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SEPARATION OF MIXTURES OF *o*-PHTHALALDEHYDE-DERIVATIZED AMINO ACIDS BY REVERSED-PHASE GRADIENT ELUTION

ACCURACY OF COMPUTER SIMULATION FOR PREDICTING RETENTION AND BAND WIDTH

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SUMMARY

The separation of seven *o*-phthalaldehyde-derivatized amino acids by reversed-phase gradient elution was studied as a function of gradient time and mobile phase flow-rate. The resulting separations were compared with those from computer simulation (Drylab G). Predictions of retention time via computer simulation were found to be quite accurate, being about ±0.7% for retention time and about ±7% for retention-time differences (resolution). Predictions of band width were accurate within about ±15% for all but the steepest gradients ($b > 1.0$). Consequently, the ability of computer simulation to predict chromatograms reliably as a function of gradient conditions and flow-rate was confirmed for a sample that is representative of "real life". For very steep gradients ($b > 1.0$), significant errors in band width were observed. The source of these errors could arise from various effects which are discussed.

INTRODUCTION

The theory of gradient elution, especially for application to reversed-phase high-performance liquid chromatography (HPLC), is now well developed[1–3]. When combined with what we know about the dependence of the column plate number on experimental conditions[4–8], it is possible to predict retention times, band widths and therefore resolution as a function of gradient conditions and other experimental variables. This in turn leads to the possibility of computer simulation for HPLC method development, as exemplified by several papers in this issue[9–14].

This study is concerned with the accuracy of these computer-simulation predictions for reversed-phase gradient elution. A specific computer program (DryLab G) was evaluated for the separation of selected *o*-phthalaldehyde (OPA)-derivatized amino acids as a representative example. However, our results can also be used to assess the applicability of present theories of gradient elution and column efficiency.

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Specifically, previous studies have shown[15–18] that predicted band widths are usually smaller than experimental values, except for very shallow gradients. For very steep gradients, the error in predicted band widths can be as large as a factor of two. This study was designed to shed additional light on the origin and predictability of these band-width errors.

THEORY

The theory behind the present comparisons of experimental and computer-simulated separations can be summarized in the following well documented[1,3,10] relationships:

$$\log k' = \log k_w - S\,\varphi \tag{1}$$

$$t_g = (t_o/b)\log(2.3k_o b + 1) + t_o + t_D \tag{2}$$

$$\sigma_t = t_o\, G[1 + (1/2.3b)]/N^{1/2} \tag{3}$$

where k' is the capacity factor of a solute for a particular mobile phase composition φ (volume fraction of the strong solvent B in a binary mobile phase A–B), k_w is the value of k' for water as mobile phase, S is a constant for a given mobile phase A–B and solute, t_g is the retention time of the solute in gradient elution, t_o is the column dead time, b is a gradient-steepness parameter, k_o is the value of k' at the beginning of the gradient, t_D is the dwell time of the gradient equipment, σ_t is the band width in gradient elution ($4\sigma_t$ = baseline band width, W), G is a gradient compression factor ($G \approx 1.0$ for shallow gradients and $G < 1$ for steeper gradients) and N is the column plate number. A fuller description of these parameters was given in other publications[1,3,10].

It has been found[3,18] that experimental band-width values, σ_{expt}, are larger than values of σ_t from eqn. 3 by an empirical factor J:

$$J = \sigma_{expt}/\sigma_t$$

$$\approx 0.99 + 1.70b - 1.35b^2 + 0.48b^3 - 0.062b^4 \tag{4}$$

Predicted values of band width in gradient elution (σ_t) can be corrected for this anomalous band broadening by multiplying by J. Alternatively, it has been noted[19] that

$$JG \approx 1.1 \tag{5}$$

which affords another means of predicting corrected band widths in gradient elution (this latter expedient is currently used in the DryLab G computer-simulation program).

A limited data set [18] suggests that J does not vary with the nature of the solute or with chromatographic conditions other than those which determine b (see eqn. 4). The origin of these anomalously large experimental values of band width in gradient

elution is so far undetermined. One objective of this study was to measure values of J for a wider range of experimental conditions than have so far been studied. This was expected to shed further light on the accuracy of band-width predictions via computer simulation (eqns. 3–5), and possibly provide insight into the cause of this band-broadening anomaly ($\sigma_t < \sigma_{expt}$).

Computer simulation based on the DryLab G software makes use of two initial experimental runs to derive values of S and k_w for each solute in the sample. These parameters then allow the calculation of k_o and b for each solute[3,19], and the prediction of retention and band width as a function of experimental conditions.

EXPERIMENTAL

Equipment

An LC/9533 ternary-gradient liquid chromatograph (IBM Instruments, Danbury, CT, U.S.A.) was used with a Model 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.) having a 20-μl loop. A circulating water-bath (Model T9; P. M. Tamson, Zoetermeer, The Netherlands) was used for temperature control (25 ± 0.2°C) of the column compartment. Two detectors were used: (a) an FS-970 spectrofluorimeter (Schoeffel/Kratos/Spectros, Ramsey, NJ, U.S.A.) with a 5-μl flow cell for the OPA–amino acid studies; and (b) a variable-wavelength UV detector (Model 9523; IBM Instruments) for the column efficiency studies with other solutes. Chromatograms were processed with a 3390A reporting integrator (Hewlett-Packard, Palo Alto, CA, U.S.A); this supplied values for retention time, peak area and peak area/peak height ratio, which were used to determine band width and column plate number.

