These improvements in computer simulation have been incorporated into the DryLab software (LC Resources) used for the present study.

Errors in computer simulation were also studied as a function of error in the values of column dead-time t_0 and dwell-time t_D required in these predictions. Error in t_0 has a negligible effect on the accuracy of predicted results. Error in t_D affects absolute values of retention time, but has less effect on relative retention and resolution.

GLOSSARY OF SYMBOLS

b	gradient-steepness parameter, defined by Eqn. 3
ET(30)	a spectroscopic function of the mobile phase; see Figure 2
F	mobile-phase flowrate (mL/min)
k'	capacity factor for a given band
k	average value of k' for a solute during gradient elution
k_o	value of k' for a given solute at the beginning of gradient elution (for starting mobile phase)
k_w	value of k' for water as mobile phase (extrapolated value)
S	equal to d(log k')/dØ; see Eqn. 1
t_D	dwell-time (min) of a given HPLC system
t_g	retention time (min) in gradient elution?
t_G	gradient time (min)
t_o	column dead-time (min)
t_R	solute retention time (min)
V_m	column dead-volume (mL)
Ø	volume-fraction organic solvent in mobile phase
$Ø_a$, $Ø_b$, $Ø_c$	values of Ø corresponding to points a, b and c in Figure 1
$Ø_1$, $Ø_2$	see Figure 1; $Ø_2$ is the value of Ø for which we desire to predict values of retention time; $Ø_1$ is the value of Ø which when used in Eqn. 1 yields correct values of k' and t_R
$Ø_I$, $Ø_{II}$	values of Ø for two starting (experimental) runs to be used for computer simulation; see Figure 1
$\Delta Ø$	change in Ø during gradient elution

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APPENDIX

Linearization of Log k' vs Ø Plots.

Reference 15 suggests that retention can be more accurately predicted as a function of mobile phase composition using the relationship

$$\log k' = \log k_w - S' [ET(30)] \qquad (i\text{-}1)$$

Here ET(30) is a function of Ø as given in Figure 2 for either methanol or acetonitrile as organic solvent. S' is a constant for a given solute and organic solute, analogous to S. Let us assume that Eqn. i-1 is exact for the purposes of our discussion. The hypothetical plot of Figure 1 can then be redrawn as in Figure i-1. For every value of Ø there corresponds a value of ET(30) as in Figure 2 -- this relationship is indicated in Figure i-1 as the dashed curve. The solid line in Figure i-1 is

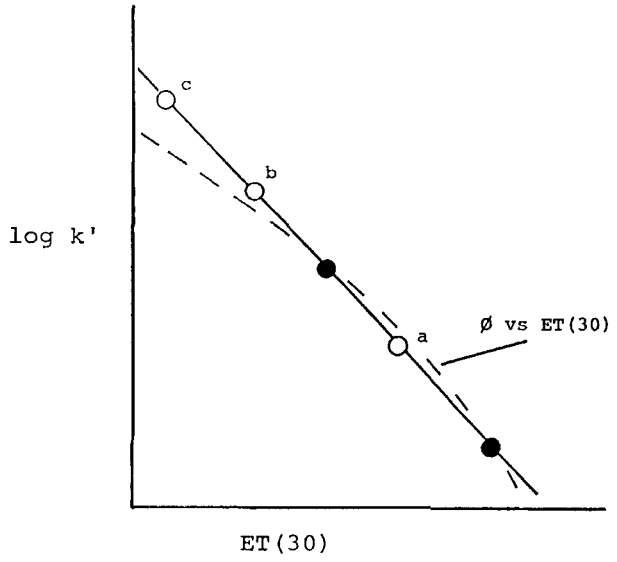

FIGURE i-1. Linear plot of log k' vs ET(30) as in Eqn. i-1. See text for details.

defined by the two experimental data-points (solid circles), which in turn allows the more accurate prediction of data points a-c because all values of k' vs Ø (or ET[30]) now fall on this straight line. If we desire a value of k' for any value of Ø, we need only convert Ø into ET(30) from Figure 2 and use Eqn. i-1 to determine k'.

The discussion of Eqn. i-1 and Figure i-1 applies for all other solutes in the sample. In each case, a given value of Ø corresponds to the same value of ET(30) for each solute. This in turn implies that the value of

(ϕ_2-ϕ_1) in Figure 1 will be the same for all solutes in a given HPLC system (same conditions, only ϕ and solute varying). So if we can estimate (ϕ_2-ϕ_1) for a given sample (Table 4), we can use Eqn. 1 (log k' vs ϕ) to calculate k' for data-points a-c as accurately as with Eqn. i-1.