The column dead volume, $V_m = t_o F$ (where F is flow-rate), was measured from the retention time for the OPA derivative of cysteic acid (55% acetonitrile as mobile phase) as V_m = 1.99 ml. The equipment dwell volume, $V_D = t_D F$, was determined by

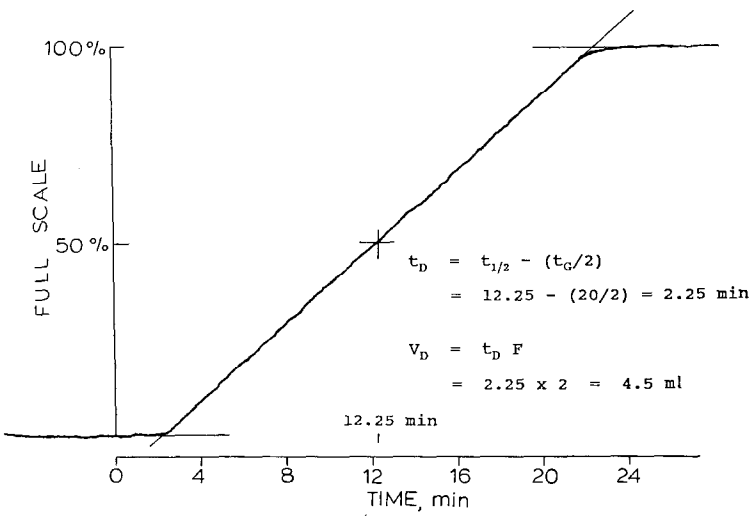

Fig. 1. Determination of gradient shape and dwell volume, V_D, for the gradient HPLC system used. Conditions: column removed from the system; flow-rate, 2 ml/min; gradient time, 20 min; 0–100% B (A is methanol; B is methanol–0.1% acetone).

running a blank gradient without the column, as illustrated in Fig. 1 (V_D = 4.5 ml); see also Refs. 20 and 21. An extra-column band broadening of $\sigma_{ec} \approx 0.01$ ml was assumed, equal to twice the volume of the spectrofluorimeter flow cell[6]. The repeatability of retention-time and band-width measurements was obtained from replicate runs under identical conditions as ±0.05% in retention time and ±0.3% in band width (average values).

Materials

Columns were 25 × 0.45 cm I.D., packed with C_{18}-bonded silica (IBM Instruments). The column packing was 5 μm in diameter, with 10–nm pores. Columns were evaluated for plate number at frequent intervals during the study; a test mixture of uracil, acetophenone, nitrobenzene, methyl benzoate and toluene was used with methanol–water (70:30, v/v) as mobile phase. Similar evaluations were also carried out with the OPA–amino acids as samples. The initial plate numbers for toluene were 16 000–18 000 for a flow-rate of 0.8 ml/min. Columns exhibiting a significant drop from the original plate number were discarded.

Chemicals and solutions were as described previously[22]. For separations of the OPA–amino acids, sample sizes were within the range of 50–200 pmol. In gradient runs, acetonitrile–aqueous buffer (5.1 m*M* NaH_2PO_4 and 12.9 m*M* Na_2HPO_4) (5:95, v/v) was used as solvent A and acetonitrile–buffer (55:45, v/v) as solvent B. Hence solvent A is acetonitrile–water (5:95, v/v) and solvent B is acetonitrile–water (55:45, v/v). All gradients were linear and were run from 0 to 100% B. Other conditions are given in the text.

Apart from the column test mixture described above (uracil, etc.), the sample used was a mixture of seven amino acids that were derivatized with OPA by the procedure in ref. 22: glutamic acid (Glu), serine (Ser), β-alanine (β-Ala), taurine (Tau), isoleucine (ILeu), phenylalanine (Phe) and fluorophenylalanine (F-Phe). These compounds were selected to be well resolved in all gradient runs, so as to allow accurate measurements of retention time and band width for every peak.

Computer simulations and band-width measurements

An IBM-compatible personal computer was used with DryLab G and DryLab I software (LC Resources, Lafayette, CA, U.S.A.) for computer simulations and related calculations of the parameters of eqns. 1–3. Predictions of band width require estimates of parameters X and Y, where X is the ratio of the volume of mobile phase outside the particle pores to the total volume of mobile phase and Y is the ratio of solute diffusion coefficients inside and outside the pores. Based on the use of a C_{18} packing of 10-nm pore size, values of $X = 0.78$ and $Y = 0.35$ were estimated[6] (see also DryLab G Users' Manual[23]).

Computer predictions of band width (Drylab G) require a value of the Knox parameter A[5,6]. This was determined for toluene as solute [methanol–water (70:30, v/v) as mobile phase, flow-rate 0.8 ml/min] by means of the DryLab G software to be $A = 1.0$.

Band widths and plate numbers (N) were measured in various ways: (a) from the half-height value, where σ_t = (half-height width)/2.35; (b) from the area/height ratio, R, of the band[24] ($\sigma_t = R/2.51$); (c) by the Dorsey–Foley method[25]. The Dorsey–Foley procedure gave N values that were about 10% lower than those obtained

by the other procedures; similar values of σ_t and N were obtained by the other two methods. The band-width data reported here are based on the area/height ratio (conveniently read from the integrator output).

RESULTS AND DISCUSSION

Retention time predictions

Several reports have dealth with the accuracy of computer simulation (DryLab G) in predicting retention times, t_g, and resolution, R_s, in gradient elution[10,19,26]. As

TABLE I

EXPERIMENTAL AND PREDICTED RETENTION TIMES FOR GRADIENT SEPARATION OF OPA–AMINO ACID SAMPLE

Conditions: standard gradient (5–55% acetonitrile) with varying gradient time, t_G; flow-rate = 1.5 ml/min.

t_G (min)	Solute	t_g (min)		Retention errors (±)	
		Exptl.	Calc.[a]	t_g[b]	Δt_g[c]
16	Glu	8.40	8.39	0.01	0.02
	Ser	11.56	11.57	0.01	0.01
	β-Ala	12.61	12.61	0.00	0.00
	Tau	13.08	13.08	0.00	0.00
	ILeu	14.21	14.21	0.00	0.00
	Phe	14.58	14.58	0.00	0.01
	F-Phe	14.86	14.85	0.01	
			Average	0.01	0.01
				(0.1%)	(1%)
20	Glu	8.98	9.00	0.02	0.00
	Ser	12.98	13.00	0.02	0.00
	β-Ala	14.28	14.30	0.02	0.01
	Tau	14.89	14.90	0.01	0.02
	ILeu	16.31	16.34	0.03	0.00
	Phe	16.78	16.81	0.03	0.02
	F-Phe	17.14	17.15	0.01	
			Average	0.02	0.01
				(0.2%)	(1%)
40	Glu	11.07	11.19	0.12	0.06
	Ser	19.18	19.24	0.06	0.02
	β-Ala	21.87	21.91	0.04	0.01
	Tau	23.12	23.15	0.03	0.00
	ILeu	26.12	26.15	0.03	0.01
	Phe	27.15	27.17	0.02	0.01
	F-Phe	27.87	27.88	0.01	
			Average	0.04	0.02
				(0.2%)	(1%)

[a] DryLab G predictions, based on experimental data for t_G = 12 and 60 min as inputs.
[b] Predicted minus experimental retention times (absolute values).
[c] Predicted minus experimental values of $(t_2 - t_1) = \Delta t_g$.

TABLE II
SUMMARY OF EXPERIMENTAL *VS.* PREDICTED RETENTION TIMES FOR SEPARATION OF OPA–AMINO ACID SAMPLE (AS IN TABLE I)

Conditions as in Table I, unless indicated otherwise.

Flow-rate (ml/min)	*Gradient time (min)*	*Av. retention errors (min/%)*[a]			
		Flow-rate constant[b]		*Flow-rate varying*[c]	
		t_g	Δt_g	t_g	Δt_g
0.4	9	0.31/1.4	0.14/28	0.95/4.1	0.22/26
	12	[d]	[d]	0.52/2.2	0.14/18
	16	0.30/1.1	0.09/9	0.87/3.3	0.21/20
	20	0.07/0.4	0.02/5	0.59/2.1	0.14/10
	40	0.10/0.3	0.06/3	0.56/1.6	0.31/10
	60	[d]	[d]	0.83/2.0	0.47/10
0.7	6	0.09/0.7	0.04/12	0.32/2.5	0.05/15
	9	0.04/0.6	0.03/5	0.28/1.9	0.03/6
	12	[d]	[d]	0.28/1.8	0.06/8
	16	0.03/0.2	0.02/2	0.22/1.2	0.09/8
	20	0.07/0.4	0.02/1	0.18/0.9	0.13/9
	40	0.31/1.1	0.06/2	0.66/2.6	0.23/8
	60	[d]	[d]	1.04/3.3	0.29/6
1.0	3	0.06/0.7	0.05/27	[e]	
	4	0.07/0.8	0.03/14		
	6	0.15/1.5	0.02/6		
	9	0.08/0.7	0.06/11		
	12	[d]	[d]		
	16	0.14/0.9	0.03/3		
	20	0.06/0.2	0.02/1		
	40	0.03/0.2	0.02/1		
	60	[d]	[d]		
1.5	12	[d]	[d]	0.08/0.6	0.14/21
	16	0.00/0.1	0.00/0	0.17/1.0	0.20/25
	20	0.02/0.1	0.01/1	0.26/1.8	0.27/22
	40	0.04/0.3	0.02/1	0.95/4.9	0.80/27
	60	[d]	[d]	1.65/6.5	1.00/14
2.5	3	0.05/1.3	0.04/14	0.06/1.4	0.01/5
	4	0.05/1.1	0.03/11	0.08/1.7	0.01/4
	6	0.07/1.3	0.05/12	0.09/1.7	0.03/7
	9	0.06/0.9	0.03/5	0.11/1.7	0.03/5
12	[d]	[d]	0.17/2.2	0.04/5	
	16	0.01/0.2	0.02/2	0.17/1.9	0.05/3
	20	0.11/0.9	0.03/3	0.11/1.1	0.06/4
	40	0.14/0.8	0.09/3	0.11/0.7	0.07/2
	60	[d]	[d]	0.43/2.1	0.15/4
	Av. error:	±0.7%	±7%	±2.2%	±11%

[a] *e.g.*, 0.95/4.1 signifies an average error of 0.95 min and a relative error of 4.1%.

[b] Predicted values of retention were obtained from input data for 12 and 60 min gradients at the same flow-rate.

[c] Predicted values of retention were obtained from input data for 16 and 60 min and a flow-rate of 1.0 ml/min in each instance.

[d] Error is zero for these runs, as they correspond to input data, *i.e.*, the input data are predicted exactly.

[e] These data correspond to the same flow-rate as input data.

R_s is proportional to the difference in retention times for two adjacent bands ($t_2 - t_1$) = Δt_g, errors in R_s can be related to the error in Δt_g. An example from the present study is shown in Table I. Here it is seen that the average error in predicted retention times is ±0.1%, and the corresponding error in resolution or Δt_g is ±1%.

Retention time predictions as in Table I are affected by the accuracy of the equipment dwell volume, V_D[26]. A value of V_D can be determined directly, as in Fig. 1, or it can be measured indirectly from experimental data for three different gradient times, t_G[23]. The latter procedure was used as a check on the value of V_D = 4.5 ml from Fig. 1. Experimental data for the OPA–amino acid sample were obtained for t_G = 12, 20 and 60 min (1.5 ml/min). The data for the 12- and 20-min runs were used as input to DryLab G, and predicted retention times for t_G = 60 min were obtained, using different values of V_D. These results were then compared with experimental times; the best agreement was obtained for V_D = 3.9 mL (average retention-time error = 0.06 min; 0.2%). The corresponding retention-time error for V_D = 4.5 ml (the value from Fig. 1) was 0.84 min (3.4%).

Our experience is that computer-calculated values of V_D (*i.e.*, 3.9 ml in this study) yield generally better predictions of retention by computer simulation. For this reason, the computer simulations reported here are for V_D = 3.9 ml. The resulting errors in predicted retention times for a wrong value of V_D are usually quite regular, so that errors in predicted band spacing (and resolution) do not depend much on the value assumed for V_D.

Table II summarizes comparisons of experimental and predicted retention data (as in Table I) for several flow-rates and gradient times. The predicted retention data were determined in each of two ways: (a) using experimental data for t_G = 12 and 60 min as input and the same flow-rate (*i.e.*, as in Table I, for F = 1.5); and (b) using experimental data for t_G = 16 and 60 min and F = 1.0 ml/min as input, and predicting data for all other runs (varying t_G and F) from these latter two runs. Previous comparisons of experimental *vs.* predicted retention times (DryLab G) have always involved the same flow-rates for input and output data. The comparisons of Table II therefore allow an assessment of the accuracy of retention predictions when the flow-rate is changed relative to the original (input) runs.

The agreement of predicted retention (t_g) and resolution (Δt_g) is seen to be quite good: ±0.7% in t_g and ±7% in Δt_g when the flow-rate is not allowed to vary, and ±2.2% in t_g and ±11% in Δt_g when the flow-rate is varied over a 6-fold range. These comparisons are similar to those reported[10,19,26] (flow-rate constant). The accuracy of predictions of resolution (Δt_g) is expected to be similar for either case (flow-rate constant or varying), and this is seen to be so.

Prediction of isocratic retention. Isocratic separations were also carried out for a slightly different OPA–amino acid sample, in order to assess the ability of DryLab I to predict isocratic separations on the basis of gradient data as input to computer simulation[a]. For the case of a constant flow-rate (1.5 ml/min) for both gradient and isocratic runs in this comparison, reasonable agreement was found between experimental and predicted retention for isocratic elutions, with average errors of ±3.4% for t_g and ±5% for Δt_g. These results are summarized in Table III.

When gradient data with a different flow-rate (0.4–1.0 ml/min) were used as input, the errors in predicted isocratic retention (for 1.5 ml/min) were larger. This is

[a] The same predictions of isocratic retention can also be made with DryLab G.

TABLE III

EXPERIMENTAL AND PREDICTED RETENTION TIMES FOR ISOCRATIC SEPARATION OF OPA–AMINO ACID SAMPLE

Conditions: flow-rate, 1.5 ml/min; mobile phase composition varies (22.5–32.5% acetonitrile); other conditions the same.

Solute	*Retention time, t_g (min)*[a]							
	$\varphi = 0.225$		$\varphi = 0.250$		$\varphi = 0.275$		$\varphi = 0.325$	
	Exptl.	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*
Thr	6.1	6.4	4.0	4.4	2.8	3.2	2.1	2.2
β-Ala	11.9	11.6	7.0	7.3	4.6	4.8	2.7	2.7
Tau	17.1	15.9	9.6	9.7	5.9	6.2	3.2	3.1
ILeu	46.8	38.6[b]	21.8	21.6	12.0	12.4	5.1	4.8

[a] Input for DryLab I calculations are 20- and 60-min gradient times, flow-rate 1.5 ml/min; other conditions as in Table I.

[b] This value deviates from the experimental run much more than other retention times; it was omitted from calculations of average error in retention.

similar to the prediction of gradient retention data when the flow-rate is allowed to vary, as summarized in Table II.

Band-width predictions

Table IV provides some illustrative data showing the comparison of experimental and predicted band widths (σ_t) for the present OPA–amino acid sample. For a flow-rate of 0.4 ml/min, it can be seen that the average error in predicted band widths is high (±56%) for a gradient time of 9 min, considerably smaller (±15%) for a gradient time of 20 min and acceptable (±6%) for a gradient time of 60 min. Also, the error in the first two bands (Glu and Ser) is generally different than for later bands. The Glu and Ser bands are subject to some "pre-elution" by the starting gradient[20], which complicates a discussion of errors in predicted band widths. For this reason, band-width data for these two bands were discarded in the average results in Table V.

Possible causes of error in band-width prediction. Table IV shows that the average error in σ_t decreases as gradient steepness (b) decreases, and this was generally true for all the data reported here (summarized in Table V). This is further shown in Fig. 2, where the ratio σ_{expt}/σ_t is plotted against b; the error in σ_t is seen to be generally small (<20%) for $b < 1$. However, relatively large errors are observed when $b > 1$. We shall consider possible causes of the errors in these predicted band widths, especially those error sources that are related to b.

Extra-column band broadening. It should be noted that band width generally decreases with b, other factors being equal, that is, errors in predicted band widths appear to be greater for narrower bands (smaller values of σ_t). If extra-column band broadening were greater than we have assumed ($\sigma_{ec} \approx 0.01$ ml), this would therefore lead to errors of the type shown in Fig. 2. However, we believe this is doubtful for two

TABLE IV

EXPERIMENTAL AND PREDICTED BAND WIDTHS FOR GRADIENT SEPARATION OF OPA–AMINO ACID SAMPLE

Conditions: standard gradient (5–55% acetonitrile) with varying gradient time, t_g; flow-rate, 0.4 ml/min.

t_G (min)	b	Solute	Band width, σ_t (min)		
			Exptl.	Calc.[a]	Error (%)
9	2.3	Glu	0.26	0.22	−16
		Ser	0.25	0.28	13
		β-Ala	0.24	0.35	45
		Tau	0.24	0.34	40
		ILeu	0.24	0.39	63
		Phe	0.24	0.42	77
		F-Phe	0.24	0.56	137
				Average	56
20	1.0	Glu	0.31	0.27	−11
		Ser	0.29	0.29	3
		β-Ala	0.27	0.30	11
		Tau	0.27	0.30	12
		ILeu	0.27	0.32	20
		Phe	0.27	0.33	22
		F-Phe	0.27	0.34	28
				Average	15
60	0.35	Glu	0.47	0.45	−4
		Ser	0.42	0.35	−18
		β-Ala	0.39	0.39	−2
		Tau	0.39	0.36	−8
		Ileu	0.38	0.39	3
		Phe	0.37	0.38	3
		F-Phe	0.37	0.38	2
				Average	6

[a] DryLab G predictions, based on experimental data for t_G of 12 and 60 min as inputs, flow-rate 1.0 ml/min.

reasons. First, errors as large as those in Table V and Fig. 2 would require a value of $\sigma_{ec} \approx 0.03$ ml, which seems unlikely. Second, the value of σ_{ec} required to explain these errors varies from 0.02 to 0.04 ml, *i.e.*, it is not (roughly) constant as required by the assumption that extra-column band broadening is responsible for these errors.

Error in the predicted value of N. Whatever model is used to predict N as a function of conditions, previous comparisons[3,6] suggest that resulting errors in band width will be of the order of ± 10–20%. The errors in σ_t implied by Fig. 2 are much larger, corresponding to errors in N as large as a factor of two. Moreover, the dependence of N on b (or $1/k'$, which is equivalent) should be greater for small values of b, which is the opposite trend from that observed in Fig. 2[a], Hence the plot in Fig. 2 does

[a] It has been shown[6,7] that the average diffusion coefficient of the solute increases with b (as $A + Bk'$ or as $A + B'/b$), owing to diffusion of solute molecules in the stationary phase. The resulting effect on N and band width increases in proportion to k' or $1/b$, which is the opposite of the trend in Fig. 2, assuming that errors in N are caused by a wrong value of B' in DryLab G.

TABLE V

SUMMARY OF EXPERIMENTAL *VS.* PREDICTED BAND WIDTHS FOR SEPARATION OF OPA–AMINO ACID SAMPLE (AS IN TABLE IV)

Conditions as in Table IV, except where indicated otherwise.

Flow-rate (ml/min)	*Gradient time, t_G (min)*	*Average band-width error (%)*[a]	*b*
0.4	9	54	2.3
	12	33	1.7
	16	17	1.3
	20	11	1.0
	40	8	0.52
	60	8	0.35
0.7	6	64	2.0
	9	52	1.3
	12	35	1.0
	16	28	0.75
	20	(31)[b]	0.60
	40	(49)[b]	0.30
	60	(53)[b]	0.20
1.0	3	30	2.9
	4	18	2.2
	6	14	1.5
	9	5	0.98
	12	2	0.73
	16	−1	0.55
	20	−2	0.44
	40	−7	0.22
	60	−14	0.15
1.5	12	18	0.45
	16	9	0.34
	20	3	0.27
	40	−6	0.14
	60	−13	0.09
2.5	[c]	[c]	[c]

[a] Excluding data for Glu and Ser; also, the small errors associated with the approximation of eqn. 5 have been corrected for.

[b] Deviant results; Not plotted in Fig. 2.

[c] Experimental band widths were much wider than predicted for all values of t_G; the column N value may have decreased in these runs.

not appear to arise from error in predicted values of N, although such errors probably contribute to the scatter of the data seen in Fig. 2.

Anomalous band broadening effect. It is possible that J increases with b faster than predicted by eqn. 4. This possibility is examined further in Fig. 3, where the dependence of J and b suggested by the plot in Fig. 2 is superimposed (dashed curve) on the plot reported previously[18], together with the data (points in Fig. 3) from ref. 18 that were originally used to determine J as a function of b. It appears that there is considerable scatter in values of J for $b > 0.5$, suggesting that the use of eqn. 4 (or a similar approximation for J) for predicting σ_t will not be very precise ($\pm$20–50%).

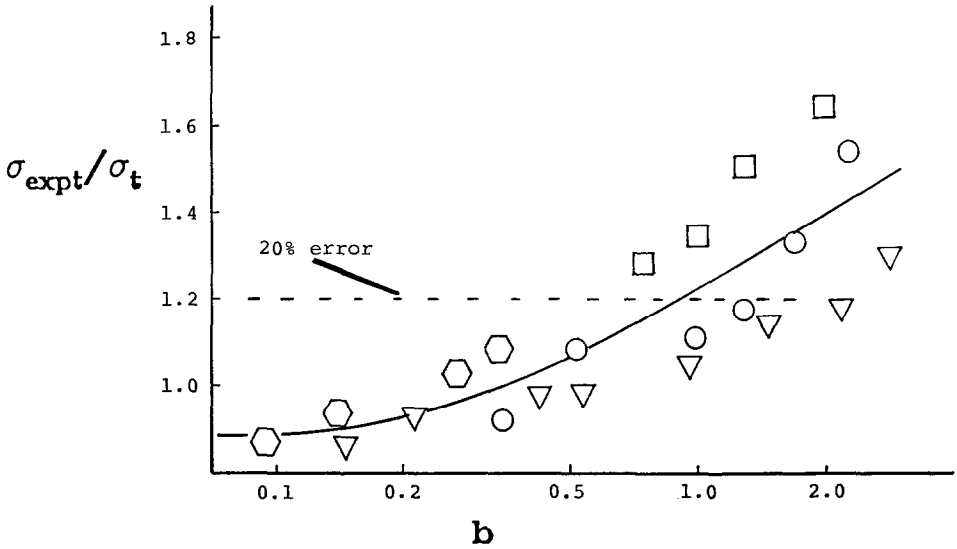

Fig. 2. Errors in predicted band widths as a function of gradient steepness, b ($\sigma_{expt}/\sigma_t = 1.00$ corresponds to no error); data from Table V. Flow-rate: ○, 0.4; □, 0.7; ▽, 1.0; ⬡, 1.5 ml/min.

Distortion of the gradient shape. Large values of b imply a small gradient volume $V_G = t_g F$, which in turn means that the gradient shape is more subject to distortion[20]. This rounding of the gradient at its beginning and end (for very small values of V_G) can result in a decrease in the effective steepness of the gradient (decrease in b) and a widening of the band relative to the case of an ideal (undistorted) gradient. This would in turn lead to wider (experimental) bands than are predicted for an ideal gradient.

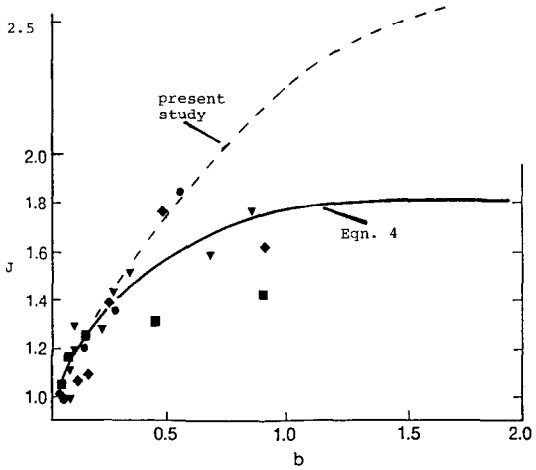

Fig. 3. Values of J *vs.* b from the present study (dashed line) and from ref. 18 (solid line). Data points from ref. 18; ◆, insulin; other data points (●,■,▼), small molecules (see ref. 18 for details).

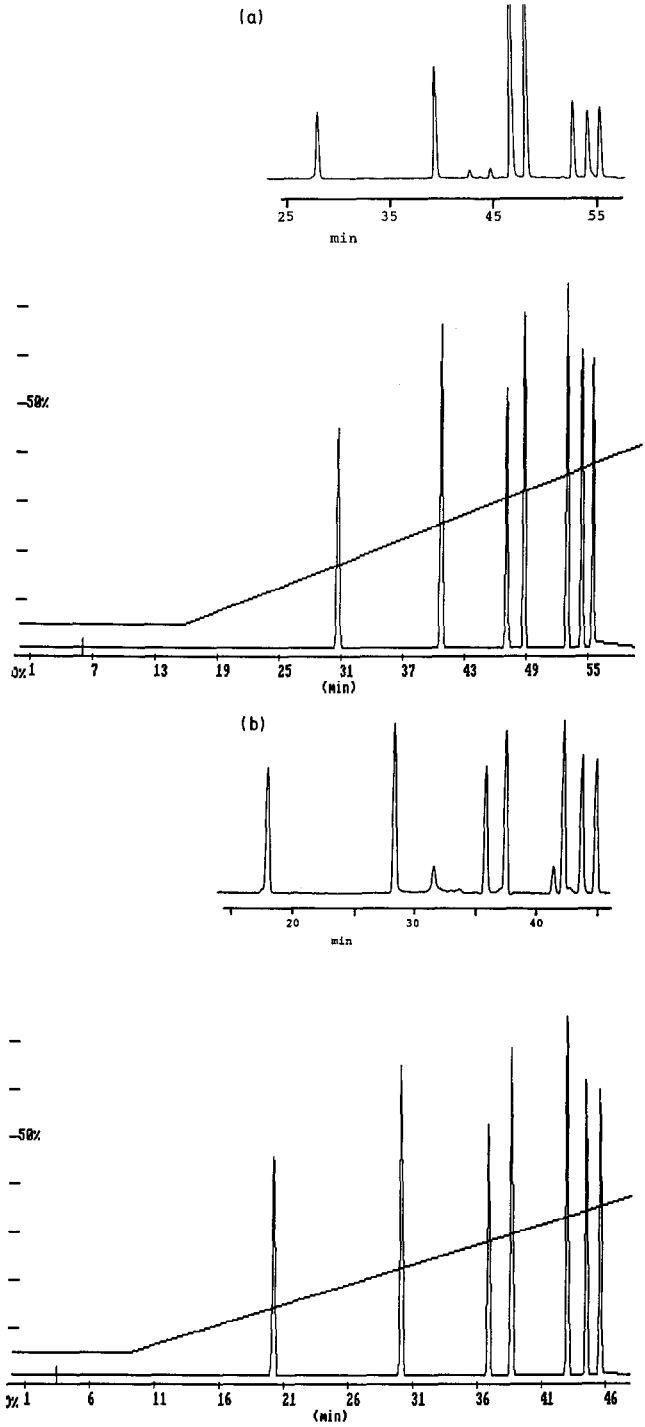
(a)
25
35
45
55
min
50%
0%
1
7
13
19
25
31
37
43
49
55
(min)
(b)
20
30
40
min
50%
0%
1
6
11
16
21
26
31
36
41
46
(min)

Fig. 4.

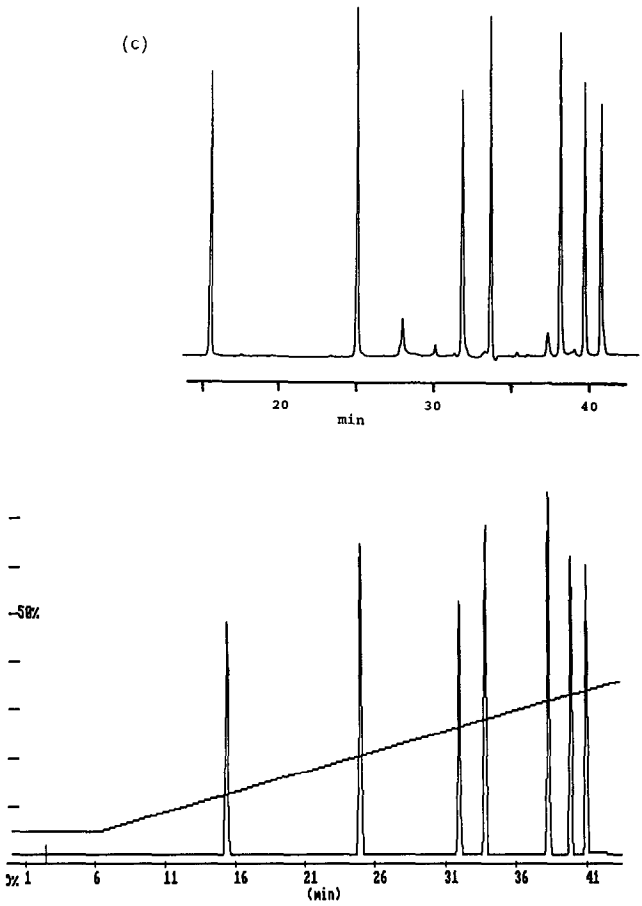

Fig. 4. Comparison of experimental and predicted (DryLab G) chromatograms. Gradient time is 60 min; other conditions except flow-rate are as in Table I. Experimental (top) and simulated (bottom) chromatograms. (a) 0.4 ml/min (b = 0.35); (b) 0.7 ml/min (b = 0.20); (c) 1.0 ml/min (b = 0.15).

The latter possibility (gradient distortion) is an intriguing explanation for "anomalous band broadening" for the case of steep gradients. However, gradient distortion for small values of b should also result in greater relative errors (as a percentage) in predicted retention times; the data in Table II do not support this proposal to any great extent. Further work will be required to verify this hypothesis, however, and we expect to report on this in a future paper. For the present, it should suffice to note that in most instances an optimum value of gradient steepness[1] corresponds to $0.2 < b < 0.5$. The resulting error in predicted value of σ_t for this case (Figs. 2 and 3) is not very great (+ 10–15%), and for practical purposes this error can be ignored. It should also be noted that errors in σ_t for steeper gradients ($b > 0.5$) can be empirically corrected by assuming a larger value of σ_{ec} than is actually measured (see below).

Predicted chromatograms

Figs. 4 and 5 illustrate the use of computer simulation for the prediction of final chromatograms as a function of experimental conditions. In Fig. 4, for shallower gradients ($b < 0.5$), several experimental chromatograms are compared with their computer simulations. There is generally good agreement between the corresponding experimental and simulated chromatograms.

In Fig. 5, for steeper gradients ($b = 0.75$ and 1.0), an experimental chromatogram is compared with two simulations: (a) for $\sigma_{ec} = 0.01$ (the assumed "best" value) and (b) for $\sigma_{ec} = 0.03$ (an artificial input value that eliminates much of the error summarized in Fig. 2). When simulations for steeper gradients are found to exhibit better overall resolution than the corresponding experimental chromatogram, we suggest that this can be empirically corrected by the expedient of adjusting the value of σ_{ec} entered into the DryLab G program.

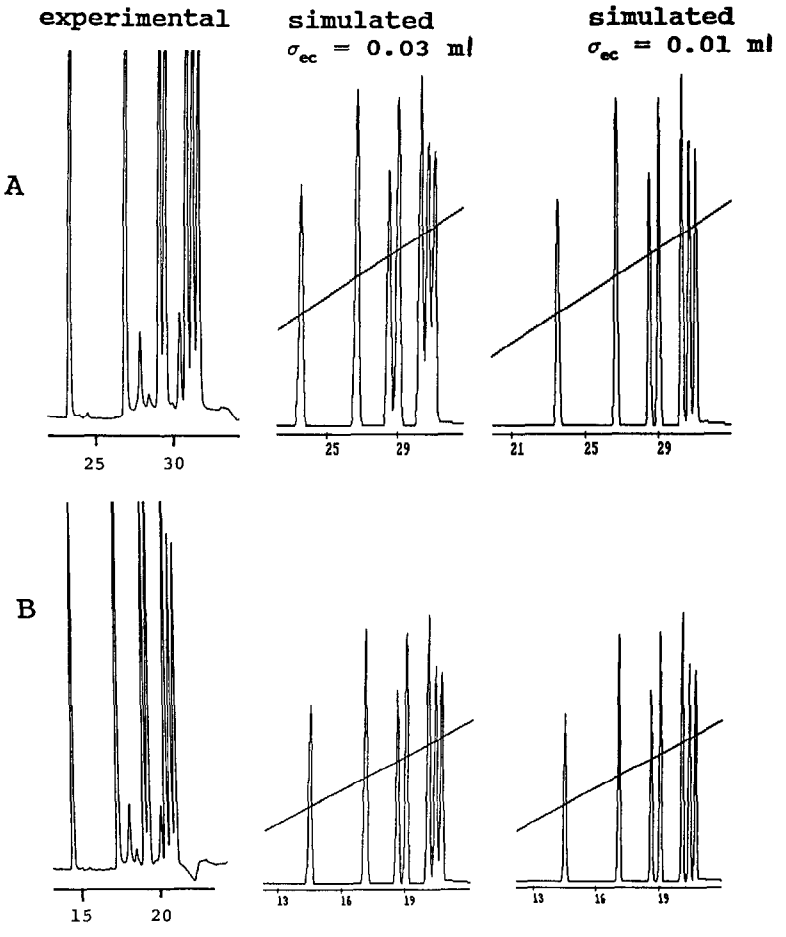

Fig. 5. Comparison of experimental and simulated (Drylab G) chromatograms. Conditions as in Table I, except gradient time and flow-rate. (A) 20-min gradient, 0.4 ml/min ($b = 1.0$); (B) 16-min gradient, 0.7 ml/min ($b = 0.75$).

CONCLUSIONS

This study has confirmed the accuracy of computer simulation for predicting separations by reversed-phase gradient elution. For a mixture of seven OPA-derivatized amino acids, retention times and band widths are each predicted with sufficient accuracy to allow accurate representations of the final chromatograms. Both gradient time and flow-rate were varied over wide limits, mimicking similar changes in a typical approach to method development.

The excess (anomalous) band broadening reported earlier[15–18] for steep gradients has been confirmed. For steep gradients ($b > 0.5$), it does not appear possible to predict the magnitude of this effect within narrow limits. This means that predicted values of band width for steep gradients can be in error by as much as ± 20–50%. However, these errors in predicted band widths are not of much practical significance, as very steep gradients are usually far from optimum for the application of gradient elution to a given sample. It appears possible to correct for these errors empirically, by artificially adjusting the extra-column band broadening of the system.

The causes of this anomalous band broadening for steeper gradients have been examined with respect to available experimental data (including those reported here). Gradient distortion and extra-column band broadening become more important for steeper gradients, and some combination of these two factors might account for wider than predicted bands as gradient steepness increases. Further work on this problem is in progress.

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